The Function of Heat Shock Protein 27 in Interleukin-1 Signalling

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A thesis submitted for the degree of Doctor of Philosophy to Imperial College of Science, Technology and Medicine

January 2009

The Kennedy Institute of Rheumatology
Imperial College London
ACKNOWLEDGEMENTS

I would like to thank my supervisors Dr. Jon Dean and Prof. Jerry Saklatvala for their advice and support during my PhD. I am very grateful for the time that Dr. Jon Dean has put in both for the discussion of experiments and thesis writing. I would also like to thank the other members of Dr. Jon Dean’s group, past and present, who offered their help, support and friendship throughout my PhD; Kate, Sarah, Lesley, Corina and Francesco. Other members of the signalling laboratory and institute who have also helped to make my time at KIR enjoyable include Annika, Louise (my running partner), Becky (another running buddy), Josie, Wing, Kathryn, Kawing and Brendan. Finally my thanks go to my friends and family, especially to my husband Damian who has always been there for me.
ABSTRACT

The p38 mitogen activated protein kinase (MAPK) pathway enhances the expression of pro-inflammatory response genes, including cyclooxygenase (COX)-2 and interleukin (IL)-6 in response to stimuli including IL-1. Heat shock protein 27 (HSP27) is a major protein phosphorylated by MAPK activated protein kinase 2 (MK2), a downstream kinase of p38 MAPK. However, the role of HSP27 in inflammation is unclear. Previous work has shown the requirement of HSP27 for stabilisation of COX-2 and IL-6 mRNAs following IL-1 treatment of HeLa cells. To examine the role of HSP27 in IL-1 signalling I depleted the protein from HeLa cells, human alveolar epithelial (A549) cells and human dermal fibroblasts (HDFs) by RNA interference (RNAi) using multiple short interfering RNAs (siRNAs).

HSP27 suppression in HeLa cells inhibited IL-1 induced activation of MK2, p38 MAPK and c-Jun N-terminal kinase (JNK), but not extracellular signal-regulated kinase (ERK). Upstream MAPK kinase (MKK) 3, 4, 6 and 7 and TGF-beta-activated kinase 1 (TAK1) were also inhibited. HSP27 depletion did not disrupt the constitutive interaction found between TAK1 and its binding protein TAB1. HSP27 appeared to impinge on signalling at a point lying between cell surface receptors and TAK1 or at TAK1 itself as activation of JNK and p38 MAPK by tumour necrosis factor (TNF) was inhibited by HSP27 knockdown.

A transfection procedure was developed for transfection of siRNA into HDF cells. In HDFs and A549 cell types HSP27 was required for COX-2 protein expression and the full activation of p38 MAPK. However, while TAK1 was shown to be responsible for sustained activation of p38 MAPK, TAK1 activity was not affected by HSP27 depletion in HDFs. MKK3 (rather than MKK6) appeared to be the major HDF activator of p38 MAPK in response to IL-1. My results demonstrate that HSP27 has a novel role in augmenting IL-1 signalling in epithelial cell lines and fibroblasts.
DECLARATION

This thesis is a result of my own work. Some figures were contributed with the help of other members of the laboratory. During the course of manuscript preparation for a paper work on the HSP27-dependent regulation of IL-1-induced MKK3,4,6,7 and TAK1 in HeLa cells was performed by Dr. Jonathan Dean, Dr. Sarah Glennie and Ms. Lesley Rawlinson and are explicitly labelled as such. All other figures are of my own work. The work was carried out at the Kennedy Institute of Rheumatology Division, Imperial College, London. This work was funded by a studentship from the Medical Research Council and the Arthritis Research Campaign.
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ABBREVIATIONS

Ab  Antibody
Akt  Protein kinase B
AP-1  Activator protein 1
ARE  Adenylate/ Uridylate-rich element
ARENBP  AU-rich element-binding protein
ASK  Apoptosis signalling kinase
ATCC  American type culture collection
ATF  Activating transcription factor
ATP  Adenosine Triphosphate
BRF  Butyrate response factor
BSA  Bovine serum albumin
C5a  Complement factor 5a
C/EBP  CAAT/enhancer binding protein
CHOP  CREB-homologous protein
CMT  Charcot-Marie-Tooth disease
COP  Caspase recruitment domain-only protein
COX-2  Cyclooxygenase-2
CRE  Cyclic AMP response element
CREB  Ca2+ cyclic adenosine monophosphate response element binding protein
CSAID  Cytokine-suppressive anti-inflammatory drug
CXCL12  Stromal cell derived factor 1
CYLD  Cylindromatosis
DMEM  Dulbecco’s modified Eagle’s medium
DMSO  Dimethyl sulfoxide
DUB  Deubiquitinating enzyme
<table>
<thead>
<tr>
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<th>Full Name</th>
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<tbody>
<tr>
<td>DUSP1</td>
<td>Dual specificity protein phosphatase 1 (MKP1)</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced Chemiluminescence</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediamine tetraacetic acid</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethylene glycol tetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunoabsorbent sandwich assay</td>
</tr>
<tr>
<td>EMEM</td>
<td>Eagle’s minimum essential medium</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular-signal Regulated Kinase</td>
</tr>
<tr>
<td>FADD</td>
<td>Fas-associated death domain protein</td>
</tr>
<tr>
<td>FCS</td>
<td>Foetal calf serum</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte monocyte colony-stimulating factor</td>
</tr>
<tr>
<td>GST</td>
<td>Glutathione S-transferase</td>
</tr>
<tr>
<td>HA</td>
<td>Hemagglutinin</td>
</tr>
<tr>
<td>HDF</td>
<td>Human dermal fibroblast</td>
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<tr>
<td>HEPES</td>
<td>4-2(hydroxyethyl)-1-piperazineehenesulphonic acid</td>
</tr>
<tr>
<td>hnRNP</td>
<td>Heterogeneous nuclear ribonucleoprotein</td>
</tr>
<tr>
<td>dHMN</td>
<td>Distal heritary motoneuropathies</td>
</tr>
<tr>
<td>HRP</td>
<td>Horse radish peroxidise</td>
</tr>
<tr>
<td>HSF</td>
<td>Heat shock transcription factor</td>
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<tr>
<td>HSP27</td>
<td>Heat shock protein 27</td>
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<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IκB</td>
<td>Inhibitor of NF-κB</td>
</tr>
<tr>
<td>IKK</td>
<td>IκB kinase</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
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<td>IL-1R</td>
<td>IL-1 receptor</td>
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<td>IL-1RAcP</td>
<td>IL-1R accessory protein</td>
</tr>
<tr>
<td>IRAK</td>
<td>IL-1 receptor associated kinase</td>
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<td>JIP</td>
<td>JNK-interacting protein</td>
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<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
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<tr>
<td>LB</td>
<td>Luria Bertani</td>
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<tr>
<td>LNA</td>
<td>Locked nucleic acid</td>
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<td>LPS</td>
<td>Lipopolysaccharide</td>
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<td>LRR</td>
<td>Leucine-rich repeats</td>
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<tr>
<td>Lys</td>
<td>Lysine</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
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<td>MBP</td>
<td>Myelin basic protein</td>
</tr>
<tr>
<td>M-CSF</td>
<td>Macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>MEF</td>
<td>Mouse embryonic fibroblast</td>
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<tr>
<td>MEK</td>
<td>MAPK ERK kinase</td>
</tr>
<tr>
<td>MEKK</td>
<td>Mitogen ERK kinase kinase</td>
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<td>miRNA</td>
<td>MicroRNA</td>
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<td>MK</td>
<td>MAPK activated protein kinase</td>
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<td>MAPK kinase</td>
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<td>MAPK kinase kinase</td>
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<td>MKP1</td>
<td>MAPK phosphatase 1</td>
</tr>
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<td>MLK</td>
<td>Mixed lineage kinase</td>
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<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
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<tr>
<td>MNK</td>
<td>MAPK-interacting kinase</td>
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<tr>
<td>MP-1</td>
<td>MEK Partner 1</td>
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<tr>
<td>MSK</td>
<td>Mitogen- and stress-activated kinase</td>
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<td>MTTT</td>
<td>3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide</td>
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<tr>
<td>MyD88</td>
<td>Myeloid differentiation primary response gene 88</td>
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<tr>
<td>NF-κB</td>
<td>Nuclear factor-kappaB</td>
</tr>
<tr>
<td>NSAID</td>
<td>Non-steroidal anti-inflammatory drug</td>
</tr>
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<td>NIK</td>
<td>NF-κB-inducing kinase</td>
</tr>
<tr>
<td>OA</td>
<td>Osteoarthritis</td>
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<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
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<tr>
<td>PAK</td>
<td>p21-activated protein kinase</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen associated molecular patterns</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<td>PBS-T</td>
<td>PBS 0.1 % Tween 20</td>
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<td>PCR</td>
<td>Polymerase chain reaction</td>
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<td>Prostaglandin</td>
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<tr>
<td>PKC</td>
<td>Protein kinase C</td>
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<td>PMA</td>
<td>Phorbol 12-myristate 13-acetate</td>
</tr>
<tr>
<td>PP</td>
<td>Protein phosphatase</td>
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<tr>
<td>PRAK</td>
<td>p38-regulated/activated kinase</td>
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<td>PRR</td>
<td>Pathogen recognition receptor</td>
</tr>
<tr>
<td>PVDF</td>
<td>polyvinyl difluoride</td>
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<tr>
<td>RA</td>
<td>Rheumatoid arthritis</td>
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<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>RIP</td>
<td>Receptor interacting protein</td>
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<td>RISC</td>
<td>RNA-induced silencing complex</td>
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<td>RNAi</td>
<td>RNA interference</td>
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<td>ROS</td>
<td>Reactive oxygen species</td>
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<td>SAPK</td>
<td>Stress-activated protein kinases</td>
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<tr>
<td>S.E.M.</td>
<td>Standard error of the mean</td>
</tr>
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<tr>
<td>UV</td>
<td>Ultraviolet</td>
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<tr>
<td>VCAM</td>
<td>Vascular cell adhesion molecule</td>
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Chapter 1

Introduction
The aim of this project was to investigate the role of heat shock protein (HSP) 27 in inflammation, notably interleukin (IL)-1 signalling. This followed on from previous work in our laboratory that had shown a role for HSP27 in IL-1-induced expression and post-transcriptional regulation of the inflammatory mediators; cyclooxygenase (COX)-2 and IL-6 (Alford, 2006; Alford et al., 2007). This introduction focuses on the innate immune system, inflammation, IL-1 signalling and the reported functions of HSP27 to provide a background for the experiments in this thesis.

PART 1: Immunity and inflammation

1.1 Innate immunity

Innate immunity is the first line of defence of the human body against the invasion of pathogens. Innate immunity is an immediate response and does not produce a long-lasting defence. If the innate immune system proves unsuccessful then the adaptive immune system is activated. However, the activation of the adaptive immune response takes several days during which time the innate immune response is required to keep infection under control (Janeway Jr., 2001). Neutrophils and macrophages are the main cells involved in the innate immune response. These cells are activated by pattern recognition receptors (PRRs) on the cell surface that recognise pathogen associated molecular patterns (PAMPs) on the invading microorganism. Neutrophils and macrophages can then ingest the pathogen by phagocytosis and break it down. Macrophages can then secrete cytokines and other mediators to induce inflammation.

1.2 Inflammation

Inflammation is the response of tissues to injury, infection or autoimmune diseases. It is a defence mechanism against microorganisms and initiates the repair process. Inflammation is an effector mechanism of both innate and adaptive immunity, it has rapid onset and is generally of short duration. There are five signs of inflammation; redness, swelling, heat, pain and loss of function. Inflammation is triggered by many events, for example the inflammatory cytokines IL-1 and tumour necrosis factor (TNF), complement activation, antigen-antibody interactions and signalling through PRRs (Janeway Jr., 2001). One example of PRRs is the toll-like receptors (TLR), for example TLR4, which binds to lipopolysaccharide (LPS). The binding of PAMPs, IL-1 and TNF to their receptors activates a common set of intracellular signalling pathways resulting in the production of many cytokines and chemokines, enzymes and adhesion molecules. Chemokines act as chemoattractants to phagocytic cells, such as monocytes and neutrophils. Inflammation causes vasodilation which is partly due to production of
prostaglandins by COX-2, an induced enzyme. The increase in the expression of adhesion molecules on the surface of the vascular endothelial cells of the capillaries causes leucocytes to adhere and their migration then follows chemotactic gradients to the site of injury or infection.

Under normal circumstances inflammation resolves once the cause has been removed. This requires the synthesis of glucocorticoids, anti-inflammatory cytokines such as IL-10 and other factors. If this process is not able to occur then chronic inflammation may ensue and can lead to serious tissue damage, destructive inflammation and loss of function. Persistent inflammation can be caused by certain microorganisms that are effective at evading the immune response. Chronic inflammation may also occur in certain autoimmune diseases such as rheumatoid arthritis (RA) and Crohn’s disease. Acute inflammatory reactions are often characterised by the presence of polymorphonuclear leucocytes whereas chronic inflammatory diseases are characterised by mononuclear cellular infiltrate (Kunkel et al., 1994).

The thorough understanding of the regulation of signalling pathways such as IL-1 should lead to the better understanding of current anti-inflammatory drug mechanisms (for example glucocorticoids) and help identify new drug targets. This is important both for the minimisation of episodes of chronic and destructive inflammation and to increase the number of treatment options for patients, with the consequent hope that less patient inflammation is refractory to treatment options. Heat shock proteins have been reported to both dampen and exacerbate the immune response and it is possible that heat shock proteins could fine-tune the immune response and have potential as drug targets. In this project I will investigate the importance of a specific heat shock protein, HSP27, in IL-1 signalling.

1.3 Cytokines and pro-inflammatory mediators

Cytokines are a large group of small peptides, proteins and glycoproteins (>25 kDa) that have potent biological effects. Cytokines are released principally from macrophages and T helper cells in response to an activating stimulus and mediate their actions through binding to cell surface receptors. Most cytokines are secreted but some may be expressed on the cell surface or held in reservoirs in the extracellular matrix.

1.3.1 Interleukin-1

IL-1 is a pro-inflammatory cytokine that has two forms: IL-1α and IL-1β. Both forms of IL-1 are expressed as propeptides by a variety of cells in response to different stimuli. The propeptides are approximately 31 kDa. IL-1 was identified originally as the fever inducing
endogenous pyrogen (Auron et al., 1984). The two forms of IL-1 signal through the same receptor (Kilian et al., 1986) and therefore have similar biological actions. There are three IL-1 receptors which can either be expressed as cell-surface or soluble receptors: the type I IL-1 receptor (IL-1RI), type II IL-1 receptor (IL-1RII) and IL-1 receptor accessory protein (IL-1RACP) (Braddock and Quinn, 2004; Janssens and Beyaert, 2003). IL-1RI and IL-1RACP bind to IL-1 and instigate the downstream signalling. The signalling pathways activated through these receptors will be discussed later. IL-1RII acts as a decoy receptor, which competes with IL-1RI for IL-1 and thus acts as a buffer for excess IL-1 (Neumann et al., 2000). Soluble IL-1RACP has been shown to act as an endogenous inhibitor of IL-1 (Smeets et al., 2003). The IL-1R antagonist (IL-1Ra), shares only about 30% sequence identity to IL-1α and IL-1β (Eisenberg et al., 1991), however it has the same β-barrel tertiary structure (Vigers et al., 1994). IL-1Ra can bind to the IL-1RI with extremely high affinity (Dripps et al., 1991). The structure of IL-1RI bound to IL-1Ra revealed that the C-terminal domain is rotated by 170º compared to the IL-1RI in complex with IL-1β (Vigers et al., 2000) and this complex cannot bind to the IL-1RACP. Thus IL-1Ra acts as a competitive inhibitor (Arend et al., 1998).

IL-1 expression is induced by a wide variety of stimuli for example LPS (Burchett et al., 1988), phorbol 12-myristate 13-acetate (PMA) (Fenton et al., 1988), UV light (Kupper et al., 1987), TNF and IL-1 itself (Dinarello et al., 1987; Warner et al., 1987a; Warner et al., 1987b). Some stimuli require synergy to induce IL-1 expression for example IL-3 requires co-stimulation with LPS (Frendl et al., 1990).

IL-1α is thought to be principally involved in autocrine signalling (Dinarello, 1998). It is active in both the precursor and processed mature forms. Once expressed it is mostly retained in the cell and only appears in the circulation in severe disease states. Pro-IL-1α may be released from a dying cell that is undergoing apoptosis or necrosis and subsequently cleaved to the mature form by circulating proteases. Alternatively, IL-1α may be cleaved by the calcium activated cysteine protease, calpain (Kobayashi et al., 1990), associated with the cell membrane. In contrast, the IL-1β propeptide has very little activity and needs to be cleaved into its mature form to exert its potent effects. Pro-IL-1β is cleaved by the IL-1β converting enzyme (ICE or caspase-1), a part of the inflammasome (Cerretti et al., 1992; Martinon et al., 2002; Thornberry et al., 1992). A group of proteins termed COPs (caspase recruitment domain-only proteins) can interact with pro-caspase-1 and prevent its activation and therefore the unnecessary activation of IL-1β (Druilhe et al., 2001; Lamkanfi et al., 2004). Characterisation of IL-1β-null mice revealed that IL-1β does not have a role in the healthy individual, however it acts as a pyrogen and an acute phase response mediator in a subset of inflammatory disease models, for example when challenged with turpentine (Zheng et al., 1995).
IL-1 has a similar wide range of actions to the cytokine TNF, often associated with inflammation, infection and autoimmune disorders (Dinarello, 1986). IL-1 has a role in many chronic, often systemic, inflammatory and auto-immune diseases such as RA, osteoarthritis (OA), Crohn’s disease (Braddock and Quinn, 2004), familial Mediterranean fever (Dinarello, 2005) and some diseases of the central nervous system such as multiple sclerosis (Simi et al., 2007). IL-1 has therefore been an attractive drug target for disease treatment (Braddock and Quinn, 2004). The most important drug to date is the soluble IL-1Ra, Kineret ® (Anakinra), which has been approved for use to treat RA.

### 1.3.2 Interleukin-6

IL-6 is a pleiotropic cytokine that has multifunctional roles in inflammation, the immune response, hematopoiesis, the endocrine system and the nervous system (Hirano, 1998). IL-6 requires the IL-6 receptor (IL-6R) and the signal transducer gp130 (Taga et al., 1989). IL-6-/- mice exhibit severely compromised inflammatory acute phase response after tissue damage or infection (Kopf et al., 1994). IL-6-/- mice develop normally, however they fail to overcome infection with either Vaccinia virus, Listeria monocytogenes or vesicular stomatitis virus.

### 1.3.3 Interleukin-8

IL-8 (CXCL1) was a founding member of the chemokine superfamily. Inside the cell it is generated as a precursor and secreted after the cleavage of the signal peptide. It is released by phagocytes and a variety of other cell types in response to inflammatory stimuli such as IL-1 (Kunkel et al., 1994). Once released, IL-8 acts as a potent chemoattractant for neutrophils. IL-8 causes neutrophils to undergo calcium translocation, shape change, chemotaxis, actin polymerisation, exocytosis of storage proteins and may trigger the respiratory burst (Hoch et al., 1996). IL-8 causes neutrophils to shed L-selectin and up-regulate the adhesion-promoting receptor CD11b/CD18 (CR3).

### 1.3.4 Tumour necrosis factor

The pleiotropic cytokine TNF is a member of a large family of proteins (Pasparakis et al., 1996). TNF has a central role in immune and inflammatory processes including the induction of local inflammatory response to contain infections. However, TNF can also lead to systemic effects such as the acute-phase response, fever and septic shock (Dinarello, 1987). TNF has been implicated in many autoimmune diseases, for example overexpression of TNF has been reported in RA (Brennan et al., 1990) and Crohn’s disease (Papadakis and Targan, 2000). In
contrast underexpression of TNF has been reported in type I diabetes (Christen et al., 2001) and systemic lupus erythematosus (Kontoyiannis and Kollias, 2000). A murine model with TNF overexpression was generated by the deletion of its adenylate/uridylate-rich element in its 3’ untranslated region. These mice exhibit inflammatory arthritis and a Crohn’s-like inflammatory bowel disease as a result of increased TNF mRNA stability and TNF production (Kontoyiannis et al., 1999). The importance of TNF in autoimmune diseases has led to the development of anti-TNF as a therapeutic for RA and Crohn’s disease, for example the chimeric human-mouse anti-TNF monoclonal antibody cA2 (infliximab or Remicade). Anti-TNF therapies are delivered by subcutaneous injection and are expensive.

1.3.5 Cyclooxygenase-2

There are two isoforms of cyclooxygenase (COX), COX-1 and COX-2. These enzymes catalyse the first committed step in the conversion of arachidonic acid to prostaglandin (PG) $\text{H}_2$ (Langenbach et al., 1999). PGH$_2$ can then be metabolised further into the biologically active prostaglandins, PGE$_2$, PGI$_2$ (prostacyclin), PGF$_{2\alpha}$, PGD$_2$ and thromboxane A$_2$. COX-1 is constitutively expressed in cells of most tissues and is considered to be the “housekeeping” isoform. In contrast COX-2 is not normally expressed in most tissues but is induced by mitogens, cytokines and certain other inflammatory agents.

The main biological functions of COX-1 are thought to include platelet aggregation, renal water balance and gastric cytoprotection. The functions of COX-2 are thought to include platelet disaggregation, inflammation, vasodilation and bone resorption (Morita, 2002). It is thus thought that COX-2 is responsible for the upregulation of prostaglandins in response to inflammatory stimuli (Smith et al., 2000). Non-steroidal anti-inflammatory drugs (NSAIDs) pharmacologically target the COX isoforms and are a common therapeutic for chronic inflammatory disorders. NSAIDs can cause gastrointestinal side effects as a result of the inhibition of COX-1. COX-2 inhibitors have an increased risk of thrombosis and cardiovascular complications (Mitchell and Warner, 2006).

PART 2 Inflammatory cell signalling

1.4 The Interleukin receptor/ Toll-like receptor superfamily

Toll-like receptors (TLRs) are pattern recognition receptors expressed on many cells. The first mammalian TLR to be identified was TLR4, cloned on the basis of its homology to Drosophila
protein Toll (Medzhitov et al., 1997). Other laboratories identified TLR4 as a result of trying to identify the single gene product that caused insensitivity to LPS (Poltorak et al., 1998). Since then ten other TLR homologues have been identified in mammals (Arancibia et al., 2007). TLRs are highly conserved transmembrane proteins that possess a Toll / IL-1R (TIR) domain and an extracellular domain with leucine-rich repeats (LRRs). The LRRs vary between each TLR and are responsible for the recognition of the TLR ligands. Examples of ligands include lipopolysaccharide (LPS) from Gram-negative bacteria (TLR4), lipoteichoic acid (TLR2), flagellin (TLR5) and single-stranded RNA (TLR7 and 8). Conditioned medium from RA cultures has been found to contain a potential TLR ligand (Sacre et al., 2007). Recently TLR8 has been reported to be an important contributor to TNF production in RA (Sacre et al., 2008). It is thought that the inhibition of TLR8 would not interfere with signalling triggered by other TLRs or inhibit cytokine expression by other mechanisms. Therefore it has been postulated that TLR8 would be a good new drug target in RA, with less side effects than anti-TNF therapy (Sacre et al., 2008). The IL-1R is also a homologue of the TLRs and contains the TIR domain.

1.5 IL-1R and TLR signalling

Cell signalling is triggered when a ligand binds a receptor for example IL-1 to the IL-1R. The IL-1 receptor can bind to IL-1α, IL-1β and IL-1Ra. The conservation of the cytoplasmic TIR domain in the IL-1R and TLRs ensure that many of the downstream signalling components of these receptors are shared. This thesis focuses on the signalling downstream of the IL-1R and therefore this is described here. However, there are 33 well-characterised members of the interleukin family, related to IL-1 (Arend et al., 2008), IL-34 (Lin et al., 2008) and IL-35 (Collison and Vignali, 2008) have also been very recently discovered. The interleukin family members stimulate related signal transduction pathways, therefore it is likely that work to discover the importance of HSP27 in IL-1 signalling will have relevance for the regulation of signal transduction pathways downstream of the receptors of other interleukin family members and the toll-like receptor family.

IL-1RAcP is required for downstream signalling (Greenfeder et al., 1995). Upon IL-1 binding, IL-1R and IL-1RAcP form a complex which recruits myeloid differentiation primary response gene 88 (MyD88) (Fig. 1) and a second accessory protein Toll-interacting protein (Tollip) (Burns et al., 2000). MyD88 contains a TIR domain and a death domain. The death domain on MyD88 interacts with the death domain on the Ser / Thr kinases termed IL-1 receptor associated kinases (IRAK) 1 and IRAK4. IRAK4 is the protein kinase responsible for the phosphorylation of IRAK1 (Lye et al., 2004). The kinase activity of IRAK1 is not required for signalling (Vig et al., 1999). Phosphorylated IRAK1 is released from the receptor complex (Fig. 1).
Phosphorylated IRAK1 then recruits and binds to TNF receptor associated factor (TRAF) 6, which forms homooligomers (Cao et al., 1996).

TRAF proteins are E3 ubiquitin ligases through their N-terminal RING domain. TRAF6 oligomerisation is thought to activate its ligase activity (Chen, 2005). The RING domain contains zinc fingers (Aravind et al., 2003; Rothe et al., 1994). TRAF6, with the aid of the E2 heterodimeric ubiquitin conjugating enzyme (Ubc13) / ubiquitin-conjugating enzyme variant human 1A (Uev1A) complex, catalyzes the formation of lysine 63 (Lys63)-linked polyubiquitin chains on TRAF6 (Fig. 1). Lys63 polyubiquitin chains form very different configurations compared to the Lys48 polyubiquitin chains that target proteins for proteasomal degradation (Varadan et al., 2004). The Lys63 polyubiquitin chain is thought to act as a scaffold to recruit downstream signalling proteins, including the MAPK kinase kinase (MKKK), TAK1. The MKKKs and downstream kinase cascade will be discussed in more detail later.

Recently IRAK1 and IRAK4 have been reported to phosphorylate Pellino isoforms. This activates the E3 ubiquitin-ligase activity of Pellino and together with Ubc13-Uev1A Pellino can catalyse the formation of Lys63-linked polyubiquitin chains on IRAK1 (Ordureau et al., 2008). This polyubiquitination of IRAK1 has been proposed to act as a scaffold for the recruitment of the IκB kinase (IKK) complex through IKKγ (Windheim et al., 2008). As IRAK1 and TRAF6 interact this brings TAK1 and the IKK complex into close proximity, facilitating the phosphorylation and activation of the IKK complex by TAK1.

1.6 TNF-induced cell signalling

TNF can bind to two receptors termed TNF receptor (TNFR)-1 (p55) and -2 (p75) (Idriss and Naismith, 2000). TNFR-1 is the receptor which is most important in signalling to nuclear factor-kappaB (NF-κB) and in the production of IL-6 (Yamada et al., 1998). TNFR-1 exists as a monomer in the cell membrane. It is activated by forming a trimer on binding TNF, which is a trimer (Fig. 1.1). This leads to the recruitment of the TNFR associated death domain protein (TRADD), which, as its name suggests, contains a death domain. Signalling through the TNF receptor can transduce apoptotic, inflammatory and survival signals (Sheikh and Huang, 2003). The apoptotic and inflammatory pathways diverge at the next proteins recruited to the receptor complex. The binding of Fas-associated death domain protein (FADD) to TRADD through their death domains leads to apoptotic signalling through caspase 8. The recruitment of the intracellular adaptor proteins TRAF2 and TRAF5 to the receptor complex mediates the inflammatory response and provides survival signals (Chung et al., 2002) (Fig. 1). Receptor interacting protein (Rip)1 is also recruited to TRADD (Hsu et al., 1996) and is ubiquitinated by
Figure 1.1: IL-1 and TNF signalling pathways. IL-1 and TNF signalling occurs through different plasma membrane receptors. However, the signalling pathways converge at the level of the MKKKs, most notably TAK1.
Chapter 1

Introduction

TRAF2 (Lee et al., 2004). TAB2 can bind to the Lys63-linked polyubiquitin chains on Rip1 (Kanayama et al., 2004), leading to the activation of TAK1.

1.7 MKKKs

MKKKs are serine/threonine protein kinases that phosphorylate and activate the downstream MAPK kinases (MKKs). A large variety of candidate MKKKs have been identified and there is some controversy over which MKKKs are important in pro-inflammatory signalling.

1.7.1 TAK1

TAK1 is the most widely cited MKKK in the literature for activation of the MAPK cascades in response to pro-inflammatory stimuli. TAK1 was identified as important in the TGF-β signal transduction pathway (Yamaguchi et al., 1995). TAK1 (Map3K7 gene) knockout mice are early embryonic lethal and therefore TAK1−/− mouse embryonic fibroblasts (MEFs) were generated (Shim et al., 2005). Experiments using TAK1−/− MEFs have shown that TAK1 is essential for JNK and NF-κB signalling in response to TNFα and IL-1β (Shim et al., 2005). Overexpression of TAK1 with TAK1 binding protein (TAB)1 in HeLa cells activated NF-κB, JNK and p38 MAPK pathways and led to the expression of IL-8 from a reporter construct (Holtmann et al., 2001). TAK1 phosphorylates MKK3, MKK4 and MKK6 (Moriguchi et al., 1996a; Shirakabe et al., 1997). TAK1 can also activate MKK7 (Hammaker et al., 2007; Zhou et al., 1999).

TAK1 forms a constitutive complex with TAB1 and binds to TAB2 / TAB3 in response to cell stimulation. TAB1 is a pseudophosphatase (Conner et al., 2006), which is critical for the activation of TAK1 because TAK1 could not be activated in TAB1−/− MEFs in response to IL-1 or TNF (Mendoza et al., 2008). Ectopically expressed TAK1, co-expressed with TAB1 becomes autophosphorylated (Kishimoto et al., 2000). IL-1 induces the translocation of TAB2 from the membrane to the cytosol where it binds to TAK1 and TRAF6 (Takaesu et al., 2000; Takaesu et al., 2001). A functional interaction between TAK1 and TAB2/3 is required for TAK1-dependent signalling (Besse et al., 2007; Wang et al., 2001). It is thought that the FP motif in the CUE domain of TAB2/3 allows the proteins to bind to polyubiquitinated TRAF6 (Kishida et al., 2005). The polyubiquitinated TRAF6 is able to act as a scaffold to which multiple TAK1 complexes can bind through TAB2/3 (Kanayama et al., 2004). TAB2 and TAB3 are thought to be partially redundant. The proximity of the multiple TAK1 complexes is thought to allow TAK1 subunits to cross-phosphorylate residues including Thr187 (Singhirunnusorn et al., 2005) in the activation loop, leading to the activation of TAK1 and downstream signalling.
Overexpressed TAK1 has been shown to be polyubiquitinated and TAB1 monoubiquitinated in response to IL-1, however the functions of these ubiquitinations are unknown (Thiefes et al., 2006).

Surprisingly, TAK1−/−, TAB1−/− and TAB2−/− mice have very different phenotypes. TAK1−/− embryos have neural fold dysmorphogenesis and begin to die around E10. TAB1−/− embryos have cardiovascular and lung dysmorphogenesis and are embryonic lethal at late stages of gestation (Komatsu et al., 2002). TAB2−/− mice had embryonic lethality about E12.5 due to liver degeneration and apoptosis (Sanjo et al., 2003). Although work in TAK1−/− MEFs suggested an important role for TAK1 in JNK and NF-κB signalling there have been several reports that this is not the case for TAB1−/− (Mendoza et al., 2008; Shim et al., 2005) or TAB2−/− mice (Sanjo et al., 2003; Shim et al., 2005). It may be that TAB2 and its homologue TAB3 are redundant. However, the role of TAB1 in inflammatory signalling is still controversial because the original paper using TAB1−/− MEFs found that TAB1 was essential for TAK1 phosphorylation of MKK6 and important for TGF-β signalling (Komatsu et al., 2002).

TAB1 has also been reported to cause p38 MAPK activation independently of TAK1 and MKK3 (Ge et al., 2002). This is through the autophosphorylation of p38 MAPK. The splice variant TAB1beta is only able to interact with p38 MAPK alpha but not TAK1 (Ge et al., 2003). The importance of this mechanism of p38 MAPK activation is unclear.

Another protein has recently been identified as a possible TAB, type 2A phosphatase-interacting protein, TIP (TAB4) (Prickett et al., 2008). TAB4 was found to co-precipitate with TAK1 and stimulate TAK1 phosphorylation in the linker region, above the levels induced by TAB1. TAB4 was shown to have an FP sequence motif like TAB2 / TAB3 that can bind to polyubiquitin chains.

1.7.2 Other MKKKs

It is possible that different MKKKs could contribute to the downstream activation of the three MAPK cascades and NF-κB to different extents dependent upon which cell type or stimuli is used. Other MKKKs that could be involved include mitogens ERK kinase kinase (MEKK)1-4, apoptosis signal-regulating kinase 1(ASK1), p21-activated protein kinase (PAK)1/2, mixed lineage kinases (MLKs), NF-κB-inducing kinase (NIK) and tumour progression locus 2 (TPL-2 / COT).
The MEKK family consists of four kinases with conserved catalytic domains and variable N-terminal regions; MEKK1-4 (Hagemann and Blank, 2001). MEKK1 is usually considered the MKKK primarily responsible for the activation of ERK. However, in embryonic stem cells MEKK1 has been reported to be responsible for IL-1- and TNF-induced JNK activation (Xia et al., 2000) in cells expressing a MEKK1-β-galactosidase fusion protein with the entirety of the kinase domain missing. There could however, be the potential that the rest of the MEKK1 protein was sufficient to interfere with the function of a potential other MKKKs. MEKK1 can bind to MKK4. MEKK1 is thought to link to signalling through TRAF6 through the evolutionarily conserved signalling intermediate in Toll pathways (ECSIT) (Kopp et al., 1999). Interaction of ECSIT with TRAF6 and MEKK1 leads to the cleavage of MEKK1 and the release of active MEKK1, which can then lead to the downstream activation of activator protein 1 (AP-1) and NF-κB (Kopp et al., 1999). Cheng et al., suggest that MEKK2 is responsible for the phosphorylation and activation of JNK through MKK7 (Cheng et al., 2000). This was examined by co-transfecting the kinases into COS-1 cells. MEKK3 has been reported to have a critical role in TNF signalling to NF-κB (Yang et al., 2001). MEKK3 (Map3K3 gene) knockout mice are embryonic lethal at E10.5 - 11.0 with disrupted blood vessel development and defects of the structural integrity of the yolk sac (Yang et al., 2000). Yang et al., conclude that MEKK3 must be intrinsically important in endothelial cells because there is a defect in embryonic not maternal blood vessel development in the placentas of the MEKK3−/− embryos. An interaction was found between TAK1 and MEKK3 by tandem affinity purification (Di et al., 2008). The MEKK3-TAK1 complex consists of non-phosphorylated forms of the two proteins. TAK1 binding to MEKK3 interfered with the phosphorylation of MEKK3. In the presence of TAB1, TAK1 was able to autophosphorylate and this reversed the inhibitory effects upon MEKK3 phosphorylation in human 293 cells and TAK1−/− MEFs (Di et al., 2008). In contrast to MEKK1, 2 and 3, MEKK4 has been shown to activate only the JNK pathway (Gerwins et al., 1997).

ASK1 has been implicated in TNF activation of JNK (Nishitoh et al., 1998). Overexpression of TRAF2, 5 or 6 was found to activate HA-tagged ASK1. A truncated derivative of TRAF2 shown to inhibit TNF-induced JNK activation also inhibits ASK1 activation in 293 cells. ASK1 was found to bind to TAK1 and interfere with the TRAF6-TAK1 interaction, hence inhibiting IL-1-, TRAF6- or TAK1-induced NF-κB activity but not NIK-induced activity (Mochida et al., 2000).

PAK1 / PAK2 are MKKKs that can phosphorylate and activate MKK3 / MKK6 and hence p38 MAPK in response to stimuli going through small GTPases of the Rho family such as Rac and CDC42. RNA interference (RNAi) to deplete cells of PAK1 / PAK2 has been found to block the
activation of p38 MAPK and HeLa cell migration in response to stromal cell derived factor 1 (CXCL12) (Rousseau et al., 2006). They found that p38α MAPK activation mediated cell migration in response to the chemo-attractants; platelet-derived growth factor (PDGF-BB), hepatocyte growth factor (HGF), CXCL12 and complement factor 5a (C5a). Depletion of HSP27 by RNAi blocked C5a induced cell migration and thus they infer that the p38 MAPK mediated cell migration was mediated by HSP27. CXCL12- and C5a-induced cell migration is critical for many processes including inflammation.

A further family of MKKKs that have been implicated in the activation of MAPKs is the MLKs. There are currently seven known mammalian members of this family, of which probably MLK3 is the most characterised. All of the MLKs have been shown to activate JNK (Gallo and Johnson, 2002). The importance of the various MLKs for the activation of JNK and the other MAPK cascades in response to different stimuli and cell types has not been fully elucidated. However, MLK3 has been shown to activate NF-κB in response to T-cell co-stimulation but not TNF or IL-1 (Hehner et al., 2000). There are no reports of MLKs as key components in IL-1 signalling.

Cells that express a kinase deficient mutant of NIK have been reported to fail to activate NF-κB-mediated transcription in response to IL-1, TNF, TRADD, Rip or MORT1/FADD (Malinin et al., 1997). It has been proposed that the activation of NF-κB by IL-1 stimulation may be partially dependent upon hydrogen peroxide-induced activation of NIK leading to the phosphorylation and activation of IκB kinase (IKK)α (Li and Engelhardt, 2006). However, NIK is thought to only activate NF-κB through the IKK complex and not lead to the activation of the MAPK, JNK (Song et al., 1997).

TPL-2 / Cot−/− MEFs exhibit defective ERK, JNK and NF-κB signalling in response to stimulation with TNF (Das et al., 2005). However, TPL2−/− MEFs only show defects in ERK activation in response to IL-1, indicating that it is not important in the activation of other MAPKs or NF-κB in response to IL-1 stimulation (Das et al., 2005).

1.8 MKKs

MKKs are phosphorylated and activated by the MKKKs mentioned above. MKKs are dual-specificity protein kinases, which can phosphorylate MAPKs at their Thr-Xxx-Tyr motif in the activation loop. MAPK ERK kinase (MEK)1 / 2 has been shown to activate ERK 1 / 2, MEK5
to activate ERK5, MKK4 / MKK7 to activate JNK1 / JNK2 and MKK3 / MKK6 to activate p38 MAPK (Chang and Karin, 2001).

### 1.8.1 MKK3 and MKK6

Two MKKs are believed to be the direct activators of the p38 MAPK isoforms; MKK3 and MKK6. However, although these kinases have been known for some time it is still controversial which MKK or both is principally responsible for this activity in vivo. It may be that this depends upon the cell type and stimuli used. MKK3 has been reported to selectively activate p38α MAPK and p38γ MAPK, whereas MKK6 is a common activator of p38α, p38β2 and p38γ MAPK isoforms in COS-7 cells (Ensen et al., 1998). In response to osmotic shock MKK6 has been reported to be the most important activator of p38 MAPK (Moriguchi et al., 1996b). MKK4 has also been demonstrated to possess the ability to phosphorylate p38 MAPK in vitro (Derijard et al., 1995), however it is widely thought to be more important as an activator of JNK (Brancho et al., 2003).

MKK3−/− and MKK6−/− mice are viable and exhibit no obvious morphological defects (Tanaka et al., 2002; Wysk et al., 1999). However, MKK3−/− MKK6−/− compound knockout mice die during mid-gestation at embryonic day 11.0-11.5 (Brancho et al., 2003). These mice have delayed development, severe anoxia and resemble the p38 MAPK−/− mice (Adams et al., 2000). This suggests that MKK3 and MKK6 selectively activate p38 MAPK and serve redundant, essential roles for survival. p38 MAPK signalling in response to UV radiation, osmotic shock and IL-1 is not affected (although osmotic shock caused by sorbitol appeared to increase p38 MAPK activity) in the MKK3−/− MEFs. In contrast, TNF-induced activation of p38 MAPK was substantially reduced in the MKK3−/− MEFs (Wysk et al., 1999) and completely abolished in MKK3−/− MKK6−/− MEFs (Brancho et al., 2003). JNK activation was unaffected in MKK3−/− mice compared to wild type mice in response to any of the stimuli. Work in MKK3−/− mice has shown that LPS-induced IL-1 and IL-6 production was unaffected however, TNF-induced IL-1 and IL-6 production was ablated (Inoue et al., 2006). This has lead to the speculation that MKK3 could prove to be a more selective drug target than p38 MAPK in disease models such as RA (Thalhamer et al., 2008).

### 1.8.2 MKK4 and MKK7

JNK is phosphorylated and activated synergistically by MKK4 and MKK7. MKK4 preferentially phosphorylates the Tyr of the Thr-Pro-Tyr motif and MKK7 preferentially phosphorylates the Thr (Lawler et al., 1998). MKK7 is more distantly related to the highly
homologous MKK4 (48 % sequence identity), MKK3 (39 % sequence identity) and MKK6 (41 % sequence identity) (Lawler et al., 1997). The gene encoding murine MKK7 has 14 exons and six MKK7 isoforms can be produced by alternative splicing (Tournier et al., 1999). MKK4 and MKK7 are believed to be distributed in both the cytoplasm and the nucleus and this localisation is not altered by stimulation of cells with IL-1α or UV-C radiation (Tournier et al., 1999).

1.9 Mitogen activated protein kinases (MAPKs)

There are three parallel MAPK pathways in the cell (Fig. 1.2), which mediate the changes in gene expression in response to extracellular signals. The classical MAPK, ERK, was the first MAPK to be discovered and is activated in response to mitogens. p38 MAPK and JNK, known as stress-activated protein kinases (SAPKs), are activated in response to pro-inflammatory or stress stimuli (Clerk et al., 1998; Johnson and Lapadat, 2002). The three MAPKs are activated by dual phosphorylation on Thr and Tyr within the motif Thr-Xaa-Tyr (Derijard et al., 1995). For ERK the central amino acid of the motif is glutamate, for p38 MAPK family it is glycine and for the JNK family it is proline.

1.9.1 p38 MAPK

The p38 MAPK pathway is important in several cellular processes including cell growth, differentiation, apoptosis and immune cell activation. p38 MAPK was discovered by several different laboratories independently at a similar time. p38 MAPK was identified as the IL-1-induced protein kinase responsible for the phosphorylation of p50 upon threonine and some serine residues. p50 was found to be MK2. MK2 in turn phosphorylated HSP27 upon serine (Freshney et al., 1994). p38 MAPK was also identified as the kinase tyrosine phosphorylated in response to LPS stimulation (Han et al., 1994). In another study p38 MAPK was the protein responsible for the kinase activity that phosphorylated MK2 in response to sodium arsenite, heat shock and osmotic stress (Rouse et al., 1994). p38 MAPK was also identified as a target for a class of pyridinyl-imidazole compounds, known as cytokine-suppressive anti-inflammatory drugs (CSAIDs). These CSAIDs inhibited the expression of IL-1 and TNF in LPS-stimulated monocytes (Lee et al., 1994).

Four isoforms of p38 MAPK have been identified; p38α, p38β, p38γ and p38δ, which share 60-70 % sequence identity. Only p38α MAPK and p38β MAPK are inhibited by the pyridinyl-imidazole drugs, which bind in the ATP-binding pocket. p38α MAPK is thought to be ubiquitously expressed and abundant in placenta, brain (cerebellum) and lymphoid tissues.
Figure 1.2: Overview of IL-1 and TNF signalling downstream of the MKKK, TAK1. TAK1 can activate three main pathways downstream of IL-1 and TNF; JNK, p38 MAPK and NF-κB. This leads to the up-regulation of pro-inflammatory mediators through regulation of transcription, mRNA stability and translation.
p38β is most abundantly found in brain tissues, p38γ has restricted expression and is found principally in skeletal muscle and p38δ MAPK is found in the salivary gland, pituitary gland and adrenal gland (Wang et al., 1997). There appears to be some differences in the role, importance and substrates of the different p38 MAPK isoforms. Knockout of p38α MAPK is embryonic lethal in midgestation due to a placental defect (Adams et al., 2000). Studies in p38α MAPK−/− mice highlight the importance of p38α MAPK as a kinase for MK2 and as a key mediator of IL-1 signal transduction but not apoptosis (Allen et al., 2000). A conditional knockout of p38α MAPK in macrophages found a significant inhibition of LPS-induced TNF, IL-12 and IL-18 (Kang et al., 2008). However, surprisingly the p38α MAPK conditional knockout macrophages had no effect on the LPS-induced production of IL-6 (Kang et al., 2008), previously shown to be dependent on p38 MAPK through inhibitor studies (Chae et al., 2001), which may be cell-type specific. The p38β MAPK−/− mice are viable with no apparent health problems (Beardmore et al., 2005). The activation of p38 MAPK substrates MK2 and MSK1 was normal in p38β MAPK−/− mice, as was the serum level of LPS-induced inflammatory cytokines (Beardmore et al., 2005). The findings from the knockout mice indicate that p38α MAPK, not p38β MAPK is the major isoform in the immune response.

p38 MAPK is activated by a variety of stimuli including pro-inflammatory cytokines such as IL-1 and TNF and environmental stresses such as UV light, osmotic stress and heat shock (Freshney et al., 1994; Han et al., 1994; Rouse et al., 1994). The activation of p38 MAPK depends upon the cell type as well as the stimulus. p38 MAPK is thought to be nuclear. Its activators MKK3 and MKK6 are believed to be present in both the cytoplasm and the nucleus. p38 MAPK itself does not contain a nuclear localisation sequence or nuclear export sequence. MK2 has a nuclear localisation sequence which is required for activation in response to a wide range of stimuli. Phosphorylation of MK2 by p38 MAPK unveils a nuclear export signal in its C-terminus (Engel et al., 1998) that triggers the export of MK2 in a complex with p38 MAPK into the cytoplasm (Ben-Levy et al., 1998). The export of the complex requires p38 MAPK phosphorylation but not MK2 activity. These experiments were performed using indirect immunofluorescence in 293T cells. The p38 MAPK substrate, MK5 (PRAK), has also been shown to act as a chaperone for p38α MAPK into the nucleus (Li et al., 2008; New et al., 2003) in the murine embryonic fibroblast cell line, NIH 3T3 cells. TAB1, which can lead to p38 MAPK autophosphorylation and activation in the absence of TAK1, has also been found to bind to p38 MAPK and exclude it from the nucleus (Lu et al., 2006).

p38 MAPKs have a variety of substrates, some of which have been mentioned above. These include the kinases, MAPK-activated protein kinase (MK)2 (Stokoe et al., 1992a), MK3
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(Sithanandam et al., 1996), MK5 (New et al., 1998), MAPK-interacting kinase (MNK)1, MNK2 (Waskiewicz et al., 1997), mitogen- and stress-activated kinase (MSK)1 and MSK2 (Deak et al., 1998). A brief summary of these kinases is included in this section and the importance of p38 MAPK activation in inflammation through the activation of this substrate is discussed later.

The generation of MK2−/− MEFs uncovered a role of MK2 in the stabilisation of p38 MAPK through its C-terminus in a manner independent of the activity of MK2 (Kotlyarov et al., 2002). MK2 is also the kinase responsible for the phosphorylation of HSP27 and a protein termed tristetraprolin (TTP). MK3 has some redundancy with MK2 (McLaughlin et al., 1996). MK2−/− mice showed an impaired inflammatory response. MK2−/− mice challenged with Listeria monocytogenes exhibited an increase in bacterial load associated with an attenuation in TNF production (Lehner et al., 2002). Spleen cells from MK2−/− mice had interferon (IFN)-γ synthesis after stimulation with Listeria monocytogenes (Lehner et al., 2002). The half-life of IL-6 is reduced more than 10-fold in MK2-deficient macrophages (Neininger et al., 2002). MK3−/− mice had no defect in the production of inflammatory cytokines or IL-6 stability (Ronkina et al., 2007). In wild type cells MK3 was expressed at much lower levels and displayed reduced kinase activity compared to MK2 (Ronkina et al., 2007). MK2 / MK3 double knockout mice are viable however, they do exhibit a further decrease in TNF production and the expression of p38 MAPK and TTP compared to the MK2−/− mice. MK5 had previously been identified as a kinase that could phosphorylate HSP27 (New et al., 1998), however, this has proven not to be reproducible (Shi et al., 2003). The MK5−/− mice were viable, fertile with no abnormalities in tissue morphology or behaviour (Shi et al., 2003). Spleen cells from MK5−/− show no defect in LPS-induced synthesis of TNF, IL-6 or IFN-γ, in contrast to the MK2−/− mouse cells. This indicates that MK2 is the more important kinase for this function.

MNK1 and MNK2 are essential for the phosphorylation of eukaryotic translation initiation factor 4E (eIF4E) in embryonic fibroblasts and adult tissues (Ueda et al., 2004). However, the role of this phosphorylation is unclear. MNK1+/− and MNK2+/− mice are viable, fertile and show no defect in translation (Ueda et al., 2004). The phosphorylation of heterogeneous nuclear ribonucleoprotein A1 (hnRNPA1) by MNKs has been proposed to have a role in TNF translation (Buxade et al., 2005).

MSK1 and MSK2 have been shown to have important roles in transcription, discussed later. Studies in MSK1−/− and MSK2−/− mice show that these kinases are required for the phosphorylation of the transcription factors Ca2+/cyclic adenosine monophosphate response element binding protein (CREB) and activating transcription factor (ATF)-1 (Wiggin et al.,
2002) and the phosphorylation of histone H3 and high mobility-group protein HMG-14 (Soloaga et al., 2003). The CREB phosphorylation was shown to be required for the full induction of c-Fos and junB in response to UV-C radiation and anisomycin. MSK1 has also been proposed to phosphorylate the p65 subunit of NF-κB at Ser 276, leading to the selective activation of NF-κB-dependent gene expression (Vermeulen et al., 2003).

1.9.2 JNK

The JNKs are SAPKs activated in response to pro-inflammatory stimuli such as IL-1, TNF and LPS and environmental stresses, for example UV radiation. JNK is expressed from three genes; JNK1, JNK2 and JNK3. JNK1 and JNK2 are expressed ubiquitously, however, JNK3 has limited tissue expression that is largely confined to brain, testis and heart. Alternative splicing of these genes produces 10 or more JNK isoforms (Gupta et al., 1996). JNK1, 2 and 3 are 85% identical within their core catalytic domains (Pearson et al., 2001). The three genes have been deleted from mice by homologous recombination and the JNK-deficient mice are viable but have defects in apoptosis and immune responses. Less is known about the potential role of JNK in inflammation because of the lack of specific inhibitors, in contrast to p38 MAPK. However, JNK activates transcription factors that are involved in the inflammatory response, for example Elk1 and potential components of AP-1 (c-Jun, JunB, JunD and ATF-2) (Davis, 2000), as discussed below.

1.9.3 ERK

Extracellular signal-regulated kinase (ERK) was the first MAPK identified and is known as the classical MAPK. There are many isoforms of ERK, however, only ERK1, ERK2 and ERK5 (big MAPK 1) are conventional MAPK that are activated by MKKs. The most potent stimuli of ERK are mitogens and in response to these ERK is activated through the Ras-Raf-MEKK-MEK pathway. Originally ERK1 and ERK2 were believed to be most important in cell proliferation, however, more recently ERK1 and ERK2 signalling has also been implicated in inflammatory and immune responses (Dumitru et al., 2000; Eliopoulos et al., 2002). ERK may be phosphorylated and activated by inflammatory stimuli such as IL-1 in many cell types. However, the mechanism of IL-1-induced activation of ERK is likely to not involve TAK1 and is therefore different to that of p38 MAPK and JNK.

The ERK kinase MEK may be activated by the MKKK, TPL-2 (Beinke et al., 2004). TPL-2 is found in a complex with p105, an inhibitor of NF-κB, in resting cells (macrophages) (Beinke et
al., 2003; Belich et al., 1999). In this complex TPL-2 is stabilised but prevented from phosphorylating and activating MEK. However, upon LPS stimulation p105 is phosphorylated at two serine residues, polyubiquitinated and digested by the proteasome (Beinke et al., 2004). This also leads to the release of TPL-2, which can then phosphorylate and activate MEK, which in turn can phosphorylate and activate ERK (Beinke et al., 2004). LPS-induced TNFα and COX-2 production was substantially reduced in TPL-2-deficient macrophages due to a lack of ERK1/2 activation (Dumitru et al., 2000; Eliopoulos et al., 2002). In the case of TNF this is due to defective transport of TNF mRNA from the nucleus to the cytoplasm (Dumitru et al., 2000). In contrast, for COX-2 the inhibition is due to a reduction in CREB phosphorylation (Eliopoulos et al., 2002).

ERK1 and ERK2 have been reported to phosphorylate and activate certain substrates shared with p38 MAPK including MNK1, MNK2 (Waskiewicz et al., 1997), MSK1 (Deak et al., 1998; Eliopoulos et al., 2002), as well as other substrates such as p90 ribosomal S6 kinase isozymes (Smith et al., 1999). Therefore ERK1 and ERK2 may have a similar function to p38 MAPK in the regulation of the transcription, translation and stability of pro-inflammatory mediators. As ERK is less strongly activated by pro-inflammatory cytokines, such as IL-1, than p38 MAPK, it is believed not to be as important as p38 MAPK in these systems. ERK has been shown to phosphorylate and activate MK2 in vitro (Coxon et al., 2003). However, the action of pyridinyl imidazole compounds such as SB203580 at concentrations specific for the inhibition of p38 MAPKα/β isoforms showed that activation of MK2 is mainly dependent on p38 MAPK (Clifton et al., 1996).

ERK5 was initially reported to be the member of the ERK family activated by stress stimuli including oxidative stress and hyperosmolarity (Abe et al., 1996). ERK5 can also phosphorylate some of the same substrates as ERK1 and ERK2 including Sap1a, c-Myc and ribosomal S6 kinase (Rsk), although the latter is controversial (Nishimoto and Nishida, 2006). ERK5 can also induce intermediate-early genes such as c-Jun and c-Fos as well (Kato et al., 1997). However, there has been no documented evidence of a role of ERK5 in IL-1 signalling.

1.10 NF-κB

The NF-κB family of transcription factors are also activated downstream of a wide variety of pro-inflammatory stimuli including IL-1 that also activate p38 MAPK and JNK (Fig. 1.2). In resting cells NF-κB is held in the cytoplasm by a family of inhibitors of NF-κB termed IκBs, of which the most important is IκBα. The activation of NF-κB downstream of IL-1 is through the
canonical pathway, which involves an IKK complex consisting of IKKα, IKKβ and IKKγ (NEMO) (Li et al., 1999). TAK1 can directly phosphorylate the activation loop serine residues on IKKβ and activate the IKK complex (Wang et al., 2001). The canonical pathway involves the IKKβ and NEMO-dependent degradation of the IκB proteins. This results in the release and translocation to the nucleus of mostly RelA containing-heterodimeric NF-κB (Scheidereit, 2006).

NF-κB can also be activated via the non-canonical pathway. This pathway has slower kinetics, takes place over several hours rather than minutes, usually results in long-lasting NF-κB activation and is sensitive to ribosomal inhibition (Scheidereit, 2006). The non-canonical pathway is activated by a limited number of stimuli including 12 members of the TRAF-binding receptors of the TNF superfamily including CD40 and receptor activator of NF-κB (RANK) (Hauer et al., 2005) and LPS (Mordmuller et al., 2003). In the non-canonical pathway NIK phosphorylates IKKα (Xiao et al., 2001). Active IKKα phosphorylates p100 and promotes C-terminal processing to produce p52. The NF-κB dimers generated by this pathway are p52-containing complexes, usually p52/RelB (Scheidereit, 2006). The inducers of the non-canonical pathway also stimulate the canonical pathway and the two pathways are linked because the canonical pathway is required to drive the production of p100 and RelB (Scheidereit, 2006).

Part 3: Inflammatory gene regulation by the MAPKs and NF-κB

1.11 Transcriptional regulation

The expression of pro-inflammatory mediators usually involves a burst in transcription in response to an inflammatory stimulus. The promoters of the inflammatory genes contain indispensable cis-elements for several trans-activating factors. The most common sites are for NF-κB, AP-1, CAAT/enhancer-binding proteins (C/EBP) and Ets.

A wide range of pro-inflammatory mediators require NF-κB to bind to sites in their promoters for transcription to be initiated, for example TNF and COX-2 (Smith et al., 2000). Transfection experiments with reporter plasmids found that mutations in the cis-acting regulatory sequences attenuate the transcription of COX-2 after stimulation of cells with TNF (Yamamoto et al., 1995). NF-κB is important in the transcription of adhesion molecules on leucocytes and endothelial cells, which allows the extravasation of leucocytes from the circulation to the site of infection (Eck et al., 1993). RelA-deficient mice have a severe defect in the recruitment of leucocytes to the site of inflammation (Alcamo et al., 2001). NF-κB is also required for the
expression of matrix metalloproteinases (MMPs), which are important mediators of local inflammation and leucocytes chemotaxis (Hayden et al., 2006). p38 MAPK and ERK may control the transactivation of NF-κB at the transcriptional level (Vanden Berghe et al., 1998).

The AP-1 family of transcription factors consist of homo- and hetero-dimers that may contain c-Jun, JunD, JunB, ATF-2, c-Fos, Fra-1 and Fra-2. ATF-2 possesses histone acetyltransferase activity that is controlled by phosphorylation. Phosphorylation enhanced its histone acetyltransferase activity and cyclic AMP response element (CRE)-dependent transcription (Kawasaki et al., 2000). Expression of c-Fos and c-Jun may be p38 MAPK-dependent. Fra-1, Fra-2 and JunB are ERK-dependent. JNK can phosphorylate ATF-2 and c-Jun and hence enhance their trans-activation activity and stability (Han et al., 2001; Kallunki et al., 1994). Treatment of cells with the JNK inhibitor SP600125 blocked the induction of c-Jun transcription in synoviocytes and completely suppressed AP-1 binding and collagenase mRNA accumulation in fibroblast-like synoviocytes (Han et al., 2001). However, subsequent work has found that SP600125 is not a specific inhibitor of JNK, which casts the previous results into doubt (Bain et al., 2003). The accumulation of collagenase mRNA can lead to joint destruction in RA. It is thought that pathways involving the phosphorylation and trans-activation of c-Fos and Fra-1/2 may involve ERK. p38 MAPK has also been recently reported to cause c-Jun, FosB, c-Fos and JunB phosphorylation downstream of protein kinase C in T-lymphocytes (Humar et al., 2007).

C/EBPα-ζ are leucine zipper transcription factors that contribute significantly to the expression of acute-phase proteins, chemokines and COX-2 (Kracht and Saklatvala, 2002). The p38 MAPK inhibitor, SB203580 inhibits the transactivation of C/EBP (Baldassare et al., 1999). CREB-homologous protein (CHOP) is a member of the C/EBP family that has been shown to be a substrate of p38 MAPK (Wang and Ron, 1996). p38α MAPK conditional knockout macrophages also had significantly inhibited C/EBP-β activation (Kang et al., 2008). Ets-domain transcription factors bind to the Ets DNA-binding domain and comprise a large family of proteins. Elk-1 and Ets-1 have been shown to bind and promote transcription of the TNFα gene (Tsai et al., 2000). Elk-1 and SAP1 form ternary complexes with serum-response factor (Whitmarsh et al., 1995) and bind to the serum-response element (SRE) on c-Fos (Ray et al., 1989) and JunB (Perez-Albuerne et al., 1993). There is also a serum-response element in the promoter of IL-6. ERK, JNK and p38 MAPK have been shown to activate Elk1 whereas ERK and p38 MAPK but not JNK activate SAP1 (Whitmarsh et al., 1997).
1.12 mRNA stabilisation by p38 MAPK

Many proteins involved in the inflammatory response are encoded by short-lived mRNAs. Activation of the p38 MAPK pathway by pro-inflammatory stimuli such as IL-1 or TNFα stabilises the short-lived inflammatory mRNAs. Stabilisation of these mRNAs increases their abundance and sustains their expression, leading to increased expression of inflammatory mediator proteins. SB203580, a specific inhibitor of p38 MAPK, when applied to human fibroblasts or umbilical vein endothelial cells inhibited IL-1 induced COX-2 protein and mRNA levels (Dean et al., 1999; Ridley et al., 1997). This was later reported to be due to an effect on COX-2 mRNA stability, rather than on transcription (Dean et al., 1999; Ridley et al., 1998). In human monocytes SB203580 also inhibited LPS-induced COX-2 expression (Dean et al., 1999). In this case the inhibition observed was found to be due to reduced COX-2 mRNA stability and transcription (Dean et al., 1999). p38 MAPK has been found to increase the stability of a large number of inflammatory mRNAs including TNFα (Brook et al., 2000; Rutault et al., 2001; Wang et al., 1999), IL-1β and Gro-α (Sirenko et al., 1997) and IL-6 (Miyazawa et al., 1998). However, recently the conditional knockout of p38α MAPK found no effect on TNF mRNA stability after LPS stimulation (Kang et al., 2008). This is likely to be a result of the requirement of p38 MAPK for the induction of the mRNA destabilising protein, tristetraprolin (TTP) (Mahtani et al., 2001). MK2 has been shown to be involved in p38 MAPK-mediated mRNA stabilisation (Lasa et al., 2000; Winzen et al., 1999).

Many mRNAs encoding proteins involved in the inflammatory response including TNF, IL-1, IL-6, IL-8, granulocyte macrophage colony stimulating factor (GM-CSF), c-Fos and COX-2 contain AU-rich elements (AREs) in their 3´ untranslated regions (UTRs) (Caput et al., 1986). The AREs from GM-CSF and c-Fos were inserted into the 3´ UTR of rabbit β-globin gene and this led to the otherwise stable β-globin mRNA becoming highly unstable (Kabnick and Housman, 1988; Shaw and Kamen, 1986). The p38 MAPK pathway has been shown to stabilise mRNAs otherwise labile due to the presence of AREs (Dean et al., 2004; Winzen et al., 1999), for example COX-2 (Dean et al., 2003; Lasa et al., 2000). AREs have been shown to target mRNAs for rapid deadenylation (Shyu et al., 1991; Wilson and Treisman, 1988; Xu et al., 1997), however AREs have also been implicated to cause 5´ decapping (Gao et al., 2001) and promote 3´-5´exonuclease decay (Chen et al., 2001; Mukherjee et al., 2002). p38 MAPK has been shown to regulate the deadenylation of reporter mRNAs containing either the TNF or COX-2 AREs (Dean et al., 2003).

It is likely that p38 MAPK–dependent mRNA stabilisation involves one or more ARE binding proteins (AREBPs). Many potential AREBPs have been identified, including TTP (Carballo et
al., 1998; Dean et al., 2004). TTP is the AREBP for which there is the strongest evidence of a role in inflammatory regulation of mRNA stability and it has also been linked to the p38 MAPK pathway (Fig. 1.2). TTP binds AREs including the ARE of TNF (Carballo et al., 1998). TTP+/− mice display an inflammatory phenotype including arthritis because of elevated protein levels of TNF. TTP promotes *in vitro* deadenylation of RNA containing the TNF ARE, but not RNA containing a mutated version (Lai et al., 2003). Phosphorylation of TTP by MK2 may prevent TTP from targeting the ARE-containing mRNA to the decay machinery through the sequestration of TTP by 14-3-3 proteins (Stoecklin et al., 2004). This is supported by work with the MK2/TTP double knockout mice and TTP mutants (Hitti et al., 2006).

1.13 Translational regulation

Early work indicated that p38 MAPK had an important role in the translation of inflammatory cytokines. The inhibition of p38 MAPK with pyridinyl imidazole compounds caused a reduction in TNF and IL-1 protein, with little effect on the mRNA levels (Young et al., 1993). TNF mRNA was shown to be associated with single ribosomes rather than polysomes with ribosomal density-gradient profiling of cytoplasmic extracts from cells treated with LPS and p38 MAPK inhibitor (Prichett et al., 1995). More recent work however, found that when a different inhibitor, SB203580, was used p38 MAPK inhibition caused a commensurate decrease in TNF protein and mRNA levels in LPS-treated monocytes (Dean et al., 1999). This suggested that in human monocytes p38 MAPK mainly regulates TNF mRNA levels. However, there is some controversy because there is evidence that murine TNF expressed by macrophages is regulated mainly at the level of translation (Kotlyarov et al., 1999). There has also been some evidence that ERK and p38 MAPK have a role in the translation of pro-inflammatory mediators, such as TNFα, through the activation of Mnk1 (Buxade et al., 2005).

**Part 4: Regulation of inflammatory signalling**

IL-1 is a potent pro-inflammatory stimulus and as mentioned previously it is important that this pathway is tightly regulated to ensure that the response does not lead to chronic inflammatory conditions. The regulation of the IL-1 signalling pathway occurs at many levels, to ensure that both the initial stimulus and the downstream signalling and gene expression are switched off when appropriate.
1.14 Deubiquitinating enzymes

Lysine 63-linked polyubiquitination is increasingly recognised as an important mechanism for the propagation of the signal from the IL-1, TLRs and TNF receptors. Ubiquitination, like phosphorylation is a reversible process and can be removed through the use of deubiquitinating enzymes (DUBs). Three DUBs, which have so far been implicated in the regulation of IL-1 and TNF signalling include cylindromatosis (CYLD), A20 and Cezanne (Sun, 2008). CYLD has been shown to interact with and deubiquitinate Lys63-linked chains on TRAF6 (Kovalenko et al., 2003; Yoshida et al., 2005), Rip1 (Wright et al., 2007) and NEMO (Kovalenko et al., 2003). A20 has been reported to deubiquitinate TRAF6 (Boone et al., 2004), Rip1 (Wertz et al., 2004) and NEMO (Mauro et al., 2006). A20 is unique because it also possesses E3 ubiquitin ligase activity and is thought to remove Lys63-linked chains and add Lys48-linked polyubiquitin chains to Rip1 at the same time, causing Rip1 to be targeted to the proteasome and degraded (Wertz et al., 2004). A20<sup>−/−</sup> mice have persistent activation of NF-κB by TNF causing multiorgan inflammation, cachexia and neonatal lethality (Lee et al., 2000). Cezanne is a homologue of A20 and has been reported to deubiquitinate Rip1 leading to the negative regulation of the IKK complex (Enesa et al., 2008).

1.15 Phosphatases

A key component of IL-1 signalling is the protein kinase cascades. The signal is propagated through the pathway through a series of phosphorylations, which lead to the activation of the substrate proteins. If the activation of proteins occurs through phosphorylation it would therefore follow that dephosphorylation of the pathways would lead to inactivation. Different phosphatases have been implicated in the dephosphorylation of various points in the signalling cascade. For example there have been many phosphatases reported to possess TAK1 dephosphorylation activity. Protein phosphatase (PP) 2A has also been implicated as a negative regulator of TAK1, through dephosphorylating TAK1 at the activation site (Thr183) in mesangial cells (Kim et al., 2008). PP6, a type 2A phosphatase, has reported to dephosphorylate TAK1 at Thr187 (Kajino et al., 2006). PP2C isoforms have also been implicated in TAK1 dephosphorylation (Li et al., 2003).

The importance of phosphorylation at both the tyrosine and threonine residues mean that dephosphorylation by tyrosine-specific phosphatases, serine/threonine-specific phosphatases or dual-specificity (threonine/tyrosine) protein phosphatases can inactivate the MAPKs (Keyse, 2000). PP2A has also been demonstrated to interact with and dephosphorylate p38 MAPK (Lee et al., 2003; Sundaresan and Farndale, 2002). The dual-specificity phosphatase (MAP kinase
phosphatases/ MKP) family are thought to be the primary phosphatases responsible for the dephosphorylation of the MAPKs. There are 10 mammalian MKPs, which exhibit different substrate specificity, subcellular localisation and regulation. MKP1 is the archetype (Wang and Liu, 2007). MKP1 preferentially dephosphorylates p38 MAPK and JNK (Franklin and Kraft, 1997).

1.16 Protein scaffolds

The regulation of signalling specificity is thought to occur is through the formation of specific signalling complexes, with the aid of scaffold proteins. Scaffold proteins can bind to more than one component of the pathway simultaneously, bringing the pathway components together. This also increases the signalling efficiency and speed in response to a signal. In the upstream signalling pathways polyubiquitination of TRAF6 or Rip1 is thought to act as a scaffold on which the downstream signalling complexes can form, as discussed above. Other scaffold proteins that have been postulated to have important roles in these signalling pathways include JNK-interacting protein (JIP)-1. JIP1 sequesters JNK in the cytosol and selectively enhances JNK activation mediated by MLK3 (Whitmarsh et al., 1998). JIP-1 forms complexes that contain MLK3, M KK7 and JNK. MEK Partner 1 (MP-1) is a scaffold protein that specifically binds MEK1 and ERK1. The Elk1 transcription factor was used to activate a reporter to measure ERK activation (Schaeffer et al., 1998). MP-1 did not interact with MEK1 previously phosphorylated by B-Raf, indicating that this complex, as well as that mediated by JIP-1, is dissociated upon phosphorylation and activation of the components. Several scaffold proteins have also been proposed for p38 MAPK including Sin1, which has been shown to bind to ATF-2 and p38 MAPK (Makino et al., 2006) and JIP4 by a mechanism involving MKK3 and MKK6 (Kelkar et al., 2005).

1.17 Protein stability

Protein turnover is the balance between the synthesis and degradation of a particular protein in the cell. Protein turnover is highly variable. Chaperones are often required to ensure that a newly synthesised protein is folded correctly. However, some proteins also require the presence of a chaperone to stabilise the protein and once removed from the chaperone these proteins are often rapidly degraded. Heat shock proteins are a highly conserved family of chaperones. Some family members are constitutively expressed in certain cell types but the expression of many is upregulated in response to stress, such as heat shock. One family member, HSP90 has been shown to act as a chaperone and regulate protein stability for many proteins involved in signal transduction cascades.
In the IL-1 signalling pathway HSP90 has been shown to be a chaperone for IRAK1, TAK1 and has been reported to be part of the IKK complex. The HSP90-Cdc37 chaperone module has been shown to be required for the maintenance of IRAK1 (De Nardo et al., 2005) in macrophages. Although HSP90 expression increases upon stressful stimulation of the cells (LPS) Cdc37 expression does not and this is believed to tip the balance leading to the degradation of IRAK1 and subsequent downstream signalling. HSP90 can regulate IL-1β-induced signalling through the interaction and maintenance of stability of TAK1 in HEK293 and HeLa cells (Shi et al., 2008) as demonstrated through the use of the HSP90 inhibitor, geldanamycin, RNAi and overexpression studies. However, HSP90 is not required for the activation of TAK1 and did not alter its activity in response to IL-1 or LPS (Liu et al., 2008). TAK1 cannot bind to TAB1 and HSP90 simultaneously and it is believed that when there is a pool of free TAB1 this displaces HSP90 and binds to TAK1 (Liu et al., 2008).

**Part 5: Heat shock protein 27 (HSP27)**

### 1.18 The small heat shock protein family

There are five families of heat shock proteins; the 110 kDa HSPs, 90 kDa HSPs, 70 kDa HSPs, 60 kDa HSPs and the small heat shock proteins (sometimes the 40 kDa HSPs are also classed as a family). HSP expression is induced by various stresses including heat shock (Landry et al., 1982; Landry et al., 1989), oxidative (Iwaki et al., 1993) and osmotic stress (Garmyn et al., 2001). There are ten members of the small heat shock protein (sHSP) family in the human genome; HSPB1-10 (Kappe et al., 2003). This is a ubiquitous family of molecular chaperones of typical size 16 – 25 kDa. Members of the sHSP family include HSP27 (HSPB1) αA-crystallin (HSPB4) and αB-crystallin (HSPB5) (Kappe et al., 2003). These proteins are characterised by a conserved C-terminal region, the α-crystallin domain, a variable N-terminal domain and occasionally a variable C-terminal tail. Members of the small heat shock family are widely spread throughout all kingdoms and their existence has been reported from bacteria to humans. The only notable absence of small heat shock protein family members is in some parasites and / or pathogens of humans and animals (Narberhaus, 2002).

### 1.19 HSP25, the murine orthologue of HSP27

The murine orthologue of HSP27, HSP25, is expressed in a wide variety of tissues (Gernold et al., 1993; Klemenz et al., 1993). HSP25 is phosphorylated on sites equivalent to the human Ser15 and Ser82 (Ser86) (Stokoe et al., 1992b). HSP25 has been analysed in MK2⁻/⁻ mice
(Kotlyarov et al., 1999; Vertii et al., 2006). In these mice there is no detectable phosphorylation of HSP25 at Ser86 and no detectable \textit{in vitro} binding to 14-3-3 protein (Vertii et al., 2006). This paper gives evidence that the phosphorylation of HSP25 plays a role in the disaggregation of the HSP25 complex. There was no detectable effect of MK2-dependent phosphorylation of HSP25 on actin reorganisation in MEFs.

1.20 Phosphorylation and oligomerisation states of HSP27

IL-1 and TNF have long been known to cause the phosphorylation of HSP27 (Kaur et al., 1989; Saktatvala et al., 1991), although both the function of the protein, and its phosphorylation are not well understood. The functions of HSP27 are likely to be influenced by its phosphorylation state. Human HSP27 is phosphorylated at three sites with the sequence Arg-Xxx-Xxx-Ser by MK2; serine 15 (Ser15), Ser78 and Ser82 (Landry et al., 1992; Stokoe et al., 1992b). MK3 (McLaughlin et al., 1996), the delta isoform of protein kinase C (PKC) (Maizels et al., 1998) and protein kinase D (Doppler et al., 2005) have all been shown to phosphorylate HSP27 on at least one of the sites phosphorylated by MK2. cGMP-dependent protein kinase has been shown to phosphorylate HSP27 on Thr143. HSP27 phosphorylated on Thr143 has been proposed to inhibit platelet aggregation, in contrast to HSP27 phosphorylated by MK2 (Butt et al., 2001).

All the members of the sHSP family form large homo-oligomers (Haslbeck et al., 2005). Non-phosphorylated HSP27 analysed by size-exclusion liquid chromatography forms oligomers of 530 kDa, equivalent to 24 subunits (Rogalla et al., 1999). Upon phosphorylation the 24-mer dissociates into tetramers (~110 kDa), dimers or monomers (Lelj-Garolla and Mauk, 2005). The oligomerisation state of HSP27 has been demonstrated as important in certain actions of HSP27. HSP27 can not only form large oligomers with itself but is also capable of the formation of large heterooligomers with other members of the sHSP family including αB-crystallin (Fu and Liang, 2002) and HSP22 (Benndorf et al., 2001).

The importance of the phosphorylation state of HSP27 upon the oligomerisation and function of HSP27 suggests that the regulation of HSP27 through dephosphorylation is also of relevance. The only reports on HSP27 dephosphorylation suggest that PP2A is the principle HSP27 phosphatase (Cairns et al., 1994; Tar et al., 2006). PP1 has been reported to dephosphorylate HSP27 \textit{in vitro}, but only PP2A is required \textit{in vivo} (Cairns et al., 1994).
1.21 Cellular Functions of HSP27

1.21.1 Cytoprotection

HSP27 can protect cells against heat shock (Landry et al., 1989) and apoptotic agents. Many mechanisms have been suggested to account for the cytoprotective action of HSP27, however there is no clear consensus. For example HSP27 overexpression diminishes the reactive oxygen species (ROS) burst induced by TNF and increases the glutathione content of L929 cells and this has been suggested as the anti-apoptotic mechanism (Mehlen et al., 1996a). HSP27 also protects against Fas / APO-1 mediated apoptosis in L929 cells and against the PKC inhibitor, staurosporine (Mehlen et al., 1996b). Phosphorylated HSP27 dimers bind to Daxx, a mediator of Fas-induced apoptosis. This prevents Daxx from interacting with ASK1 and Fas and prevents Daxx-mediated apoptosis (Charette et al., 2000). HSP27 inhibits F-actin fragmentation caused by hydrogen peroxide and menadione (Huot et al., 1996). HSP27 is protective against various cytotoxic drugs. For example, in colorectal cancer cells HSP27 protects against apoptosis mediated by doxorubicin and cisplatin (Garrido et al., 1997). As well as reducing ROS in cells, HSP27 confers resistance to staurosporine, etoposide and Fas by inhibiting cytochrome c release from the mitochondria (Paul et al., 2002). The protection against F-actin fragmentation has been postulated to be partially responsible for the inhibition of cytochrome c release from the mitochondria and to interfere with the Bid intracellular redistribution involved in apoptosis (Paul et al., 2002).

1.21.2 Chaperone function of HSP27

HSP27, like most other heat shock proteins, possesses chaperone activity. It is thought that each small HSP complex can bind to several non-native polypeptide chains. The interactions between the chaperone and unfolded protein are thought to be stable at physiological temperatures. It is believed that other chaperones may help facilitate substrate release. HSP oligomers are dynamic and the constant recycling of subunits may expose substrate binding sites (Haslbeck et al., 2005). The chaperone activity of HSP27 is thought to be ATP independent, which is unusual. Few substrates for HSP27 chaperone activity have been identified. A Chinese hamster cell line overexpressing HSP27 was found to form heat-induced nuclear protein aggregates, however these cells exhibited an enhanced rate of recovery from nuclear protein aggregation as thermotolerant cells (Kampinga et al., 1994). At stoichiometric amounts both murine HSP25 and human HSP27 interact with and prevent the heat-induced aggregation of two model proteins; dimeric citrate synthase from pig heart and α-glucosidase from yeast (Jakob et al., 1993). HSP25 and HSP27 also promoted functional re-folding of these proteins after urea denaturation.
Chapter 1

Introduction

(Jakob et al., 1993). A change in temperature from 10 to 40 °C augmented the size of HSP27 homo-oligomers and increased the chaperone activity of HSP27 as shown by the inhibition of dithiothreitol-induced insulin aggregation (Lelj-Garolla and Mauk, 2006). The ability of HSP27 to act as a chaperone to citrate synthase was decreased upon the phosphorylation of HSP27 and reduction in oligomeric size (Rogalla et al., 1999). Therefore the chaperone activity of HSP27 is thought to depend on its phosphorylation and oligomeric state.

1.21.3 Cytoskeletal dynamics

HSP27 has also been shown to be involved in migration of endothelial cells (Piotrowicz et al., 1998), smooth muscle cells (Hedges et al., 1999) and in pinocytosis by fibroblasts (Lavoie et al., 1993b). KRIBB3 (5-(5-ethyl-2-hydroxy-4-methoxyphenyl)-4-(4-methoxyphenyl) isoxazole) (Shin et al., 2005), has been suggested to be a synthetic inhibitor of HSP27. KRIBB3 blocked cell migration and invasion in a human breast cancer cell line (MDA-MB-231) due to the inhibition of PKC-dependent HSP27 phosphorylation. Cell migration involves microfilament reorganisation. HSP27 has been proposed to be an F-actin cap binding protein which can inhibit actin polymerisation in vitro (Landry and Huot, 1995; Miron et al., 1991). Phosphorylation of HSP27 leads to an increase in the stability of actin microfilaments and a faster recovery of disrupted filaments after stress (Guay et al., 1997; Lavoie et al., 1993a). Another influence of HSP27 interacting with and affecting cytoskeletal dynamics is wound healing. A study using stably transfected fibroblast cell lines which either over- or underexpress HSP27 found a role of HSP27 in fibroblast adhesion, elongation and migration in the context of wound healing assays (Hirano et al., 2004).

1.22 Other protein-protein interactions of HSP27

A large number of proteins have been identified as potential binding partners for HSP27. In some cells, such as HeLa cells, constitutively expressed HSP27 makes up a large proportion of total cellular proteins and therefore it is not clear if all the interactions proposed are specific. A few examples other examples of HSP27 interacting partners are discussed here.

Immunoprecipitation and glutathione S-transferase (GST) pull-down studies found that Akt forms a complex with p38 MAPK, MK2 and HSP27 in neutrophils (Rane et al., 2001). HSP27 dissociates from the complex upon stimulation with formyl-methionyl-leucyl-phenylalanine. Akt was phosphorylated and activated by p38 MAPK-dependent MK2 (Rane et al., 2001). Further work went on to show that HSP27 regulates Akt activation and cellular apoptosis through mediating the interaction of MK2 with Akt (Wu et al., 2007).
A yeast two-hybrid screen from a rat kidney gromuleri identified hic-5, a focal adhesion kinase as an interaction partner for HSP27 (Jia et al., 2001). This interaction was found to inhibit the protection from heat-induced cell death conferred by the overexpression of HSP27 in 293T cells. The same group had shown an interaction with PASS1 in the rat (Liu et al., 2000).

Zoubeidi et al., report that the androgen-bound androgen receptor can lead to the phosphorylation of HSP27 (Zoubeidi et al., 2007). Phosphorylated HSP27 can then displace the androgen receptor from its complex with HSP90 and chaperone it to the nucleus where it is then able to activate transcriptional activity.

1.23 HSP27 in disease

Variations in HSP27 expression levels or mutations in the protein sequence have been implicated in a large number of diseases. A list of diseases for which HSP27 has been involved is included in Table 1.

1.23.1 Neurodegenerative disorders

HSP27 has been implicated in the pathology of many diseases, directly and indirectly. Recently, five mutations in HSP27 have been identified that cause autosomal dominant Charcot-Marie-Tooth (CMT) disease type 2F and distal hereditary motor neuropathies (dHMN) (Evgrafov et al., 2004; Ismailov et al., 2001; Tang et al., 2005). The mutations are 379C→T (R127W), 404C→T (S135F), 406C→T (R136W), 452C→T (T151I) and 545C→T (P182L). Four of the mutations occur in the conserved \(\alpha\)-crystallin domain. CMT disease is the most common inherited neuromuscular disease, affecting 1 in 2500 people. Co-transfection of neurofilament light chain together with a mutant HSP27 altered neurofilament assembly, suggesting that the mutations may affect function of the protein.

HSP27 has also been implicated in various protein conformation diseases, characterised by the build up of accumulated proteins. As shown on table 1 higher levels of HSP27 have been found in Alzheimer’s disease, Alexander disease and Parkinson’s disease patients suffering from dementia. HSP27 has been shown to bind preferentially to hyperphosphorylated tau, found in the paired helical filaments that are the hallmark of Alzheimer’s disease, and lead to its dephosphorylation and degradation. HSP27 has been shown to rescue hyperphosphorylated tau-mediated cell death (Shimura et al., 2004). HSP27 has also been shown to prevent cellular
<table>
<thead>
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<th>Disease</th>
<th>Mutation in HSP27 known?</th>
<th>Expression of HSP27</th>
<th>Reference</th>
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<td>(Chung et al., 2008; Evgrafov et al., 2004; Houlden et al., 2008; Tang et al., 2005; Zhai et al., 2007)</td>
</tr>
<tr>
<td>Protein conformation diseases (neuropathies)</td>
<td>Alexander Disease</td>
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<td>(Head et al., 1993)</td>
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<tr>
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<td>Alzheimer’s disease</td>
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<td>(Renkawek et al., 1993)</td>
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<tr>
<td></td>
<td>Parkinson’s disease with dementia</td>
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<td>(Renkawek et al., 1999)</td>
</tr>
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<td>(Love and King, 1994)</td>
</tr>
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<td></td>
<td>Gastric</td>
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<td>(Chen et al., 2004a)</td>
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<td></td>
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<td>Increased</td>
<td></td>
<td>(Langdon et al., 1995)</td>
</tr>
<tr>
<td></td>
<td>Prostate</td>
<td>Increased</td>
<td></td>
<td>(Cornford et al., 2000)</td>
</tr>
<tr>
<td>Ichthyosis</td>
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<td>Reduced</td>
<td></td>
<td>(Jonak et al., 2005).</td>
</tr>
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</table>

**Table 1: HSP27 in diseases:** List of diseases where a mutation in HSP27 or a change of expression has been reported.
polyglutamine toxicity and suppress the increase in reactive oxygen species by huntingtin, the protein that aggregates in Huntington’s disease (Wyttenbach et al., 2002).

1.23.2 Cancer

HSP27 has been implicated in the growth and invasiveness of certain cancers, because it is cytoprotective against many cell stresses including chemotherapeutic drugs used to treat cancers. It can be overexpressed in many cancers, including breast (Love and King, 1994), prostate (Cornford et al., 2000), gastric (Chen et al., 2004a) and ovarian (Langdon et al., 1995). Cells from a colon adenocarcinoma induced in a BDIX rat highlights a role for HSP27 in tumourigenicity in syngeneic animals (Garrido et al., 1998).

Work in MDA-MB-231 breast cancer cells that overexpress HSP27 show that HSP27 up-regulates MMP-9 expression and activity and down-regulates the Src-tyrosine kinase Yes expression. Their results imply that Yes may have a role in regulating MMP-9 expression and \textit{in vitro} invasion. This has been postulated as a way in which HSP27 can influence tumour metastasis (Hansen et al., 2001).

Bruey et al., show that large oligomers of HSP27 are the structural requirement for the antiapoptotic activity of HSP27. Cell-cell contact induces the formation of large oligomers of HSP27 regardless of the phosphorylation state of the serine residues and this increases cell tumourigenicity (Bruey et al., 2000).

1.23.3 Atherosclerosis and cardiovascular diseases

HSP27 has been suggested as a biomarker for atherosclerosis (Duran et al., 2006; Martin-Ventura et al., 2004). HSP27 is decreased in the plasma of atherosclerotic patients relative to healthy subjects (Martin-Ventura et al., 2004). This was supported by work suggesting that HSP27 phosphorylated at serine residues 78 and 82 was associated with healthy blood vessels and lost from vessels of patients with accelerated graft atherosclerosis (a serious long-term complication of heart transplantation) (De Souza et al., 2005). The degradation of extracellular HSP27 by plasmin in atherosclerotic plaques could reflect a pathological vascular remodelling process where the balance between proteases and antiproteases is tipped towards the degradative process (Martin-Ventura et al., 2006). This study also suggests that intracellular HSP27 could have a protective role in the vascular wall by preventing apoptosis, a determinant of plaque instability. In contrast, HSP27 has been associated with dilated cardiomyopathy (Knowlton et al., 1998), with expression levels twice that of normal hearts.
1.23.4 Other Diseases

HSP27 is expressed mainly in the upper epidermal layers of human skin. Loss of HSP27 in these layers is a rare phenomenon, associated with specific genetic defects (Jonak et al., 2005). These defects are postulated to be either in suprabasal keratins or in enzymes involved in cholesterol biosynthesis. Loss of HSP27 was found to be associated with bullous ichthyosiform erythroderma, annular epidermolytic ichthyosis, X-linked recessive ichthyosis and congenital hemidysplasia with ichthyosiform nevus and limb defects syndrome (Jonak et al., 2005).

1.24 HSP27 function in inflammation

HSP27 has been shown to be important in a lot of disease states as mentioned above. Many of these disease states involve an inflammatory process. MK2, known to phosphorylate HSP27, is activated by the p38 MAPK pathway downstream of stress signals including IL-1, TNF and LPS (Clifton et al., 1996; Kotlyarov et al., 2002). As the p38 MAPK pathway is involved in the inflammatory response HSP27 may have a function in inflammation. Phosphomimetic mutants of HSP27, in which the serine phosphorylation sites are mutated to glutamate, caused stabilisation of COX-2 ARE reporter mRNA (Lasa et al., 2000). This stabilisation is weak when compared with overexpressed MKK6 or MK2 and may indicate that HSP27 can act to fine-tune the pathway. However, other members of our laboratory were unable to reproduce these results and it was unclear if HSP27 had a role in the stabilisation of COX-2 mRNA.

There have been some suggestions in the literature that HSP27 may be involved in inflammation, however it was unclear if HSP27 had a pro- or anti-inflammatory effect. HSP27 expression has been characterised as up-regulated in certain chronic inflammatory conditions such as uveitis in the rabbit (Rocha et al., 1996). In contrast methotrexate, an effective treatment for the chronic inflammatory condition, rheumatoid arthritis, was found to increase the prostaglandin D2-stimulated HSP27 expression in osteoblasts (Yoshida et al., 2004).

It has been previously reported that HSP27 associates with the IKK complex in response to TNFα. Phosphorylation of HSP27 enhanced this association and resulted in a decrease in IKK activity (Park et al., 2003). A further study showed by co-immunoprecipitation that HSP27 had a role in suppressing NF-κB activation at an IKK-level (Kammanadiminti and Chadee, 2006). They found by co-immunoprecipitation that HSP27 interacts with IKK-α and IKK-β. This association was found to increase in response to soluble amebic protein-treated conditioned epithelial cells. HSP27 has also been proposed to have a protective anti-inflammatory effect in angiotensin-II-induced hypertension and inflammation in the aorta (Chen et al., 2004c). HSP27
and HSP70 levels are increased in response to angiotensin II, this was elevated still further in response to heat shock. HSP27 has also been suggested to suppress heart inflammation (Chen et al., 2004b) and may protect against inflammatory injury by the suppression of NF-κB and AP-1 in vascular smooth muscle cells (Chen and Currie, 2006). Addition of exogenous HSP27 to monocytes has been reported to stimulate the production of IL-10 and to a lesser extent TNF through p38 MAPK (De et al., 2000) and has therefore been postulated to act as an anti-inflammatory agent.

HSP27 has also been reported to have a pro-inflammatory role, for example HSP27 overexpression has been shown to increase NF-κB relocalisation, DNA binding and transcriptional activity in response to etoposide, TNFα and IL-1β through enhancing the degradation of phosphorylated IkBα by the proteosome (Parcellier et al., 2003). HSP27 was found to bind to polyubiquitin chains and the 26S proteosome both in vitro and in vivo (Parcellier et al., 2003). Previous work from our laboratory using RNAi to deplete HSP27 from cells showed that HSP27 is required for IL-1-induced expression of COX-2 and IL-6 protein and mRNA in HeLa cells (Alford, 2006; Alford et al., 2007). Three siRNA oligonucleotides targeted against different HSP27 mRNA each suppressed HSP27 expression. All three siRNAs inhibited COX-2, IL-6 and IL-8 protein expression. COX-2 and IL-6 reduction in IL-1-induced mRNA expression was demonstrated to be due to a decrease in mRNA stability (Alford et al., 2007). The stability of a wide variety of other mRNAs was unaffected in HSP27 siRNA-transfected cells, including GADD45, Bcl-x, c-FOS, p53 and Bax (Alford, 2006). The involvement of HSP27 in inflammatory mRNA stabilisation suggests that HSP27, together with the p38 MAPK pathway may play a role in chronic inflammatory diseases.

**PART 6: Project Aims**

Despite the identification of HSP27 as a protein that is phosphorylated in response to IL-1 stimulation in fibroblasts almost twenty years ago (Guesdon and Saklatvala, 1991) and its subsequent identification as a component of the p38 MAPK pathway it is still unclear what the role of this protein is in inflammation. As mentioned above, work by a previous student in the laboratory using RNAi had shown that HSP27 is required for the stabilisation of IL-1-induced COX-2 and IL-6 in HeLa cells. The initial aim of this project was to use HSP25−/− mice to determine if HSP27/25 has an important role in inflammation in vivo. A second aim of this project was to determine if HSP27 regulates IL-1 signalling as well as gene expression in HeLa cells using RNAi. If IL-1 signalling were regulated by HSP27 it would be important to determine the point in the pathway where the regulation occurs and the mechanism by
examining the activity of the pathway components following HSP27 depletion using RNAi. A further part of the project was to determine if the regulation of inflammatory gene expression / signalling by HSP27 is a general or HeLa cell-specific mechanism.
PART 1: MATERIALS

2.1 General reagents

All general reagents were purchased from Sigma-Aldrich (Poole, Dorset, UK) unless otherwise stated. [α-32P]-dATP was originally purchased from GE Healthcare (Amersham, UK), but for the fibroblast work was from Perkin Elmer (Waltham, Massachusetts, USA). Acrylamide 30 % (w/v) / bis-acrylamide 0.8 % was from Severn Biotech Ltd (Kidderminster, UK). Dithiothreitol (DTT) was from Alexis Corporation (San Diego, CA, USA). ‘QIAamp® RNA Blood Minikit’ was from Qiagen Ltd. (Crawley, West Sussex, UK). ‘UltraMobius 1000 Plasmid kit’ from Novagen (San Diego, CA, USA). Deionised water purified by reverse osmosis was used for all general applications.

2.2 Antibodies and antisera

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### Chapter 2: Materials and Methods

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<td>Prof. Saklatvala laboratory</td>
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| Table 2.1: List of antibodies and anti-sera used. |

The secondary antibodies used for western blotting were all horse radish peroxidise (HRP)-conjugated. These were rabbit anti-mouse, swine anti-rabbit, rabbit anti-sheep or rabbit anti-goat purchased from Dako (Glostrup, Denmark). The secondary antibodies used for confocal microscopy were labelled with the Alexa Fluor dyes from Invitrogen (Paisley, UK). Primary antibodies were stored in the presence of 0.02 % sodium azide. Most primary antibodies were diluted in phosphate buffered saline (PBS) containing 0.1 % Tween-20 and 5 % Marvel. Primary antibodies against phosphorylated proteins were diluted in Tris buffered saline (TBS) containing 0.1 % Tween-20 and 5 % bovine serum albumin (BSA).
2.3 Protein Inhibitors

<table>
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<th>Working Concentration</th>
<th>Source</th>
</tr>
</thead>
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<tr>
<td>Aprotinin</td>
<td>Serine protease</td>
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<td>Phenyl methyl sulphonyl fluoride (PMSF)</td>
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<td>Sigma-Aldrich</td>
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<td>E64</td>
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<td>Sigma-Aldrich</td>
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<td>Sigma-Aldrich</td>
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<td>N-Ethylmaleimide (NEM) *</td>
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<td>Sigma-Aldrich</td>
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<tr>
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<td>Protein phosphatase 1</td>
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<td>Alexis Corporation</td>
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<td>Protein phosphatase 2A</td>
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<td></td>
</tr>
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<td>Sodium fluoride</td>
<td>Serine/threonine protein phosphatases</td>
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<td>Sigma-Aldrich</td>
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<tr>
<td>Sodium orthovanadate</td>
<td>ATPase, alkaline phosphatase, tyrosine phosphatases</td>
<td>0.1 – 1 mM</td>
<td>Sigma-Aldrich</td>
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<tr>
<td>SB202190 *</td>
<td>p38 MAPK</td>
<td>1 µM, 10 µM</td>
<td>Calbiochem (Novabiochem, Beeston, Nottingham, UK)</td>
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<tr>
<td>MG132 *</td>
<td>Proteosome</td>
<td>10 µM</td>
<td>Sigma-Aldrich</td>
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</table>

Table 2.2: List of protein inhibitors used.
* Iodoacetamide and NEM were used in all buffers when ubiquitinated proteins were desired to be detected. MG132 was used only to determine that the anti-ubiquitin antibodies were working. SB202190 was only used in specific experiments to inhibit p38 MAPK.

2.4 Buffers

2.4.1 General buffers

*Phosphate buffered saline (PBS)* (made from a 10 x concentrated solution)
8.9 mM Na₂HPO₄, 3.6 mM NaH₂PO₄, 2.7 mM KCl and 120 mM NaCl adjusted to pH 7.0

*Tris buffered saline (TBS)*
20 mM Tris-HCl and 137 mM NaCl adjusted to pH 7.6
2.4.2 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) buffers

4 x SDS-PAGE sample buffer
250 mM Tris, pH 6.8, 35 % glycerol, 8 % SDS and 0.2 mg / ml bromophenol blue
Prior to use dithiothreitol (DTT) was added to 50 mM.

Running Buffer (National Diagnostics, Atlanta, USA)
25 mM Tris, 192 mM glycine and 0.1 % (w / v) SDS

Coomassie Blue staining solution
0.1 % (w / v) Coomassie Brilliant Blue G, 50 % (v / v) methanol and 20 % (v / v) glacial acetic acid.

Fixer and Destain
50 % (v / v) methanol and 5 % acetic acid.

Bradford Reagent
0.1 % (w / v) Coomassie Brilliant Blue G, 0.05 % ethanol and 8.5 % (v / v) H₃PO₄

2.4.3 Western blotting buffers

Whole cell lysis buffer
50 mM Tris HCl pH 7.5, 250 mM sodium chloride, 3 mM EDTA, 3 mM EGTA, 1 % Triton X-100 (v / v), 0.5 % IGEPAL CA-350 (v / v), 10% glycerol (v / v), 2 mM DTT, 1 mM sodium orthovanadate and 2 mM sodium fluoride with protease and phosphatase inhibitors

Western blot transfer buffer
25 mM Tris, 192 mM glycine and 20 % (v / v) methanol

2.4.4 Kinase assay buffers

2.4.4.1 MK2, p38 MAPK, JNK, MKK3 and MKK6 kinase assay buffers.

Kinase lysis buffer
20 mM HEPES pH 7.4, 50 mM β-glycerophosphate, 2 mM EGTA, 1 % Triton X-100 (v / v), 10 % glycerol (v / v), 10 mM sodium fluoride, 1 mM sodium orthovanadate and 2 mM DTT with protease and phosphatase inhibitors.
**2 x RIPA:**
100 mM Tris/HCl pH 7.4, 300 mM sodium chloride, 20 mM sodium pyrophosphate, 2 mM EDTA, 50 mM β-glycerophosphate, 200 µM sodium orthovanadate, 2 % Igepal CA-630 (v / v), 1 % deoxycholic acid (w / v) and 0.2 % SDS (w / v)

**3 x kinase wash buffer:**
60 mM HEPES pH 7.4, 600 mM sodium chloride, 60mM β-glycerophosphate, 30 mM sodium fluoride, 300 µM sodium orthovanadate, 1.5 mM EDTA, 1.5 mM EGTA, 30 mM magnesium chloride and 0.15 % Brij35 (v / v)

**Kinase assay reaction mix (one reaction):**
10 µl 3 x kinase wash buffer, 1 µg substrate (0.5 µg recombinant MK2 for p38 MAPK assay), 20 µM ATP, 0.4 µl $[^{32}P]$-ATP, 2 μM DTT made up to 30 µl with deionised water.

**2.4.4.2 TAK1 kinase assay buffers.**

**TAK1 lysis buffer:**
50 mM Tris-HCl pH 7.5, 0.1 mM EGTA, 1 mM EDTA, 1 % Triton X-100 (v / v), 1 mM sodium orthovanadate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 0.27 M sucrose, 2 mM DTT with protease and phosphatase inhibitors

**Buffer B:**
50 mM Tris HCl pH 7.5, 0.1 mM EGTA, 0.1 mg / ml BSA, 10 mM magnesium chloride, 0.1 mM sodium orthovanadate, 2 mM DTT and 1 μM microcystin

**2.4.5 Lysis buffer for ubiquitination experiments**

**SDS buffer:**
50 mM Tris HCl pH 7.5, 1 % (w / v) SDS, 50 mM iodoacetamide, 10 mM NEM, protease and phosphatase inhibitors

**Renaturation buffer:**
50 mM Tris HCl pH 7.5, 150 mM NaCl, 2 % (v / v) Igepal CA-630, 1 mM DTT, 50 mM iodoacetamide, 10 mM NEM, with protease and phosphatase inhibitors
2.4.6 Cell fractionation buffers

2.4.6.1 Detergent lysis

Cytoplasmic lysis buffer
10 mM HEPES pH 7.6, 3 mM magnesium chloride, 40 mM potassium chloride, 2 mM DTT, 5% glycerol (v/v), 0.5% IGEPAL CA-350 (v/v), 2 mM sodium fluoride, 1 mM sodium orthovanadate and protease and phosphatase inhibitors

Wash buffer
10 mM HEPES pH 7.6, 1.5 mM magnesium chloride, 10 mM potassium chloride, 1 mM DTT, 2 mM sodium fluoride, 1 mM sodium orthovanadate and protease and phosphatase inhibitors

Nuclear Extraction buffer
20 mM HEPES pH 7.6, 0.42 M sodium chloride, 25% glycerol (v/v), 1.5 mM magnesium chloride, 0.2 mM EDTA, 1 mM DTT, 2 mM sodium fluoride, 1 mM sodium orthovanadate and protease and phosphatase inhibitors

2.4.6.2 Dounce homogenisation

Cytoplasmic lysis buffer
10 mM HEPES pH 7.9, 1.5 mM magnesium chloride, 10 mM potassium chloride, 0.5 mM DTT, 2 mM sodium fluoride, 1 mM sodium orthovanadate and protease and phosphatase inhibitors

2.5 Cell culture reagents

Dulbecco’s modified Eagle’s medium (DMEM) was obtained from PAA (Pasching, Austria). Eagle’s Minimum Essential Medium (EMEM) was purchased from BioWhittaker, Cambrex Bio Science Walkersville, Inc. (Walkersville, USA). Foetal calf serum (FCS) was purchased from Biosera (Ringmer, UK). Opti-MEM I medium was obtained from Gibco (Paisley, UK). 50 ml aliquots of FCS were stored at -20°C until required and used at final concentrations of 5 and 10 ml in the medium. Media was supplemented with final concentrations of 0.1 U ml⁻¹ penicillin and 0.1 mg ml⁻¹ streptomycin (PenStrep) (PAA). EMEM media was also supplemented with 2 mM glutamine (PAA). Trypsin-EDTA was purchased from PAA.
PART 2: Methods

2.6 Cell Culture

All cell culture was performed in a Class II vertical laminar flow cabinet.

2.6.1 HeLa cells

HeLa (human cervical epidermal carcinoma) cells obtained from Yamanouchi Pharmaceutical Co, Ltd (Oxford, UK) were cultured in DMEM supplemented with 10 % FCS, PenStrep. American Tissue Culture Collection (Tedddington, UK), ATCC, HeLa cells were cultured in EMEM supplemented with 2 mM glutamine (PAA), 10% (v / v) FCS and PenStrep. Cells were maintained in a humidified environment at 37°C, 5% CO₂. Cells were washed with PBS and trypsinised with trypsin-EDTA for 5 min at 37 ºC, 5 % CO₂. Cells were centrifuged at 400 x g for 5 min and resuspended in the appropriate volume of fresh medium. For general maintenance the seeding ratios were 1:6 or 1:8 depending on the number of days between splitting. Cells were maintained until passage 16.

2.6.2 Human alveolar basal epithelial (A549) cells

A549 cells (cultured respiratory epithelial cells from a human lung tumour) were cultured in DMEM containing 10 % (v / v) FCS and PenStrep. Cells were maintained and subcultured according to section 2.6.1. Cells were maintained until passage 16.

2.6.3 Human dermal fibroblasts (HDFs)

Normal neonatal human dermal fibroblasts (HDF) cells from Clonetics, Cambrex Bio Science Walkersville, Inc. were grown in DMEM containing 10 % (v / v) FCS and PenStrep. Cells were maintained and subcultured according to section 2.6.1. Seeding ratios in the HDFs were 1:3. Cells were maintained until passage 12.

2.6.4 Mouse embryonic fibroblasts (MEFs)

Immortalised MK2 −/− mouse embryonic fibroblast (MEF) lines were kindly provided by Alexey Kotlyarov (Universität Hamburg, Germany). Four cells lines were gifted; mock virus MEF MK2 −/− (mock); virus MEF MK2 −/− PMMP-EGFP (Leeo) (empty vector); virus PMMP-EGFP
MK2−/− WT (WT); virus PMMP-EGFP MK2−/− K76R. Cells were cultured in DMEM supplemented with 10% (v/v) FCS and PenStrep.

2.6.5 Freezing down and thawing cells

Prior to splitting the medium required to freeze down the cells was made and put on ice. For all the cell types used, except L929, frozen storage media was DMEM containing 20% (v/v) FCS and 10% (v/v) DMSO. Cell sheets were washed with pre-warmed PBS and trypsinised. The trypsin was inactivated in normal growth medium and spun down at 400 x g for 5 min. Cell pellets were resuspended in frozen storage media and pipetted into sterile Cryovials ® (Simport plastics, Beloeil, QC, Canada). The cryovials were placed, surrounded by cotton wool in a beaker at – 70 °C overnight. This enabled slow freezing of the cells. The next day the cells were transferred to the liquid nitrogen store for long term storage.

Cells were removed from the liquid nitrogen store, placed in the 37 °C waterbath for not longer than 2 min and transferred into the appropriate size flask containing normal growth medium. The next day the medium was replaced. In the case of NHDF cells 1/3 vial from a T175 was placed in each flask to ensure the maximum use of cells was gained at a low passage.
2.7 RNA interference

2.7.1 siRNA oligonucleotides.

siRNA oligonucleotides targeted against HSP27 (Fig. 2.1) were designed according to the guidelines set out by the Tuschl Laboratory (Elbashir et al., 2002) by a former PhD student in the laboratory. Early work used protected siRNA obtained from Dharmacon (Lafayette, USA) and stored in a lyophilised form at -20 °C. The siRNA was deprotected when required following the manufacturer’s instructions. Latterly previously deprotected siRNA was purchased from Dharmacon or Eurofin MWG Operon (Ebersberg, Germany) in a lyophilised form, diluted to the stock concentration of 10 µM and stored in 50 µl aliquots at -20 °C before use. Aliquots were freeze thawed three times. Scramble (Scr) and HSP27 dsRNA oligonucleotides contained a 3΄ dTdT overhang and luciferase contained a 3΄ UU overhang. The siRNA sequences were as follows:

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<th>Sense Sequence</th>
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<td>Scramble 2 (Scr)</td>
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<td>HSP27#8</td>
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</tr>
<tr>
<td>TAK1#2</td>
<td>GUAGAUCUCAACAGACUdTdT</td>
</tr>
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</table>

HSP27#5 and HSP27#7 siRNAs modified with a 2΄ O-Me group at position 2 on the antisense strand were purchased together with the unmodified sense strand from Eurofin MWG Operon (Ebersberg, Germany). The guide and passenger strands were resuspended in the appropriate volume of 5 x siMAX™ buffer (30 mM HEPES pH 7.3, 100 mM KCl, 1 mM MgCl₂; from MWG) to 100 µM and equal volumes (200 µl of each) combined. The mixture was placed at 95 °C for 5 min and allowed to cool to room temperature in the heat block. 40 µl 10 M ammonium acetate and 1.5 ml ethanol was added to each siRNA mixed and vortexed. The siRNA duplex was incubated at -20 °C for 16 h. The siRNA was centrifuged at 14 000 x g for 30 min, 4 °C. The supernatant was removed and the pellets allowed to air dry. Pellets were resuspended in 400 µl 1 x siMAX™ buffer and siRNA duplexes were stored as for unmodified siRNAs.
Figure 2.1: Nucleotide sequence of HSP27 showing the sequences and positions of the HSP27 siRNAs. Nucleotide sequence for human HSP27 adapted from X54079. Sequence in blue is non-coding, the black sequence is the coding frame, different siRNA oligonucleotides are marked in colour and the corresponding siRNA is shown in the key. The aug start site for translation and uaa stop codon are underlined.
2.7.2 siRNA Transfection

2.7.2.1 HeLa cells and HDFs (later work)

Yamanouchi HeLa cells were seeded at 1 x 10^5 cells per well in six well plates and cultured for 24 h in DMEM containing 10 % (v / v) FCS. ATCC HeLa cells were seeded at 5.5 x 10^4 cells per well. For each transfection (1 well in a six well plate) 10 µl of 1 µM siRNA was mixed with 175 µl Opti-MEM I medium by gentle pipetting. 4 µl of Oligofectamine (Invitrogen, Paisley, UK) was mixed with 11 µl Opti-MEM I in a sterile Eppendorf tube. The solutions were incubated for 10 min, combined, mixed by gentle pipetting and incubated for 20 min at room temperature. During this incubation the medium was removed from the cells and they were washed with 2 ml PBS per well. 800 µl Opti-MEM I was added to each well. Following the 20 min incubation the transfection mixture was mixed gently by pipetting and then 200 µl of lipid / siRNA complex was added to each well. The solution in the wells was mixed by gentle horizontal back and forth and side-to-side agitation. After 4 h the medium was removed, the cells washed in PBS and 1.5 ml Opti-MEM I containing 5 % (v / v) FCS and PenStrep was added. The cells were incubated at 37 °C, 5 % CO₂ for 72 h before stimulation and lysis. The final concentration of siRNA added to the cells was 10 nM. For any dilution of siRNA, 1 x siRNA buffer from Dharmacon was used (20 mM potassium chloride, 6 mM HEPES (pH 7.5), 0.2 mM magnesium chloride). For a less potent siRNA, HSP27#2, in HeLa cells and later work in human fibroblasts a second transfection was performed using oligofectamine 24 hours after the first. The cells were lysed 48 hours after the second transfection.

2.7.2.2 HDFs (early work) and A549 cells

HDFs were seeded at 6 x 10^4 cells per well in six well plates and cultured for 24 h in DMEM containing 10 % FCS. For each transfection into a well of a six well plate 6 µl of Lipofectamine 2000 (Invitrogen) was mixed with 244 µl Opti-MEM I. 2.5 µl siRNA (final concentration of 10 nM) was mixed in a final volume of 250 µl. These solutions were incubated for 5 min at room temperature. The solutions were combined and incubated at room temperature for 20 min to allow for complex formation. During this incubation the medium was removed and the cells were washed in 2 ml Opti-MEM I. 2 ml Opti-MEM I was added to each well. After the incubation 500 µl of the lipid / siRNA complex was added to each well. After 4 h incubation at 37°C, 5 % CO₂ the media was removed, the cells washed with 2 ml Opti-MEM I and 2.5 ml Opti-MEM I containing 5 % (v / v) FCS and PenStrep was added. A second round of transfection was performed 24 h after the initial transfection. In some experiments cells were
treated with a final concentration of 20 ng ml⁻¹ IL-1α 48 h following transfection. For some signalling experiments the fibroblasts were seeded at 2.95 x 10⁵ cells per 10 cm dish and the transfection procedure was scaled up 5.9 times.

2.8 DNA transfection

2.8.1 Plasmids

pCMV FLAG 2 (Sigma-Aldrich), pCMV FLAG HSP27 (Lasa et al., 2000), pCMV FLAG HSP27 AAA (Lasa et al., 2000), pCMV FLAG HSP27 EEE (Lasa et al., 2000), pmaxGFP (Amaxa biosystems, Cologne, Germany), pSR HA-p38 (Gift from A. Clark) and MKK6E (gift from C. Tudor). pCMV FLAG HSP27 constructs containing four mismatches within the 21-nucleotide target sequence designed to be resistant to HSP27#5 siRNA were provided by JL Dean.

2.8.2 Calcium phosphate DNA transfection

1 x 10⁵ HeLa cells were seeded in each well of a six well plate and cultured for 24 h. Calcium phosphate transfection of HeLa cells was performed following the manufacturer’s (Sigma-Aldrich) instructions. Briefly, 2 h before transfection the medium was removed from the cells and 2 ml fresh DMEM, 10 % (v / v) FCS, PenStrep was added to the cells. 5 μl DNA (HSP27 DNA was made up to 5 μl using pCMV FLAG 2 (empty vector) and mixed with 49 μl distilled water and 6 μl 2.5 M calcium chloride. 2 x HEPES-buffered saline (60 µl per well transfected) was bubbled using an automatic pipette pump. The DNA solution was added dropwise to the bubbling HEPES-buffered saline and vortexed for 4 seconds before incubation at room temperature for 20 min. The DNA calcium phosphate solution was added to the cells dropwise and agitated to mix. After 16 h incubation at 37 °C, 5 % CO₂ the medium was removed and 2 ml fresh DMEM 10 % (v / v) FCS, PenStrep was added.

2.8.3 Lipofection of DNA

Lipofection using SuperFect (Qiagen, Crawley, West Sussex, UK) was performed previously (Lasa et al., 2000). Briefly, cells were seeded so that they would be 70 % confluent on the day of transfection. The total amount of DNA used per point was made up to 1 μg in 100 μl serum free medium. For each well of a six-well plate 5 μl SuperFect reagent was added to the DNA solution and gently pipetted up and down several times to mix. The solution is left 10 min for the DNA-lipid complexes to form. During this time the cells were washed once with PBS and
495 μl DMEM containing 10 % (v / v) FCS and PenStrep is added to each well. After pipetting up and down once again the 105 μl SuperFect DNA mixture was added to each well. The wells were mixed by moving the plate back and forth, and side to side. The DNA was left on the cells at 37 ºC 5 % CO2. After 3 h the medium was removed and DMEM containing 10 % (v / v) FCS and PenStrep was added to the cells. Cells were incubated for 24 h prior to lysis.

2.8.4 Co-transfection of siRNA and DNA

2.8.4.1 Co-transfection of siRNA and DNA in HeLa cells

A siRNA transfection was performed, section 2.7.2.1. The Calcium phosphate transfection kit was allowed to equilibrate to room temperature. 4 h after the siRNA / lipid complex was added to the cells, the media was replaced with 2 ml DMEM 10% FCS, PenStrep. The DNA was transfected using calcium phosphate, Section 2.8.2. 48 h after the DNA / calcium phosphate precipitate was removed, cells were stimulated with IL-1 and lysed.

2.8.4.2 Co-transfection of human fibroblasts with siRNA and DNA

3 x 10⁶ HDFs were seeded in 10 cm dishes. siRNA and DNA were simultaneously co-transfected using Lipofectamine 2000 as described above, however a total of 1 µg of plasmid DNA was added with the siRNA. 24 h after the co-transfection, a second siRNA transfection was performed. 48 h after the second addition of siRNA, the cells were stimulated with IL-1 and lysed.

2.9 Sample preparation and western blotting

2.9.1 Cell Lysis

Cells were lysed for western blot analysis using whole cell lysis buffer, unless otherwise stated. On the day of lysis the cells were stimulated either with 20 ng ml⁻¹ IL-1α or 20 / 100 ng ml⁻¹ TNFα (Promega, Southampton, UK). Cells were washed in 1 ml PBS and 100 μl lysis buffer was added to each well of a six well plate. The plates were incubated on ice for 10 min. Lysates were then scraped using a cell scraper and pipetted into 1.5 ml Eppendorf tubes. Lysates were clarified at 16 000 x g for 10 min. Lysates were transferred to fresh Eppendorf tubes and either used immediately or fast frozen in liquid nitrogen and stored at -70 ºC.
2.9.2 Mouse dissection

C57Bl/6 mice were dissected with the assistance of A. Hazlehurst. The organs were homogenised using a Power Gen 125 homogeniser (Fisher Scientific, Loughborough, UK) in 5 ml whole cell lysis buffer on ice. A Bradford assay (Bradford, 1976) was carried out and 25 µg total protein of each tissue was loaded on SDS-PAGE.

2.9.3 Bradford assay

The total protein concentration in cell lysates was measured using a Bradford assay (Bradford, 1976). 3-5 µl of sample was added to a final volume of 1 ml Bradford reagent, with the same amount of lysis buffer as the blank. The absorbance of this solution was measured at 595 nm using a spectrophotometer. The protein concentrations were estimated by comparison with a standard curve. The standard curve was made by measuring the absorbances of known concentrations of bovine serum albumin (BSA). The measurements were often performed in duplicate.

2.9.4 SDS-PAGE

SDS-PAGE sample buffer (final dilution 1 x) was added to equal amounts of protein from lysed cells. Prior to loading onto the gel the samples were incubated at 95 °C for 5 min. SigmaMarker molecular weight markers were used. Proteins were separated using a discontinuous stacking / separating gel electrophoresis system according to the method described by Laemmli (Garfin, 1990; Laemmli, 1970). The resolving gel comprised 380 mM Tris-HCl pH 8.8, 0.1 % (w / v) SDS and 10 or 12 % acrylamide. The stacking gel comprised 125 mM Tris-HCl pH 6.8, 0.1 % (w / v) SDS and 5 % acrylamide. Gels were electrophoresed in an electrophoresis apparatus (Cambridge Electrophoresis, Cambridge, UK).

2.9.5 Transfer onto polyvinylene difluoride (PVDF) or nitrocellulose membrane.

PVDF membrane (‘Polyscreen® PVDF Transfer Membrane’, PerkinElmer Life Sciences, Beaconsfield, UK) was hydrated in methanol for 3-4 min and then rinsed in deionised water (dH2O) and transfer buffer. The membrane was laid out onto three sheets of soaked Whatman® 3MM filter paper. The electrophoresed resolving gel was placed on top of the membrane and a further three sheets of filter paper placed above the gel. Air bubbles were carefully removed by rolling a 10 ml pipette over the surface. The sandwich was placed between two sponges pre-soaked in transfer buffer and placed in the transfer tank (BioRad, Hemel Hampstead, UK). The
proteins were transferred onto the membrane by electrophoresis for 1 h at 100 V in transfer buffer at 4 ºC. Where recommended, proteins were instead transferred onto a nitrocellulose membrane, for example when intending to use anti-phospho-HSP27 antibodies. When nitrocellulose was used the procedure was the same however, the membrane was hydrated in water. The PVDF membranes were stained with Coommassie Brilliant Blue G. PVDF membranes were destained using 70 % (v / v) methanol. The position of the markers was marked using a ballpoint pen.

2.9.6 Western blotting

All steps were carried out with agitation on a shaking platform. The membrane was blocked in 5 % (w / v) Marvel (non-fat dried milk powder) in PBS-T / TBS-T for 1 h at room temperature or overnight at 4 ºC. This was followed by incubation with the primary antibody at the appropriate dilution in 5 % Marvel PBS-T or BSA in TBS-T for 1 h room temperature or overnight at 4 ºC. Membranes were washed vigorously in PBS-T / TBS-T three times for 10 min and incubated with the appropriate dilution of horse-radish peroxidase conjugated secondary antibody in 5 % Marvel PBS-T / TBS-T for 1 h at room temperature. The membrane was washed again three times in fresh PBS-T / TBS-T for 10 min. Bound antibody was visualised by enhanced chemiluminescence (ECL) (GE Healthcare, Chalfont St. Giles, UK) following the manufacturer’s instructions. The antibody used to detect total p38 MAPK in western blots at the beginning of this work was the in-house antibody, Sak7b. However, due to the detection of multiple non-specific bands it was decided to use an alternative commercial antibody from Cell Signaling Technology for the remainder of the work.

2.9.7 Stripping and reprobing western blots

Membranes were rehydrated in methanol and the methanol washed off. The membranes were washed vigorously for 15 min with 1 x stripping solution (ReBlot Plus Strong, Chemicon, Hampshire, UK). Membranes were washed 3 times for 10 min before re-blocking for 30 min at room temperature. Primary antibodies used to detect phosphorylated proteins were used for immunodetection first. Membranes were reprobed for HSP27 and the loading control α-tubulin later because these proteins are very abundant and the primary antibodies are very sensitive.

2.10 Human IL-6 and IL-8 ELISAs

Concentrations of IL-6 and IL-8 in the culture medium were detected using kits of matched antibody pairs (BD Biosciences Pharmingen, Oxford, UK). All steps were carried out with 100
µl volumes. ELISAs were performed on a 96-well EIA/RIA plate (Corning Incorporated, NY, USA). IL-6 and IL-8 capture and detecting monoclonal antibodies were from BD Biosciences Pharmingen, Oxford, UK). Plates were coated with 1 µg ml\(^{-1}\) capture antibody and left at 4 °C overnight. The capture antibody was removed and the plate washed three times in PBS containing 0.1 % Tween 20. The plate was blocked in 2 % BSA in PBS-T for 1 h room temperature. The samples were diluted appropriately in Opti-MEM I because this was the culture media on the cells. The dilutions used were neat, 1:10, 1:20 and 1:40. Triplicates were made for each dilution. A standard curve was made in duplicate for IL-6 / IL-8 protein standard (BD Bioscience Pharmingen). Six serial 1:3 dilutions were made from a starting concentration of 10 µg ml\(^{-1}\) of the protein standard and a blank was used as a final point on the standard curve. The blocking solution was removed and the plate washed three times in PBS-T. The standards and samples were added to the plate and left at room temp for 2 h. The standards and samples were then removed from the plate and washed 3 times in PBS-T. The biotinylated detecting antibody was diluted to 0.5 µg ml\(^{-1}\) in 0.5 % (w/v) BSA in PBS, applied to the plate and incubated for 1 h at room temperature. The plate was then washed three times in PBS-T. The streptavidin-HRP conjugate (R & D Systems, Abingdon, UK) 1: 400 dilution was added to the plate and incubated for 1 h at room temperature. The plate was washed three times with PBS-T. Equal amounts of 3,3’,5,5’-tetramethylbezidine (TMB) substrate (Kirkegaard and Perry Limited, Maryland, USA) were combined with peroxidise substrate solution (KPL, MD, USA). 100 µl TMB mixture was added into the wells to allow colorimetric detection of the captured protein. The reaction was stopped by addition of 1 M sulphuric acid. The absorbance was measured at 450 nm on an ELISA Multiskan bichromic plate reader (Thermo Electron Corporation, Basingstoke, UK). The data was analysed using Ascent software (Thermo Electron Corporation). Statistics were not performed on the results of this study except for standard error of the mean because there was either a clear difference (even with error bars) or no clear difference (the differences were not greater than the error bars on each variable) between the variables in these experiments. Therefore it was thought that statistics would not add to the data.
2.11 Kinase assays

<table>
<thead>
<tr>
<th>Kinase assay</th>
<th>Immunoprecipitating antibody / antiserum</th>
<th>Substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>MK2</td>
<td>Anti-MK2</td>
<td>Recombinant HSP27 (StressGen, #SPP-715)</td>
</tr>
<tr>
<td>p38 MAPK</td>
<td>Anti-p38 MAPK</td>
<td>Recombinant MK2, inactive (Upstate, #14-349)</td>
</tr>
<tr>
<td>JNK</td>
<td>Anti-JNK</td>
<td>Recombinant ATF-2 (amino acids 19-96) (Upstate)</td>
</tr>
<tr>
<td>M KK3</td>
<td>M KK3 antiserum (RAW1)</td>
<td>GST-p38 MAPK (Prof. Saklatvala laboratory)</td>
</tr>
<tr>
<td>M KK6</td>
<td>Anti-MKK6</td>
<td>GST-p38 MAPK (Prof. Saklatvala laboratory)</td>
</tr>
<tr>
<td>TAK1</td>
<td>Anti-TAB1 (Prof. P. Cohen laboratory)</td>
<td>MBP-MKK6 (Prof. P. Cohen laboratory)</td>
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Table 2.3: Kinase assay immunoprecipitating antibodies and substrates

Statistics were not performed on these assays for the same reasons as 2.10.

2.11.1 MK2, p38 MAPK and JNK kinase assays

Cells were stimulated and lysed as for western blots (section 2.2.4.1) but kinase lysis buffer was used. A Bradford assay (section 2.2.4.3) was performed to determine the total protein in the lysate. All steps were carried out at 4 °C to preserve kinase activity. 30 µl of protein G beads (Sigma-Aldrich) were added into each Eppendorf and washed with 500 µl kinase lysis buffer. The beads were centrifuged at 9 600 x g for 1 min at 4°C. The supernatant was removed. Cell lysate equivalent to 100-200 µg total protein made up to 500 µl with kinase lysis buffer was added to the beads. 5 µl of the appropriate antiserum or 1 µg of purified anti-MK2 antibody was added. The beads were vortexed briefly, tubes placed on a rotating wheel and incubated overnight at 4 °C. The samples were centrifuged for 1 min at 10 000 x g, 4 °C and the supernatant removed. The beads were washed four times with 1 x RIPA and twice with fresh 1 x kinase wash buffer containing freshly added DTT (2 mM final concentration). After each wash the beads were centrifuged for 1 min at 10 000 x g, 4 °C. 30 µl of the appropriate kinase assay reaction mix was added to each sample and incubated with shaking for 30 min at room temperature with 1 µg recombinant protein substrate (except for p38 MAPK assay where 0.5 µg recombinant MK2 was used) and [γ³²P]-ATP. For MK2 kinase assays this incubation was 40 min. The reaction was stopped by the addition of 10 µl 4 x SDS-PAGE sample buffer and mixed by pipetting. The samples were centrifuged at 10 000 x g for 1 min, 4 °C and the supernatant loaded onto a 10 % SDS-polyacrylamide gel and electrophoresed. The gel was stained with Coomassie Brilliant Blue G, destained with 50 % (v / v) methanol, 5 % (v / v) acetic acid overnight and dried on 3MM paper using a gel dryer (BioRad) for 2 h at 80 °C under
vacuum. Phosphate incorporation was visualised and quantified by autoradiography using a phosphorimager Fuji FLA-2000 (Raytek Scientific Ltd, Sheffield, UK). Table 2.3 shows the immunoprecipitating antibodies and substrates for each kinase assay.

2.11.2 MKK3 and MKK6 kinase assays

The kinase assay was performed as in section 2.2.6.1 however, the samples were lysed in whole cell lysis buffer and the incubation step with the antibody was for 3 h.

2.11.3 TAK1 kinase assay

This kinase assay is based on the co-immunoprecipitation of TAK1 with its binding protein, TAB1. Cells were stimulated with IL-1α for stated times. Cells were washed twice with ice-cold PBS and lysed for 10 min in TAK1 lysis buffer. Cell lysates containing 100-300 µg total protein were made up to a volume of 300 µl with TAK1 lysis buffer and incubated for 1 h with rotation with 1 µg anti-TAB1 antibody per point. 30 µl protein G beads (Sigma Aldrich) were pre-washed with 500 µl TAK1 lysis buffer and incubated for a further 1 h. Samples were spun down at 10 000 x g for 1 min and the supernatant was removed by aspiration. Samples were washed three times with 1 ml TAK1 lysis buffer containing 0.5 M sodium chloride, in the absence of sucrose (the sucrose caused the beads to become stickier and not wash properly). The beads were washed a final time in 1 ml 50 mM Tris HCl pH 7.5. Buffer B was freshly prepared and 50 µg ml⁻¹ inactive MBP-MKK6, 0.1 mM cold ATP, 0.2 mM [α-32P] ATP was added. The reaction was performed in the hot block at 30 ºC for 30 min and stopped by the addition of 15 µl 4 x loading buffer. Samples were then incubated for 8 min at 95 ºC, centrifuged at 16 000 x g to pellet the beads. Supernatants were analysed by SDS-PAGE on a polyacrylamide gel.

2.12 Ubiquitination assays

This method was adapted from Jaffray et al., (Jaffray and Hay, 2006). Briefly, cells were serum starved for 3 h. Where used MG-132 (10 µM final concentration) pre-incubated on the cells for 1 h prior to IL-1α stimulation. Cells were washed three times with 1 ml ice-cold PBS. Cells were scraped in SDS buffer (500 µl per 10 cm dish) and heated at 90 ºC for 10 min. Samples were spun through a QiaShredder 250 column (Qiagen) at 16 000 x g for 1 min. Lysates were diluted 1 in 10 in renaturation buffer and centrifuged at 900 x g, 4 ºC for 15 min. 60 µl protein G beads were washed in 1 ml renaturation buffer. Equal amounts of total protein in cell lysates were added to the pre-washed protein G beads and samples were incubated with 1 µg antibody overnight for IRAK1 and IKKγ or 2 h for TRAF6 at 4 ºC. The beads were pelleted at 900 x g, 4
°C and the supernatant kept for analysis. Beads were washed five times in renaturation buffer, with a centrifugation step of 2 min at 10 000 x g between each wash. Each pellet was resuspended in 60 µl 2 x sample buffer, boiled for 8 min at 95 °C and analysed by SDS-PAGE and western blotting. Variations on this protocol were tried including addition of sample buffer and analysis by SDS-PAGE following the QiaShredder step.

2.13 Cell fractionation experiments

2.13.1 Detergent lysis

Cells were grown until confluent in 10 cm dishes and then stimulated with IL-1α for the times indicated. The cell sheet was washed once with 5 ml ice-cold PBS. All subsequent steps were performed at 4 °C. Cells were scraped into 1 ml PBS, and spun down at 400 x g for 5 min. Cell pellets were resuspended in 250 µl cytoplasmic lysis buffer and incubated 10 min on ice. Samples were centrifuged at 400 x g for 10 min and the supernatant (cytoplasm) removed carefully. The pellet was washed twice in 200 µl wash buffer and centrifuged at 400 x g for 5 min. The nuclei were resuspended in 50 µl nuclear extraction buffer and incubated on ice for 30 min, vortexing at medium speed for 10-15 sec, every 10 min. The nuclear extract was clarified by centrifugation at 16 000 x g for 15 min. The supernatant was carefully removed and the pellet discarded. The same proportion of cytoplasmic extract and nuclear extract (cell equivalent) was analysed by SDS-PAGE. This enabled direct comparison of the amount of protein in the cytoplasmic and nuclear fractions.

2.13.2 Dounce homogenisation

Cells were cultured until confluent in 10 cm dishes. The cells were stimulated with IL-1 and washed once with 5 ml ice-cold PBS. All subsequent steps were performed at 4 °C. Cells were scraped into 1 ml PBS, spun down at 400 x g for 5 min and the supernatant discarded. Cells were incubated on ice for 10 min with swelling buffer and lysed with 20 strokes of the tight fitting of a 1 ml Dounce homogeniser (Wheaton, USA). Samples were transferred into Eppendorf tubes on ice. The nuclei were spun down at 1 600 x g, 4 °C for 10 min. The supernatant (cytoplasmic fraction) was transferred into a fresh Eppendorf tube. The residual cytoplasmic fraction was removed by pipetting. 200 µl whole cell lysis buffer was used to resuspend the nuclear pellet. The nuclei were sonicated for 10 min in a sonicating water bath. The nuclear extract was clarified by centrifugation at 16 000 x g for 10 min at 4 °C.
2.13.3 Confocal microscopy

2.13.3.1 Immunofluorescence

200,000 HDFs were seeded into Lab-Tek Chamber Slide System (Nunc, Thermo Fisher Scientific, Langenselbold, Germany), 2 wells per glass slide. The following day these were stimulated with IL-1 and fixed. Alternatively, 30,000 cells were seeded into the slide system and an RNAi experiment performed as in section 2.7.2.1. One set of wells were lysed as a control in TAK1 / TAB1 lysis buffer as in section 2.11.3. The other set were treated for immunolocalisation by confocal microscopy. PBS was used for most antibodies however, for anti-phosphorylated p38 MAPK TBS was used. Fresh paraformaldehyde (4% w/v) was made in 100 ml PBS and heated for 15 min at 65 °C. The cells were washed with 1 ml PBS for 5 min and fixed in 4% paraformaldehyde for 10 min, room temperature. The fixer was aspirated off, cells washed with 1 ml PBS for 5 min and permeabilised by incubation in 0.1% Triton X-100 PBS for 3 min. Samples were blocked in 15% BSA PBS for 1 h, room temperature. Blocking solution was removed. The cells were re-permeabilised in 0.1% Triton X-100 in PBS for 5 min and then incubated in 500 µl primary antibody overnight at 4 °C (1:500 anti-HSP27 together with 1:100 mouse monoclonal anti-p38α MAPK in 15% (w/v) BSA in PBS or 1:100 anti-phosphorylated p38 MAPK (Thr180 / Tyr182) in 15% (w/v) BSA TBS. Primary antibody was removed and the cells washed twice in 1 ml PBS for 5 min, once in 0.1% Triton X-100 for 3 min and twice more in 1 ml PBS for 10 min. The cells were incubated in 1:1000 secondary antibody and 1:200 of the nuclear stain, propidium iodide (Invitrogen) for 90 min, room temperature, rotating, in the dark. The secondary antibody was removed and the cell sheet washed four times in 1 ml PBS for 10 min. 200 µl sterile 50% (v/v) glycerol PBS was added to the cell sheet and the cover glass (VWR International, Lutterworth, Leic, UK) added. The outside edges were sealed with nail varnish.

2.13.3.2 Image collection

Optical imaging was performed using a x 60 oil immersion lens on a Perkin Elmer Ultraview Spinning Disc confocal microscope (PerkinElmer, Waltham, Massachusetts, USA) with a Hamamatsu Orca CCD camera (Hamamatsu Photonics UK Ltd, Welwyn Garden City, UK) attached. The same laser setting was used for all slides for each stain. Intensity measurements of fluorescence inside the cytoplasm and the nucleus were obtained by defining these areas and analysing using the ImageJ software (National Institute of Health, Maryland, USA).
2.14  Methylthiazolium (MTT) assay

Cells were treated as usual and on the day of lysis the medium was removed from the wells to leave a final volume of 900 µl (per well of a 6 well plate). 100 µl MTT solution was added (final concentration of 0.5 mg ml⁻¹). The cells were incubated for 4 h at 37 °C to form crystals. 1 ml of solution containing 10 % SDS and 10 mM HCl was added to the cells to solubilise the crystals and this was incubated at 37 °C overnight. The optical density was determined by measuring the absorbance at 570 nm of 100 µl of each sample in triplicate in a 96 well ELISA plate using a Multiskan biochromic plate reader (Thermo Electron Corporation).

2.15  Flow cytometry

HeLa cells or HDFs were seeded in 6 well plates and transfected with siRNA. The cells were transfected with 50 ng pmaxGFP DNA made up to 1 µg or 5 µg respectively with pCMV FLAG DNA (Empty vector) for SuperFect or calcium phosphate transfections. The cells were then grown until confluent (96 h after seeding). Cells were washed with PBS and trypsinised for 5 min at 37 °C using 1 ml trypsin-EDTA per well. The plates were agitated and the trypsinised cells diluted in 2 ml PBS. The live cell population was identified by cell size and granularity. The argon blue laser of the BD LSR flow cytometer (BD Bioscience, Oxford, UK), which excites at wavelength 488 nm was used to identify the GFP positive cells. The fluorescence emitted by GFP positive cells was detected in the green (FITC) channel. 20 000 or 10 000 cells were counted per sample for HeLa and HDFs respectively.

2.16  Transformation of competent cells

A waterbath was pre-heated to 42 °C. 1 µl previously maxi-prepped DNA was added to 25 µl competent Top 10 cells on ice and gently tapped. The cells were incubated on ice for 30 min. The bacteria were heat shocked at 42 °C for exactly 45 sec and returned to ice. 400 µl of Luria Bertoni (LB) media without antibiotics to each tube and incubate with shaking at 37 °C for 30 min to allow the bacteria to recover. 200 µl of a 1:10 dilution was spread onto an LB agar plate containing the appropriate antibiotic.
2.17 ‘Maxiprep’ of DNA

The ‘Maxiprep’ system allows for large scale preparations of plasmid DNA using the ‘UltraMobius 1000 Plasmid kit’ from Novagen. Single colonies were picked from an LB-agar plate and cultured in 100 ml LB containing the appropriate antibiotic at 37 °C with agitation, overnight. Centrifugation at 5 000 x g for 15 min at 4 °C was used to pellet the bacteria. The medium was removed, the bacteria lysed by alkaline lysis. Neutralisation, anion-exchange chromatography with a column supplied with the kit and precipitation with isopropanol was carried out exactly according to the manufacturer’s instructions. The DNA pellet was washed with 70 % ice-cold ethanol and resuspended in 100 – 500 µl DNase free water, depending upon the pellet size. The concentration of the plasmid DNA was determined by measuring the absorbance of a dilution at 260 nm (A_{260}). The purity of the DNA was assessed by measuring the absorbance at 280 nm (A_{280}) and calculating the ratio A_{260}/A_{280}.

2.18 RNA extraction

RNA was isolated from cells using the RNA Blood Mini kit (Qiagen Ltd.) following the manufacturer’s instructions. Briefly, the cells were stimulated for the appropriate time with IL-1, washed in ice-cold PBS and lysed in RLT lysis buffer. The lysate is homogenised with a QIAshredder, solubilised with 70 % ethanol and annealed to a QIAamp spin column. The sample was washed and the RNA eluted in RNase free water. The concentration of RNA was determined by measuring the absorbance at 260 nm (A_{260}).

2.19 Real Time Polymerase Chain Reaction (qRT-PCR)

Real time PCR was used to measure the gene expression. The RNA was taken from the RNA extraction step and reverse transcription and PCR steps were performed. Following the protocol that I used these steps were combined. A Reverse Transcriptase qPCR mastermix No ROX kit from Eurogentec (Southampton, UK) was used. RNA samples were diluted 5-fold. The reaction mix per sample was 2.45 µl water, 5 µl buffer, 0.05 µl Euroscript reverse transcriptase and RNase inhibitor mix, 0.5 µl probe and 2 µl (0.1 µg) RNA. The probes used were DUSP1 (MKP1) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Applied Biosystems, Foster City, CA, USA). The cycle used was 30 min at 48 °C for the reverse transcription to generate cDNA, 10 min at 95 °C, followed by 45 cycles of 3 sec at 95 °C and 30 sec at 60 °C in a thermal cycler Rotor-Gene 6000 series machine (Corbett Life Science, Sydney, Australia). The data was analysed using ΔΔCt relative quantification on the Rotor-Gene 6000 software (Corbett Life Science).
Chapter 3

Regulation of IL-1 signalling in HeLa cells by HSP27
3.1 Introduction

p38 MAPK has long been known to play an important role in inflammation, particularly through the stabilisation of ARE-containing mRNAs of inflammatory mediators. HSP27 is a substrate of MK2, a kinase downstream of p38 MAPK. Therefore, it was thought that HSP27 may play a role in mediating regulation by p38 MAPK of mRNAs of the inflammatory response. A previous PhD student in the laboratory, Kate Alford, found that depletion of HSP27 inhibited the IL-1-induced expression of IL-6 and COX-2 protein and mRNA. A widely used strategy to examine the function of a gene / protein \textit{in vivo} is to generate a ‘knockout’ mouse. A ‘knockout’ mouse has a targeted deletion of both copies of the gene of interest. Conditional and constitutive HSP25\(^{-/-}\) mice were in the process of being generated commercially by GenOway at the beginning of my project to allow the investigation of HSP25 function in inflammation \textit{in vivo}.

3.2 Tissue specific expression of HSP25

To investigate HSP25 function in mice it was first necessary to determine the tissue distribution of the small heat shock protein. Tissues from a c57BL/6 mouse were dissected and each tissue sample was homogenised in 5 ml whole cell lysis buffer. Lysates containing 25 \(\mu\)g total protein were loaded on a SDS-PAGE gel and analysed by western blot for HSP25. This showed that HSP25 was variably expressed in different tissue types. HSP25 was constitutively expressed in many organs that contain smooth muscle, for example stomach and colon (Fig. 3.1). High levels of HSP25 were found in muscle (Fig. 3.1), which is in agreement with previous reports (Neufer and Benjamin, 1996; Pichon et al., 2004).

Unfortunately, the generation of HSP25\(^{-/-}\) mice was delayed and therefore a different strategy was needed. It was decided to try and elucidate the mechanism whereby HSP25 / 27 amplifies inflammatory gene expression. Many techniques exist to study \textit{in vitro} mechanisms including overexpression and loss of function. The strategy chosen was a loss of function approach using transient depletion of the protein from the cell by RNA interference (RNAi) with siRNAs. This strategy was chosen because it would allow a rapid analysis of the function of HSP25/27 in inflammatory gene expression and IL-1 signalling. An analysis of cell types expressing HSP25 / 27 was performed to determine which cell types would be best suited for this purpose.
Figure 3.1: Tissue distribution of HSP25 in C57BL/6 mice.

A C57BL/6 mouse was dissected and the organs / tissues homogenised in whole cell lysis buffer. For each tissue sample 25 μg of total protein was loaded and analysed by SDS-PAGE and western blot was performed for HSP25. This western blot is representative of two experiments.
3.3 The expression of HSP27 in different cell types

The murine cell types examined were mouse embryonic fibroblasts (MEFs), L929 cells (mouse areolar fibroblasts), RAW 264.7 (murine macrophage-like) cells and primary bone marrow derived macrophages. Of these HSP25 was only detected in MEFs (Fig. 3.2A/B). It has been documented in the literature that L929 cells do not express HSP25 however, it is surprising that two different fibroblast cell populations differ so greatly in the protein expression levels of HSP25.

The human cell types tested for HSP27 included a mixture of cell lines and primary cells. For the cell lines 293 (human embryonic kidney epithelial), A549 (human alveolar epithelial), HeLa (human cervical epidermal carcinoma) and THP-1 cells (human monocytic leukaemia) were examined. Neonatal human dermal fibroblasts (HDFs) were also examined. Primary cells that were elutriated from buffy coat blood packs included human monocytes and T cells. The extraction of granulocytes from the buffy coat blood packs was unsuccessful due to the age of the blood packs and the short lifespan of granulocytes. Instead fresh granulocytes were provided by laboratory members. Some monocytes were differentiated into macrophages using either macrophage colony-stimulating factor (M-CSF) or granulocyte monocyte colony-stimulating factor (GM-CSF). All cell types were lysed in whole cell lysis buffer and equal amounts of total protein in the cell lysate were analysed by western blot. This approach was used to eliminate the difficulties in the comparison of loading controls, such as tubulin, due to variable expression in different cell types.

HSP27 was constitutively expressed in all of the immortalised human cell lines tested; HeLa, 293, THP-1 and to a lesser extent A549 cells (Fig. 3.2C). HSP27 expression was comparatively weak in A549 cells in two experiments, which was unexpected because HSP27 is thought to be expressed strongly in A549 cells (Skandrani et al., 2006). These results may suggest that HSP27 is expressed during the immortalisation of the cells; however HDF cells also expressed a similar level of HSP27 protein as HeLa cells (Fig. 3.2C). Endothelial cells and chondrocytes express roughly equivalent amounts of HSP27 protein levels as HeLa cells and HDFs (Jonathan Dean, unpublished). The expression of HSP27 in all passaged human cell types tested raised a concern that HSP27 is induced upon the maintenance and passage of cells in culture. Therefore L. Rawlinson tested for HSP27 expression in freshly extracted cartilage tissue. HSP27 expression was also found in freshly extracted cartilage tissue indicating that at least for chondrocytes the expression of HSP27 is present in this human tissue.
HSP27 was not detected in granulocytes or T cells (Fig. 3.2C and D). With overnight exposures a faint band could be detected in primary human monocytes. However, there was high expression of HSP27 in M-CSF- and, to a greater extent, GM-CSF- differentiated macrophages. A titration of recombinant HSP27 protein run in parallel to the samples allowed an approximation of the protein expression levels to be determined. In the M-CSF-differentiated macrophages HSP27 makes up about 0.015 % of the total cellular protein in comparison to 0.025 % for GM-CSF differentiated macrophages and 0.125 % in HDF cells. The very high expression in HDF was not surprising: HSP27 is an extremely abundant protein and similar levels of expression have been reported previously (Sakaguchi et al., 2001).

The difference in expression levels of HSP27 in primary human monocytes and THP-1 cells may be because THP-1 cells are a monocytic leukemia cell line and HSP27 is well known to be overexpressed in many cancers. The observation that HSP27 is expressed in both M-CSF- and GM-CSF-differentiated macrophages but not murine bone marrow-derived macrophages or RAW 264.7 cells was a surprise. This may be a species difference. Alternatively, HSP27 expression could be artificially induced by the process of differentiation using either M-CSF or to a greater extent GM-CSF. The lack of constitutive expression in bone marrow-derived macrophages or RAW 264.7 cells does not mean that HSP27 is never expressed in these cells. There have been reports of induction of HSP25 / 27 in response to stresses such as heat shock (Champagne et al., 1999; Landry et al., 1982) and exposure to LPS in intestinal epithelial cells (Kojima et al., 2004).

HeLa cells were chosen as the system of choice to study the role of HSP27 in IL-1 signalling. The abundance of constitutive HSP27 in HeLa cells and their ease of transfection made the HeLa cell the ideal system to probe the function of HSP27 in inflammatory gene expression. Early work investigating the effect of HSP27 depletion on IL-6 and COX-2 gene expression in the laboratory was also performed in HeLa cells. It would be of great interest to examine HSP27 function in endothelial cells however, endothelial cells are difficult to transfec and attempts to deplete HSP27 from human umbilical vein endothelial cells have failed. The supply of endothelial cells was also limited. Human dermal fibroblasts are more difficult to transfec than HeLa cells but were identified as a potential primary cell to verify the results in HeLa cells.
Figure 3.2: Expression of murine HSP25 or human HSP27 in different cell types. A / B. Expression levels of HSP25 in murine cell types. Different cell types were harvested in whole cell lysis buffer. 15 µg total protein was separated by SDS-PAGE and western blots were performed for HSP25. C / D. Expression levels of HSP27 in human cell types. Different cell types were harvested in whole cell lysis buffer. 20 µg total protein was separated by SDS-PAGE and western blots were performed for HSP27. A titration of recombinant HSP27 was run in parallel to the cell lysates to provide an approximation for the amount of HSP27 in 20 µg total cell lysate in each cell type. These blots are representative of at least two independent experiments. Primary human monocytes, macrophages, granulocytes and T cells were provided by Lynn Williams and Bernard Gregory.
3.4 HSP27 depletion inhibits IL-1-induced COX-2 protein expression in two sources of HeLa cells

Previous work in our laboratory has shown that depletion of HSP27 by RNAi inhibited the induction by IL-1 of COX-2, IL-6 and IL-8 in HeLa cells. These experiments were performed in cells obtained as a gift from Yamanouchi Ltd. Given the variability in the properties of HeLa cells from different laboratories it was decided to test whether these results could be repeated in HeLa cells from the American Tissue Culture Collection (ATCC). Six siRNA oligonucleotides that target HSP27 mRNA (HSP27#1-#6) had been used previously in our laboratory. The three that had been found to deplete HSP27 protein most strongly (#1, #2, #5) were chosen for this analysis to control for off-target transcript silencing.

HeLa cells were transfected with a relatively low dose (10 nM) of HSP27 siRNAs, to minimise off-target effects. A non-targeting control siRNA (Scramble) was also used, or the cells were left untransfected. 72 h after the addition of the siRNA / lipid complexes cells were stimulated with IL-1 (20 ng ml⁻¹) for 4 h and lysed. Western blots were performed on the lysates for COX-2, HSP27 and α-tubulin (as a loading control). A single siRNA transfection with Oligofectamine in ATCC HeLa cells did not inhibit COX-2 protein induction by IL-1 (Fig. 3.3A). The lack of inhibition of COX-2 expression in the ATCC HeLa cells may have been a result of only a weak depletion of HSP27 in these cells. Other transfection reagents were used to try to improve the depletion but to no avail (data not shown); therefore a different strategy was adopted. Cells were subjected to two rounds of siRNA transfection using Oligofectamine, 24 h apart. 48 h after the second transfection the cells were stimulated with IL-1 for 4 h and lysed. Sequential transfection of ATCC HeLa cells with HSP27 siRNA improved the degree of depletion of HSP27 protein (Fig. 3.3B) to such an extent that only a residual HSP27 was detected for HSP27#2 and #5 with an overnight exposure (data not shown). IL-1-induced COX-2 protein was inhibited by all three siRNAs. HSP27#5 inhibited COX-2 protein expression the most strongly (Fig. 3.3B).

These results suggest that strong depletion of HSP27 causes inhibition of IL-1-induced COX-2 protein expression but a poor knockdown is insufficient. One explanation may be that there is a threshold of HSP27 required in the cell for the induction of COX-2 expression by IL-1. Alternatively, if few cells were successfully transfected the HSP27 present in the non-transfected cells could mask any regulation in cells where HSP27 had been depleted. The results in this section also indicate that the inhibition of IL-1-induced COX-2 protein is not an artefact associated with Yamanouchi HeLa cells or an off-target effect of the siRNA. Cells from Yamanouchi were used for the rest of the experiments on HeLa cells because in these cells only one siRNA transfection is required to achieve strong depletion of HSP27.
Figure 3.3: HSP27 depletion following sequential but not single transfection inhibits IL-1-induced COX-2 protein expression in American Tissue Culture Collection HeLa cells. ATCC HeLa cells were subjected to one round of siRNA transfection (A) with Scramble dsRNA or three siRNA oligonucleotides targeting different sequences in HSP27 mRNA; HSP27#1, HSP27#2 and HSP27#5. After 72 h cells were stimulated with 20 ng ml⁻¹ IL-1 for 4 h, harvested and lysed in whole cell lysis buffer. SDS-PAGE and western blotting were performed to detect COX-2, HSP27 and the loading control, α-tubulin. A second round of transfection 24 h after the first was performed to improve HSP27 depletion (B). These blots are representative of four independent experiments.
3.5 IL-1-induced phosphorylation of HSP27

The actinomycin D chases that had shown the role of HSP27 in the stabilisation of IL-1-induced COX-2 mRNA had been performed at 1.5 hr post-IL-1 stimulation (Alford, 2006). At this time after stimulation the activity of a kinase involved in mRNA stabilisation, MK2, is believed to have returned to a basal level. However, HSP27 is a major substrate of MK2 and the actinomycin D chases suggested that HSP27 may mediate the stabilisation of COX-2 mRNA. It was therefore pertinent to determine if HSP27 is still phosphorylated at this relatively late time point, 90 min, post-IL-1 stimulation. HSP27 is phosphorylated in response to IL-1 stimulation on three serine residues; 15, 78 and 82. Phosphorylation of HSP27 at serine 78 was detected at 15 min and peaked at 30 min post-IL-1 (Fig. 3.4). However, residual Ser78 phosphorylation of HSP27 was still detectable at 90-120 min post-IL-1 stimulation. This would suggest that phosphorylation of HSP27 may mediate the regulation of IL-1-induced COX-2 mRNA stabilisation if only a small amount of HSP27 is required. The phosphorylation at serine 15 and serine 82 was not detected with the antibodies tried (data not shown). There are many reports in the literature that phosphorylation of small heat shock proteins such as HSP27 alters their oligomerisation state (Lelj-Garolla and Mauk, 2005; Rogalla et al., 1999). Therefore the oligomerisation state of HSP27 may be important for the regulation of IL-1-induced mRNA stabilisation.

3.6 HSP27#5 siRNA inhibits IL-1-induced MK2 activity in HeLa cells

Previous work in our laboratory using electrophoretic mobility shift assays (EMSAs) did not succeed in detecting HSP27 bound directly to RNA. However, it has been shown that p38 MAPK stabilises ARE-containing mRNAs, such as COX-2, in response to inflammatory stimuli. Kotlyarov et al., observed that MK2 was important for the stabilisation of p38 MAPK and that p38 MAPK expression and activity was reduced in MK2-/- cells (Kotlyarov et al., 2002). HSP27 is a substrate of MK2 and therefore may be involved in this feedback. It was decided to examine whether HSP27 depletion caused impairment of signalling by the p38 MAPK pathway. It was not practical to perform all experiments using a large panel of siRNAs because of the need for detailed time courses, therefore a single siRNA was used. In previous work HSP27#5 was generally the most effective siRNA oligonucleotide for the depletion of HSP27 and therefore it was the principal HSP27 siRNA used in this study. Once the mechanism for HSP27 was elucidated the aim was to verify the results using rescue experiments or with alternative siRNAs.
Figure 3.4: Time course of IL-1-induced HSP27 phosphorylation at serine 78. Yamanouchi HeLa cells were stimulated with IL-1 for the times shown and lysed in whole cell lysis buffer, a Bradford assay was performed and the samples normalised. Proteins were separated by SDS-PAGE on a 12% polyacrylamide gel. Western blot analysis was performed to detect HSP27 phosphorylated at serine 78 (P-HSP27) and total HSP27. This figure is representative of two experiments.
To investigate if HSP27 regulates MK2, the kinase downstream of p38 MAPK responsible for mRNA stabilisation, HeLa cells were transfected with either Scramble dsRNA or HSP27#5 siRNA. 72 h later the cells were stimulated with IL-1 for different times and then lysed with kinase lysis buffer. MK2 was immunoprecipitated and its ability to phosphorylate recombinant HSP27 with $\gamma^{32}$P]ATP was measured. Western blots were also performed on the lysates to determine the level of HSP27 depletion achieved. IL-1 induced a 20-fold increase in MK2 activity in Scramble-treated cells (Fig. 3.5). The peak IL-1-induced MK2 activity occurred 30 min after IL-1 was added to the cells (Fig. 3.5). MK2 activity returned to basal levels after 2 h. In three experiments peak MK2 kinase activity was reduced by an average 57 % in cells treated with HSP27#5 (Fig. 3.5). At 1 h post-IL-1 there was an average 65 % inhibition of MK2 activity (Fig. 3.5). The large error bar in Fig. 3.4 was due to different kinetics for peak IL-1-induced MK2 activity. In two experiments the peak was 60 min post-IL-1 stimulation. These results suggest that the expression of HSP27 may be necessary for full activation of MK2 by IL-1. Four hours post-IL-1 stimulation MK2 activity started to increase again in Scramble transfected cells and HSP27 depletion still inhibited the IL-1-induced activity at this time. This suggests that depletion of HSP27 with HSP27#5 inhibits both phases of MK2 activity and may link the regulation of IL-1 signalling to the inhibition of gene expression. It was not possible to examine MK2 expression because there is no suitable anti-MK2 antibody for western blotting human MK2 protein.

3.7 HSP27#5 inhibits IL-1-induced p38 MAPK activation in HeLa cells

HSP27 is required for full IL-1-induced MK2 activity therefore HSP27 may act on MK2 directly or an upstream component of the IL-1 signalling pathway such as p38 MAPK. The effect of HSP27 depletion upon IL-1-induced p38 MAPK activation was investigated by immunoprecipitation of p38 MAPK and measurement of its ability to phosphorylate a recombinant MK2 substrate. The activation of MAPKs is triggered by phosphorylation and therefore phosphorylation can be used as a guide to the activation state of the MAPK. Activation of p38 MAPK was also analysed by western blot to detect phosphorylation of Thr180 and Tyr182, the phosphorylation sites essential for the activation on p38 MAPK. IL-1-induced p38 MAPK activity peaked at 30 min post-IL-1 stimulation and returned to basal levels by 1 h (Fig. 3.6). In cells transfected with HSP27#5 siRNA, peak p38 MAPK kinase activity was reduced by 59 % (Fig. 3.6). A western blot showed that p38 MAPK phosphorylation was strongly inhibited in cells treated with HSP27#5 siRNA. p38 MAPK expression, as detected by western blot, was unchanged in HSP27#5-transfected compared with Scramble-transfected cells (Fig. 3.6). Therefore, the inhibition of detected p38 MAPK activity and activation was not
Figure 3.5: HSP27#5 siRNA inhibits IL-1-induced MK2 activity. Yamanouchi HeLa cells were transfected with either Scramble dsRNA or HSP27#5 siRNA. 72 h later the cells were stimulated with 20 ng ml\(^{-1}\) IL-1 and lysed with kinase lysis buffer at the times shown. MK2 was immunoprecipitated and a kinase assay was performed using recombinant HSP27 as the substrate. In the first two lanes of the phosphorimage a non-immune (anti-COX-2) antibody was used as a control. A. shows a phosphorimage of \(^{32}\text{P}-\text{HSP27}.\) Western blot analysis was carried out on the cell lysates, using \(1/10^\text{th}\) the amount as for the kinase assay, to detect HSP27 and \(\alpha\)-tubulin. B. Graph of mean MK2 activity induced by IL-1 in Scramble and HSP27#5-transfected cells from three separate experiments. Each experiment was normalised to the maximum MK2 activity for that experiment. Error bars are standard error of the mean (S.E.M.).
Figure 3.6: HSP27#5 siRNA inhibits IL-1-induced p38 MAPK activation in HeLa cells. HeLa cells were transfected with either Scramble or HSP27#5 siRNA. Cells were incubated for 72 h then stimulated with IL-1 for 30 min before lysis in kinase lysis buffer. p38 MAPK was immunoprecipitated and a kinase assay was performed on the immunoprecipitates by the measurement of recombinant MK2 phosphorylation. One tenth of the lysate used in the kinase assay was analysed by western blot. A. shows the phosphorimage of $^{32}\text{P}\text{-MK2}$ and western blots for HSP27, phosphorylated p38 MAPK and p38 MAPK. The western blots shown are representative of the three independent experiments. B. Graphical representation of phosphorimage results for Scramble and HSP27#5-transfected cells. Error bars are S.E.M. for three independent experiments. The data was normalised as for Fig. 3.5.
due to a decrease in p38 MAPK expression. The observed reduction in p38 MAPK activity and activation upon HSP27 depletion suggests that HSP27 targets p38 MAPK, or an upstream kinase and this causes the inhibition of IL-1-induced MK2 activity in HSP27-depleted cells. The extent of MK2 and p38 inhibition was similar therefore it appeared unlikely that HSP27 exerts a direct / additional effect on MK2.

### 3.8 In HeLa cells activation of JNK by IL-1 is inhibited by HSP27#5 siRNA

The JNK and p38 MAPK pathways share some upstream components including the MKKK, TAK1. It was possible that HSP27 regulated IL-1-induced JNK as well as p38 MAPK activity. Cells were transfected with Scramble dsRNA or HSP27#5 siRNA. After 72 h incubation the cells were stimulated with IL-1 and lysed in kinase lysis buffer. Western blots were performed to identify JNK phosphorylated on its activatory residues, Thr183 and Tyr185 (Fig. 3.7). There were difficulties encountered in the detection of JNK phosphorylated at Thr183 and Tyr185 by western blot and the blot shown in Fig 3.7 is an overnight exposure. JNK activation was induced by IL-1 and peaked at 30 min post-IL-1 in Scramble-transfected cells. IL-1-induced JNK activation was strongly inhibited in HSP27-depleted cells (Fig. 3.7). There were no suitable anti-total JNK antibodies for western blot analysis therefore α-tubulin was used. The α-tubulin blot confirmed that the inhibition of IL-1-induced JNK activity was not caused by a decrease in the total cellular protein or uneven loading of the gel.

IL-1-induced JNK activity was assessed by kinase assay using recombinant ATF-2 as the substrate. The peak of JNK activity was at 30 min post-IL-1 stimulation and the activity had returned to basal levels by 1 h (Fig. 3.7). Peak kinase activity of JNK was inhibited by an average of 64 % in HSP27#5-transfected cells (Fig. 3.7). This result confirmed that the regulation of IL-1-induced JNK activation by HSP27 corresponded to a decrease in IL-1-induced JNK activity. These results suggest that HSP27 may target a common upstream signalling pathway component or a phosphatase that can act on both JNK and p38 MAPK.
Figure 3.7: HSP27#5 siRNA inhibits IL-1-induced JNK activity in HeLa cells. HeLa cells were transfected with either Scramble dsRNA or HSP27#5 siRNA, as before. The cells were stimulated with IL-1 and lysed in kinase lysis buffer. JNK was immunoprecipitated and a kinase assay was performed on the immunoprecipitates by measurement of its ability to phosphorylate recombinant ATF-2. One tenth of the sample used for the kinase assay was analysed by western blot to detect phosphorylated JNK, HSP27 and α-tubulin (A). A shows the phosphorimage of ATF-2 phosphorylation and western blots representative of three separate experiments. B is a graph of phosphorimage data from three independent JNK kinase assays. Error bars show S.E.M. The data was normalised as for Fig. 3.5.

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Figure 3.7: HSP27#5 siRNA inhibits IL-1-induced JNK activity in HeLa cells. HeLa cells were transfected with either Scramble dsRNA or HSP27#5 siRNA, as before. The cells were stimulated with IL-1 and lysed in kinase lysis buffer. JNK was immunoprecipitated and a kinase assay was performed on the immunoprecipitates by measurement of its ability to phosphorylate recombinant ATF-2. One tenth of the sample used for the kinase assay was analysed by western blot to detect phosphorylated JNK, HSP27 and α-tubulin (A). A shows the phosphorimage of ATF-2 phosphorylation and western blots representative of three separate experiments. B is a graph of phosphorimage data from three independent JNK kinase assays. Error bars show S.E.M. The data was normalised as for Fig. 3.5.
3.9 HSP27#5 siRNA does not inhibit IL-1-induced ERK activation

IL-1 strongly activates p38 MAPK and JNK, however it can also activate the NF-κB pathway and to a lesser extent ERK. However, while the p38 MAPK, JNK and NF-κB pathways are activated by TAK1, ERK is activated by an alternative MKKK. ERK has been implicated in COX-2 expression, for example PMA has been shown to induce COX-2 expression through the activation of protein kinase C and subsequently ERK (Chang et al., 2005). However, PMA induced COX-2 expression was unaffected by HSP27 depletion (Alford, 2006). The effect of HSP27 depletion on IL-1-induced ERK activity was investigated to try and elucidate the HSP27 signalling mechanism further.

HSP27 was depleted in HeLa cells with HSP27#5 siRNA, as before, and activation of ERK by IL-1 was measured. Initially kinase assays were performed in an attempt to quantify ERK activity by following phosphorylation of recombinant myelin basic protein (MBP). However, these assays were unsuccessful due to non-specific phosphorylation of the substrate (data not shown) and therefore western blots to detect phosphorylated ERK were performed. ERK activation was assessed using an antibody that recognises ERK phosphorylated on its activatory residues Thr202 and Tyr204. As for p38 MAPK, IL-1 strongly induced phosphorylation of ERK at 30 min post stimulation. Transfection of cells with HSP27#5 siRNA had no effect on peak IL-1-induced ERK phosphorylation at these sites (Fig. 3.8). The expression of ERK was unaltered by transfection of the cells with HSP27#5 (Fig. 3.8). However, the ERK band underwent a small shift in mobility probably caused by phosphorylation at 30 min post-IL-1. The lack of HSP27-dependent regulation of ERK suggests that HSP27 may regulate a common upstream pathway component for JNK and p38 MAPK that is not shared with ERK, for example TAK1.
### Figure 3.8: HSP27#5 siRNA does not inhibit peak IL-1-induced ERK phosphorylation.

HeLa cells were depleted of HSP27 by RNAi with HSP27#5 siRNA as before. The cells were stimulated with IL-1 for the times shown. The cells were lysed in kinase lysis buffer and western blots were performed to detect phosphorylated ERK and HSP27. The blot was stripped and re-probed for total ERK as a loading control. Similar results were obtained in four separate experiments.
3.10 HSP27 depletion inhibits IL-1-induced MKK3 / MKK4 / MKK6 / MKK7

JNK and p38 MAPK are both activated by dual phosphorylation of Thr and Tyr of the phosphorylation motif Thr-Xaa-Tyr by MKKs. MKK3 and MKK6 are thought to be relatively specific activators of p38 MAPK whereas MKK4 and MKK7 phosphorylate JNK. However, MKK4 has been reported to be able to phosphorylate both p38 MAPK and JNK *in vitro* (Derijard et al., 1995). MKK3, 4 and 6 are highly homologous and therefore it would be possible for HSP27 to regulate both of them through the same mechanism. Regulation of the MKKs would directly affect the activation of p38 MAPK and JNK but not ERK.

Other researchers in the laboratory used RNAi to determine if HSP27 regulates MKK3, MKK4, MKK6 and MKK7 activity and activation (Alford et al., 2007). Scramble and HSP27#5 transfected cells were stimulated with IL-1, lysed and analysed by western blot to detect HSP27 and MKK3 / MKK6 phosphorylated at their activation sites (Fig. 3.9A, Dr. J. Dean). Cell lysates were analysed by kinase assay to examine MKK4 (Fig. 3.9B, L. Rawlinson) and MKK7 (Fig. 3.9C, L. Rawlinson) activity. Western blots were performed to detect HSP27 and α-tubulin. The activities of all four MKKs were reduced in HSP27 depleted cells (Fig. 3.9). Peak MKK4 mean activity was reduced by 65 % and peak MKK7 mean activity by 60 % (Fig. 3.9B and 3.9C). The inhibition of the MKK activities is similar to that of p38 MAPK and JNK and therefore may account for the inhibition observed for these MAPKs. All four MKKs are thought to be activated by a common upstream activator, TAK1, in response to IL-1 signalling, whereas ERK is not activated by this MKKK. Therefore these results suggested that an upstream activator MKKK may be regulated by HSP27.
Figure 3.9: HSP27#5 siRNA inhibits IL-1-induced activation of the p38 MAPK (MKK3 and MKK6) and JNK (MKK4 and MKK7) activators. A. Western blot analysis of MKK3 and MKK6 activation using an antibody to detect MKK3 phosphorylated on Ser189 and Thr193 and MKK6 phosphorylated on Ser207 and Thr211. The blot was stripped and re-probed for total MKK3 and MKK6. B. Kinase assay of MKK4 activity using a GST-JNK (K55R) mutant as the substrate. On the right is a graphical representation of the kinase assay phosphorimage data. Western blots were performed to detect HSP27 and α-tubulin. C. Kinase assay of MKK7 activity and western blots of HSP27 and α-tubulin (as B). MKK3 / MKK6 experiments were performed by JL Dean and MKK4 / MKK7 experiments were performed by L. Rawlinson. Figure from taken from (Alford et al., 2007).
3.11 HSP27 depletion inhibits IL-1-induced TAK1 activity

There are a number of MKKKs reported in the literature as important for the activation of MAPK signalling in response to pro-inflammatory stimuli. There has been some controversy over which MKKK is the most important. However, there is compelling evidence, particularly from work using TAK1 deficient mice, to suggest that TAK1 is physiologically important in MAPK signalling (Sato et al., 2005). If TAK1 was the MKKK responsible for the propagation of the IL-1 signal to p38 MAPK and JNK in HeLa cells it was possible that HSP27 may regulate IL-1 signalling through it. Work by other members of the laboratory added further credence to the possibility that TAK1 may be the target of HSP27 regulation when it was found that IL-1-induced IKKβ activity was inhibited in HSP27 depleted cells (Alford et al., 2007). To investigate this possibility an assay was used to measure TAK1 activity in lysates from HSP27 depleted and Scramble-transfected cells. The kinase assay used was modified from Cheung et al., (Cheung et al., 2003).

It is not possible to immunoprecipitate TAK1 directly in order to determine if there is any effect on TAK1 activity. However, TAK1 is found in a constitutive complex with TAB1 and TAB2 / TAB3. TAK1 was co-immunoprecipitated using an anti-TAB1 antibody. The immunoprecipitate was incubated with the recombinant substrate MBP-MKK6 and [32P]ATP for 30 min. The incorporation of 32P in MBP-MKK6 was then measured. Dr. S. Glennie found that total TAK1 protein was unaffected in HSP27 depleted cells (Fig 3.10A). TAK1 activity was only weakly inhibited (53 % at 5 min and 30 % at 10 min, the time post-stimulation with maximal TAK1 activity) in HSP27 depleted cells. The mean inhibition of IL-1-induced TAK1 activity was lower than the 60-70 % inhibition of IL-1-induced MKK4, MKK7, p38 MAPK or JNK activity in HSP27-depleted cells. This would suggest that TAK1 may not be entirely responsible for the HSP27 regulation of p38 MAPK or JNK activity. It was possible that HSP27 may regulate two points in the IL-1 signalling pathway, one at the level of TAK1 complex and another at the level of the MKKs.
Figure 3.10: HSP27 depletion partially inhibits IL-1-induced TAK1 activity in HeLa cells.

Cells were transfected and stimulated with IL-1 as shown. An antibody raised against TAB1 was used to immunoprecipitate TAK1 from Scramble (Scr) or HSP27#5-transfected cells. TAK1 activity was measured through the incorporation of $^{32}$P in the recombinant substrate MBP-MKK6. A. A portion of the samples used for the kinase assay was analysed by western blot for HSP27, TAK1 and $\alpha$-tubulin. B is a graphical illustration of mean TAK1 activity (± S.E.M.) for four independent experiments as a percentage of that in Scramble (Scr)-transfected cells at 10 min post-IL-1 stimulation. The experiments were carried out by S. Glennie and the figure was from Alford et al., (Alford et al., 2007).
3.12 Discussion

Initial work was performed to investigate the expression of the murine orthologue of HSP27, HSP25, in different tissue types, with the aim to examine HSP25 function in inflammation in HSP25−/− mice. Constitutive expression of HSP25 was found to be highly variable. Strong expression of HSP25 was found in muscle. This variation could be partly due to technical difficulties in the dissection and homogenisation of tissues, including variations in extracellular matrix dissected with each tissue, with a potential to give misleading results. A recent paper replaced part of the hspb1 coding sequence with a lacZ reporter gene and immunohistochemistry was performed in order to determine the tissue-specific expression of HSP25 by immunohistochemistry (Huang et al., 2007). Huang et al., found that HSP25 was expressed in stratified squamous epithelium of the skin, oronasal cavity, tongue, esophagous, uterine cervix and most prominently in the musculature of adult mice (Huang et al., 2007). These observations correlate strongly with those found in this study (Fig. 3.1) where the same tissues were examined.

HSP25 expression was highly variable under standard conditions in mice, however, it is believed that most signalling pathway molecules are similarly expressed between different tissues. When it was clear that the arrival of HSP25−/− mice were delayed, it was decided to examine the importance of HSP25/27 in inflammation and IL-1 signalling in particular. Previous work in the laboratory had highlighted that HSP27 may have a role in regulating IL-1-induced protein and mRNA expression of various inflammatory mediators and therefore it was thought that HSP27 may be involved in fine-tuning IL-1/i inflammatory signalling. This mechanism of regulation may be of different importance in various cell types, depending upon HSP25 constitutive expression and in certain cell types it may be delayed until HSP27 expression is induced in response to cellular stress stimuli.

RNAi was decided as the strategy to investigate the function of HSP25/27 in inflammatory gene expression and IL-1 signalling in vitro. The constitutive expression of HSP25/27 in cell types was therefore analysed to determine which cell type to use in these studies. HSP25/27 were expressed at high levels in all the immortalised cell lines tested except RAW cells. HSP27 was also expressed in human dermal fibroblasts (HDFs) and human M-CSF- and GM-CSF-differentiated macrophages. Macrophages play a pivotal role in chronic and acute innate immune responses. Functions of macrophages include the phagocytosis of invading microorganisms, antigen presentation for T cells and the secretion of cytokines such as TNF, a potent cytokine largely responsible for rheumatoid arthritis. Therefore the expression of HSP27 in these cells may be important because it suggests that HSP27 may play a role in these...
processes *in vivo*. It was unexpected that human macrophages would express HSP27 because no HSP25 could be detected in murine bone-marrow-derived macrophages. However, it is possible that there are differences in expression between species. Alternatively, the difference in expression may be due to the extraction and differentiation methods used. In the absence of HSP25−/− mice RNAi was used as the method of choice to examine the function of the small heat shock protein. HeLa cells were used for this because of the ease of transfection in these cells and as this work was a continuation of a previous PhD student’s work in HeLa cells. An alternative approach to determine which cell type to use would have been to compare the tissue expression of HSP27 with that of the pro-inflammatory markers such as COX-2 and IL-6.

Previous work in the laboratory had shown that IL-1-induced COX-2 and IL-6 protein and mRNA expression was inhibited in HSP27-depleted HeLa cells, but a direct interaction between HSP27 and these mRNAs could not be detected. An alternative hypothesis was that HSP27 enhances IL-1-induced pro-inflammatory gene expression through positive regulation of the IL-1 signalling pathway. HSP27 is itself phosphorylated by MK2 in response to IL-1 stimulation and therefore could be involved in a positive feedback mechanism. Low concentrations of siRNA were used to try and reduce potential off-target effects. Previous work in the laboratory had shown that several siRNAs targeted to HSP27 had inhibited the stabilisation of COX-2 and IL-6 mRNAs. It was decided to continue with the most potent siRNA, HSP27#5, for the remainder of the experiments in HeLa cells.

Depletion of HSP27 from the cells caused a strong inhibition in the activity of many different kinases. This included MK2, p38 MAPK, JNK, the upstream kinases MKK3/4/6/7, IKKβ and TAK1 summarised in Fig. 3.11. However, HSP27 depletion did not inhibit IL-1-induced ERK activation. ERK is not directly activated by TAK1 and this could be the reason for the lack of regulation of ERK in this system. Raf1 is thought to be the principle activator of ERK in response to growth factors. However, recently Tpl2 has been suggested to be an alternate MKKK involved in the activation of ERK in response to pro-inflammatory stimuli (Dumitrul et al., 2000). A recent report found that in IKK-deficient murine fibroblasts IKKβ was required for the activation of Tpl2 (Waterfield et al., 2004). If the IL-1-induced IKK complex activated ERK via Tpl2 in HeLa cells it would be expected that ERK activation would be inhibited in HSP27-depleted cells similarly to IKKβ. However, this was not found, which may suggest that a different MKKK is important for the IL-1-induction of ERK in HeLa cells or that feedback of IKK on Tpl2 does not occur in these cells.

The strong HSP27-dependent regulation of IL-1-induced MK2, p38 MAPK and JNK activities may account for the inhibition of IL-1-induced COX-2 and IL-6 protein and mRNA expression.
Figure 3.11: Simplified schematic of IL-1- and TNF-induced signalling downstream of TAK1. IL-1 and TNF stimuli activate TAK1. Active TAK1 can lead to the activation of MKK3, MKK4, MKK6 and MKK7 and the IKK complex leading to JNK, p38 MAPK and NF-κB activation. The activation of p38 MAPK leads to the phosphorylation of HSP27 and also to the stabilisation of genes that encode pro-inflammatory mediators such as IL-6 and COX-2.
shown previously. MK2 and p38 MAPK activity has been reported to lead to the stabilisation of inflammatory mRNAs through the phosphorylation and subsequent inactivation of the destabilising AREBP, TTP, as discussed in the introduction. p38 MAPK also activates transcription via MSK-1 (Ananieva et al., 2008). JNK is known to be involved in the induction of transcription factors that are important in the inflammatory response, such as AP-1 (Manning and Davis, 2003). The experiments in this chapter do not rule out the possibility that other, additional pathways may contribute to the regulation of inflammatory gene expression by HSP27 in response to IL-1. Such mechanisms could include the direct enhancement of an AREBP involved in the stabilisation of pro-inflammatory mRNAs, or the sequestration of a destabilising AREBP.

HSP27 is a member of the small heat shock protein family and has been reported to act as a chaperone like other family members for some proteins as discussed in the introduction (Lelj-Garolla and Mauk, 2006; Rogalla et al., 1999). Chaperone activity is required for the correct folding of proteins after translation. If a protein is misfolded then it would usually be targeted to the proteasome for degradation. If HSP27 chaperone activity was required for the correct folding of a signalling component then in HSP27-depleted cells the total protein that is dependent upon this ability of HSP27 would be decreased compared to cells which express HSP27. HSP27 depletion from HeLa cells did not result in the decrease of any total protein expression tested, so therefore HSP27 is unlikely to act as a chaperone for these proteins. However, it is possible that HSP27 may act as a chaperone for a component of the IL-1 signalling pathway between the IL-1R and TAK1, which had yet to be tested.

The HSP27-dependent regulation of the IL-1-induced activity of downstream MKKs, p38 MAPK and JNK was significantly greater than that of TAK1, found by Dr. Sarah Glennie. Data from longer exposures (not shown) revealed that the depletion of HSP27 I achieved was much greater than that by Dr. Sarah Glennie (data not shown) and this may account for the difference in the degree of regulation found. Alternatively, HSP27 may augment IL-1 signalling at more than one stage in the pathway for example at the level of the MKKs / IKKβ and also at the level of TAK1 or above. Or another MKKK expressed in HeLa cells may be more important for the propagation of the IL-1 signal to p38 MAPK and JNK in the HeLa cells and is more strongly regulated than TAK1 in cells depleted of HSP27. If the regulation was at the level of the activation of the MKKs / IKKβ this is could involve the ability of TAK1 to phosphorylate and activate its substrates either in vivo or in vitro, for example HSP27 may act as a scaffold protein to bring the substrates into close proximity to TAK1 and facilitate phosphorylation, or it could sequester or inhibit the expression of a phosphatase that acts upon TAK1.
Chapter 4

Regulation of signalling by HSP27 between the IL-1 receptor and TAK1 in HeLa cells
4.1 Introduction

In the previous chapter it was shown that depletion of HSP27 in HeLa cells inhibited both IL-1 signalling downstream of TAK1 as well as IL-1-induced gene expression. Various mechanisms were proposed which may explain how HSP27 might regulate different components of the signalling pathways activated by IL-1. In this chapter the aim was to find the mechanism whereby HSP27 regulates kinases activated by IL-1 through the examination of signalling in the pathway between the IL-1R and TAK1 in HSP27-depleted HeLa cells.

4.2 HSP27 depletion inhibits IL-1-induced TAK1 activation

HSP27 knockdown appeared to inhibit IL-1-induced TAK1 activity however the effect was weak and the mechanism whereby HSP27 regulates TAK1 was not examined (chapter 3). In response to IL-1 stimulation TAK1 is thought to oligomerise. The close proximity of TAK1 subunits in the complex causes phosphorylation of Thr187 (Singhirunnusorn et al., 2005) and this may cause subsequent autoactivation of the kinase. A western blot to detect the levels of TAK1 phosphorylated at Thr187 revealed that TAK1 activation was inhibited following HSP27 depletion (Fig. 4.1). The band corresponding to phosphorylated TAK1 ran at the same position as total TAK1 on the gel. Peak IL-1-induced TAK1 activation occurred at 30 min post-IL-1 stimulation (Fig. 4.1) compared to 10 min post-IL-1 stimulation for TAK1 activity (Fig. 3.10). This was a surprise because both sets of experiments used the same preparation of IL-1 and the same source of HeLa cells (Yamanouchi). To address this disparity the same samples used to determine TAK1 phosphorylation (Fig. 4.1) were examined by immunocomplex kinase assay. The kinetics of TAK1 activity induced by IL-1 in three experiments correlated with those for TAK1 phosphorylation (Fig. 4.1) as would be expected if this phosphorylation was responsible for activation.

In my hands, IL-1-induced TAK1 activity was inhibited by 53 % at 30 min post-IL-1 stimulation in HSP27-depleted cells. However, significant TAK1 activity was detected in resting cells and IL-1 induced activity was actually inhibited by (60 %). In Fig. 4.1 at 30-60 min post-IL-1 the TAK1 total protein levels appear to be reduced in HSP27#5-transfected cells. However, this was not seen in an additional two independent experiments in which TAK1 activity was inhibited following HSP27 depletion and therefore is unlikely to account for the reduced TAK1 activity. TAK1 activity appeared to be sustained at all time points tested including 60 min post-IL-1 and at all time points the activity was inhibited by HSP27 depletion.
Figure 4.1: HSP27#5 siRNA inhibits TAK1 activation and activity in HeLa cells. HeLa cells were transfected with Scramble dsRNA or HSP27#5 siRNA using OligoFectamine. 72 h post transfection the cells were stimulated with IL-1 for the times shown and lysed in TAK1 lysis buffer. An antibody against TAB1 was used to immunoprecipitate TAK1 from Scramble- or HSP27#5-transfected HeLa cell lysates. A kinase assay was performed using MBP-MKK6 as a substrate. A proportion of the lysates were analysed by western blot to detect total TAK1, HSP27 and α-tubulin. A. The phosphorimage data is shown from a representative kinase assay together with the corresponding western blot analysis. B. The graph shows the mean TAK1 activity for three independent experiments as a percentage of Scramble-transfected cell TAK1 activity 30 min post-IL-1. Error bars show S. E. M. The data was normalised as for figure 3.5.
The strong inhibition of IL-1-induced TAK1 activity that I observed following HSP27 depletion is consistent with the blockade of downstream signalling and suggests that HSP27 may only act at the level of TAK1 or above.

4.3 HSP27 enhances TNF-induced COX-2 protein expression, p38 MAPK and JNK activation

It is thought that the major MKKK that activates the p38 MAPK and JNK pathways in response to TNF is also TAK1, therefore, from TAK1 downstream, the TNF and IL-1 signalling pathways are the same. However, TNF and IL-1 have different receptors and signal through different adaptor proteins and TRAF proteins to activate TAK1. Therefore it was decided to stimulate HSP27-depleted cells with TNF and determine if the same effects were seen on downstream pathway components as was the case for IL-1 signalling. This would provide an insight into whether the regulation of IL-1 signalling was likely to be at the level of TAK1 or common elements of the TNF or IL-1 signalling pathways, or alternatively if HSP27 specifically regulates signalling in response to IL-1.

HeLa cells were left untransfected, mock-transfected, transfected with Scramble dsRNA or HSP27#5 siRNA. Cells were stimulated with both and 20 ng ml\(^{-1}\) and 100 ng ml\(^{-1}\) TNF compared with 20 ng ml\(^{-1}\) IL-1. Cell lysates were analysed by western blot for COX-2 protein expression (Fig. 4.2). Depletion of HSP27 was found to inhibit both IL-1 and TNF-induced COX-2 protein expression (Fig. 4.2). As can be seen in Fig. 4.2 TNF was found to be a much weaker stimulus than IL-1 in Yamanouchi HeLa cells and this was why the dose of 100 ng ml\(^{-1}\) was used. One possible explanation for this is that HeLa cells have fewer TNF receptors at the cell surface than IL-1 receptors.

TNF-induced p38 MAPK and JNK activation was also examined. p38 MAPK activation was analysed using a western blot for p38 MAPK phosphorylated at the sites necessary for its activation. JNK activity was examined by performing kinase assays. The results are shown in Fig. 4.3 and are representative of two experiments with a direct comparison of 20 ng ml\(^{-1}\) IL-1, 20 ng ml\(^{-1}\) TNF and 100 ng ml\(^{-1}\) and an additional experiment only comparing the two concentrations of TNF. TNF-induced p38 MAPK activation and JNK activity was inhibited in HSP27 depleted cells (Fig. 4.3). However, in these experiments the mean inhibition of TNF-induced JNK activity using 20 ng ml\(^{-1}\) or 100 ng ml\(^{-1}\) was 58 and 50 % respectively in comparison to 80 % for IL-1-induced activity. Despite these discrepancies, these results suggest that there is a common mechanism whereby HSP27 regulates signalling triggered by IL-1 or TNF. HSP27 may play a more minor role in TNF signalling although the reason for this is
**Figure 4.2: HSP27 depletion inhibits TNF-induced COX-2 protein expression.** HeLa cells were depleted of HSP27 using HSP27#5 siRNA, transfected with control Scramble dsRNA, mock transfected or left untransfected. The cells were stimulated with either 20 ng ml$^{-1}$ IL-1, TNF or 100 ng ml$^{-1}$ TNF for 4 h. Cells were lysed in whole cell lysis buffer and equal amounts of protein were analysed by western blot for COX-2, HSP27 and the loading control, α-actin. This figure is representative of two experiments.
Figure 4.3: HSP27 depletion inhibited TNF-induced p38 MAPK activation and JNK activity. HeLa cells were left untransfected, mock transfected, transfected with the non-targeting Scramble dsRNA control, or with HSP27#5 siRNA. Cells were stimulated with 20 ng ml$^{-1}$ IL-1 or TNF or 100 ng ml$^{-1}$ TNF for the times shown and lysed in kinase lysis buffer. JNK was immunoprecipitated from the lysate and a kinase assay performed using recombinant ATF-2 as the substrate. A shows the phosphorimage from the kinase assay and western blot analysis of a portion of lysate for p38 MAPK phosphorylated at its activatory sites, HSP27 and total p38 MAPK. B, C and D are graphical representations of mean JNK activity from at least two independent experiments for IL-1 and three for TNF (± S.E.M.) as a percentage of activity in Scramble-transfected cells 30 min post-IL-1 stimulation.
unclear. Both IL-1 and TNF pathways use upstream kinases to signal to TNF receptor associated proteins (TRAFs) that propagate the signal to TAK1 and the TAK1 binding proteins. Therefore it was possible that HSP27 may regulate one of these points in the two signalling pathways.

4.4 HSP27 depletion may delay IL-1-induced TAB1 phosphorylation

TAK1 is thought to oligomerise following the binding of its partner proteins, TAB2 / 3 to Lys63-linked polyubiquitin chains. It was possible that in HSP27-depleted cells TAK1 failed to oligomerise following IL-1 stimulation of the cells. This might occur as a result of reduced expression of either TAB2 or TAB3. The importance of TAB1 expression for TAK1 activity has been shown recently because neither IL-1 nor TNF was able to activate TAK1 in TAB1^−/− MEFs (Mendoza et al., 2008). TAB1, TAB2 and TAB3 are phosphorylated in cells (Mendoza et al., 2008). Phosphorylation of these proteins causes distinct mobility shifts on SDS-PAGE. p38 MAPK is known to form a complex with TAB1 (Ge et al., 2002) and phosphorylate it (Cheung et al., 2003). This phosphorylation causes inhibition of TAK1 activity (Cheung et al., 2003). p38 MAPK has also been suggested to form a complex containing MK2 and HSP27 (Zheng et al., 2006). In the absence of HSP27 p38 MAPK may be liberated and therefore the interaction with TAB1 might be promoted. Despite p38 MAPK activity being lower than normal in HSP27-depleted cells increased interaction with TAB1 might actually result in an increase in the phosphorylation of TAB1 upon HSP27 depletion with subsequent inhibition of TAK1 activity. Therefore the expression and putative phosphorylation (mobility shifts) of TAB1, TAB2 and TAB3 in Scramble- and HSP27#5-transfected cells were examined by western blot.

Proteins were separated on an 8 % polyacrylamide gel prior to western blotting to maximise resolution of unphosphorylated and phosphorylated forms of TAB1. TAB1 has a predicted relative molecular mass of 60 kDa. A non-specific band caused by a protein of slightly higher mobility runs above the TAB1 band, corresponding to a protein of relative molecular mass of about 70 kDa. There did not appear to be a difference in TAB1 protein expression between HSP27#5- and Scramble-transfected cells (Fig. 4.4A). TAB1 protein from Scramble-transfected cells underwent a shift in mobility on SDS-PAGE at 30 min and 60 min post-IL-1 stimulation. Interestingly, this putative phosphorylation was delayed in HSP27#5-transfected cells (Fig. 4.4A). The data are consistent with reduced TAB1 phosphorylation due to the lower p38 MAPK activity in the HSP27-depleted cells.

At the same time these experiments were performed a different approach was taken involving the use of p38 inhibitor, SB202190. The idea was to see if any increase in IL-1-induced
phosphorylation of TAB1 that may have been caused by HSP27 depletion was mediated by p38 MAPK. HeLa cells were depleted of HSP27 using HSP27#5 siRNA with the control of Scramble dsRNA. Cells were then either treated with a low dose (1 µM) of SB202190 or the vehicle control, DMSO, or left untreated and 1 h later cells were stimulated with IL-1 for the appropriate times.

In the presence of SB202190 a form of TAB1 with lower mobility than that seen in resting cells was detected with the anti-TAB1 antibody (Fig. 4.4B). This implies that TAB1 undergoes basal phosphorylation by p38 MAPK in resting cells, which is prevented in the presence of SB202190. Upon stimulation of cells with IL-1 TAB1 appeared to shift in mobility as observed previously (Fig. 4.4A) and most of the TAB1 had shifted up through three bands by 60 min post-IL-1 stimulation. In agreement with Fig. 4.4, depletion of HSP27 from the cells resulted in an apparent delay in the mobility shift of TAB1 and at 60 min post-IL-1 stimulation the TAB1 was still more dispersed in these samples (Fig. 4.4B). In the presence of SB202190 the shift in mobility appeared to be slightly delayed. Since putative TAB1 phosphorylation was actually found to be delayed or inhibited in HSP27-depleted cells, regulation of TAB1 by HSP27 is unlikely to be the mechanism responsible for the inhibition of IL-1-induced TAK1 activity. The effects of HSP27 depletion and SB202190 appeared to be additive, arguing that HSP27 knockdown inhibits the phosphorylation of TAB1 via inhibition of p38 MAPK or another TAB1 kinase.

TAB2 protein was difficult to detect and on the 8 % polyacrylamide SDS-PAGE mini-gel used it appeared to resolve as two bands (Fig. 4.4A). In Scramble-transfected cells the upper band was fainter after 20-60 min IL-1 stimulation. Interestingly, the upper band did not disappear or become fainter in the HSP27#5-transfected cells. From these results it was unclear if HSP27 regulates IL-1-induced TAK1 activity by acting on TAB2. The difficulty in the detection and determination of which band was TAB2 meant that the interpretation of TAB2 in this and future experiments was unclear and subsequent work on TAB2 has not been included in this thesis. Western blots to detect TAB3 protein expression were unsuccessful because of low antibody sensitivity.
Figure 4.4: The effect of HSP27 depletion upon TAB1 and TAB2. A. Proteins in Scramble- and HSP27#5-transfected HeLa cell lysates were separated by SDS-PAGE on an 8% polyacrylamide mini-gel. The samples were then analysed by western blot for TAB2, HSP27 and the loading control α-tubulin. The same samples were run on a separate gel and analysed by western blot for TAB1. The relative molecular mass markers shown are Abcam rainbow markers. A. is representative of four independent experiments. B. HeLa cells were depleted of HSP27 using HSP27#5 (#5) siRNA or transfected with the control, Scramble (S) dsRNA. Cells were pre-treated with 1 µM SB202190, a p38 MAPK inhibitor, or the vehicle control DMSO, stimulated with IL-1 and lysed in TAK1 lysis buffer. Samples were separated by SDS-PAGE and analysed by western blot for TAB1, HSP27 and α-tubulin. B. represents one experiment.
4.5 HSP27 depletion does not disrupt the interaction between TAK1 and its binding partner TAB1

In the experiments to analyse TAK1 activity an anti-TAB1 antibody was used to immunoprecipitate the TAK1 complex. In one experiment TAB1 was confirmed to co-immunoprecipitate with its binding partners TAK1 and TAB2. This interaction was present in untreated cells and was not disrupted upon stimulation with IL-1 at 30 min post-IL-1 stimulation (data not shown). If the TAK1 complex was disrupted in HSP27-depleted cells this would be expected to inhibit TAK1 and downstream signalling.

To test if the TAK1 complex is disrupted in HSP27-depleted cells the same time course of IL-1 treatment was used as for the TAK1 kinase assay and the cells were lysed as before. The TAK1 complex was immunoprecipitated with anti-TAB1 antibody as previously and the immunoprecipitates were run on a SDS-polyacrylamide gel and analysed by western blot. Immunoprecipitation was either performed using 1μg anti-TAB1 antibody (raised in sheep) or 1μg of sheep IgG as a control. The anti-TAB1 antibody readily immunoprecipitated both TAB1 and TAK1 (Fig. 4.5). The sheep IgG did not immunoprecipitate any detectable TAB1 or TAK1, which indicates that the interaction is likely to be specific (Fig. 4.5). TAB1 clearly interacted with TAK1 and this was not disrupted in HSP27#5- or Scramble-transfected cells at any of the times post-IL-1 stimulation tested (Fig. 4.5). There was little difference in the TAB1 band intensity in the supernatants that had been immunodepleted with anti-TAB1 antibody or sheep IgG, therefore only a small proportion of cellular TAB1 was successfully immunoprecipitated. It is therefore unsurprising that there was no difference in amount of TAK1 detected in the supernatants (Fig. 4.5). A faint smear was detected above the TAK1 protein band in Scramble-treated cells stimulated with IL-1 for 30 min (Fig. 4.5) and there was a suggestion that this was inhibited in HSP27-depleted cells. There have been a few instances in the literature of TAK1 polyubiquitination being reported (Thiefes et al., 2006) which may account for this smear.

In the same experiment co-immunoprecipitation of p38 MAPK and HSP27 with TAB1 were also tested. The HSP27 western blot shows that the HSP27 depletion was effective in the HSP27#5 cells compared to Scramble-transfected cells (Fig. 4.5). However, it was not possible to detect any HSP27 that immunoprecipitated with either the TAB1 antibody or sheep IgG. Probing the membrane with anti-phosphorylated p38 MAPK, showed that the depletion of HSP27 inhibited p38 MAPK activation as detected in previous experiments. However, both the blots for phosphorylated and total p38 MAPK failed to detect any p38 MAPK that co-immunoprecipitated with TAB1 in either resting cells or 30 min post-IL-1 stimulation.
Figure 4.5: HSP27 depletion does not disrupt the constitutive interaction between TAK1 and its binding partner TAB1. HeLa cells were depleted of HSP27 with HSP27#5 (#5) siRNA or Scramble (Scr) dsRNA as used as a control. Cells were stimulated with IL-1 for the times shown and lysed in TAK1 lysis buffer. Equal amounts of total protein from cell lysates were immunoprecipitated with either 1 μg anti-TAB1 antibody or sheep IgG. The pellets were washed extensively and then separated by SDS-PAGE with wide range SigmaMarkers™. Western blot analysis was performed for TAB1, TAK1, HSP27, phosphorylated p38 MAPK and total p38 MAPK. The antibody chains are marked as *Ab. This experiment was performed once.
In conclusion TAK1 forms a constitutive interaction with TAB1 in HeLa cells. This interaction is not disrupted in HSP27-depleted cells. There was no detectable interaction between TAB1 and p38 MAPK or HSP27 in this experiment (Fig 4.5). These experiments suggest that HSP27 does not enhance IL-1-induced TAK1 activation through the regulation of the TAK1 complex and therefore the function of HSP27 in upstream signalling was investigated.

4.6 HSP27 does not regulate the expression of upstream signalling proteins TRAF6, IRAK1, IRAK4 and MyD88

HSP27 is a member of the small heat shock protein family and like many other heat shock proteins there have been reports that it has chaperone properties (Jakob et al., 1993; Lelj-Garolla and Mauk, 2006; Rogalla et al., 1999). It is therefore possible that in the absence of HSP27 one or more components of the IL-1 signalling pathway could be misfolded. Most misfolded proteins are detected and targeted for degradation by the proteasome. If HSP27 had chaperone properties for an upstream component of the IL-1 pathway its expression would be expected to be diminished in HSP27-depleted cells. The protein expression of several key components in the IL-1 signalling pathway which propagate the signal from the receptor (IL-1R) to TAK1 was analysed.

MyD88 binds to and recruits the kinases IRAK4 and IRAK1 in response to IL-1 stimulation. Therefore it was important to test whether depletion of HSP27 affected IRAK1 or IRAK4 protein expression. HSP27 was depleted from HeLa cells and IRAK1 and IRAK4 expression was examined by western blot. The IRAK1 antibody detected two bands of a relative molecular mass of about 80 kDa, the expected size of IRAK1, when compared to the SigmaMarker electrophoresed on the mini-gel. It was not clear which band was IRAK1 however neither of these bands were regulated in HSP27-depleted cells (Fig. 4.6B). The relative molecular mass of IRAK4 is 50 kDa. Although, two bands were detected by the anti-IRAK4 antibody used, it was
not clear which, if either corresponded to IRAK4. Neither of these bands were regulated (Fig. 4.6B).

The current dogma in the IL-1 signalling field is that TRAF6 becomes Lys63- polyubiquitinated in response to IL-1 signalling and this leads to the recruitment of multiple TAK1 complexes through the TAB2 / TAB3 subunits. TAK1 is then able to autophosphorylate other TAK1 subunits and this leads to its activation and subsequent downstream signalling. To investigate if HSP27 knockdown resulted in reduced TRAF6 expression a western blot was performed for TRAF6. TRAF6 protein expression was unchanged after HSP27 depletion (Fig. 4.6C). In conclusion reduced expression of MyD88, TRAF6, IRAK1 and possibly IRAK4 did not appear to be responsible for the defect in IL-1 signalling in the absence of HSP27.

4.7 Examination of Lys63-polyubiquitination of TRAF6 and IRAK1

Polyubiquitination has recently been demonstrated to be critical in the signalling pathways from both the IL-1 and TNF receptors (Lamothe et al., 2007; Wang et al., 2001; Windheim et al., 2008; Wu et al., 2006a). Initially for IL-1 signalling it was believed that TRAF6 possessed E3 ubiquitin ligase activity and could autoubiquitinate in response to IL-1 stimulation of cells (Wang et al., 2001). If TRAF6 polyubiquitination is inhibited by the absence of HSP27 then the TAK1 complex, through TAB2, would be unable to bind to the polyubiquitin chains of TRAF6. In this situation the TAK1 complexes would be unable to come into close proximity, oligomerise, autophosphorylate and become activated. In order to examine this possibility it was first necessary to detect endogenous IL-1-induced TRAF6 polyubiquitination in HeLa cells. Polyubiquitination, in contrast to monoubiquitination, usually results in a laddering of proteins of higher relative molecular mass and lower mobility than the band of the unmodified protein on the SDS-PAGE gel.

The method used to examine endogenous ubiquitinated proteins was adapted from Jaffray et al., (Jaffray and Hay, 2006). Samples were lysed in a highly denaturing buffer containing 2 % SDS. Ubiquitin ligases remove ubiquitin from proteins and are highly active even in sample preparations at low temperatures. Therefore the ubiquitin ligase inhibitors iodoacetamide and N-ethylmaleimide were included in the buffers used. Alkylating agents such as iodoacetamide can achieve complete and irreversible inhibition of most deubiquitinating enzymes, which are cysteine proteases, in denaturing conditions. The viscosity of the sample was reduced by sonication and the SDS was diluted in a renaturation buffer.
Figure 4.6: HSP27 depletion had no effect on MyD88, IRAK1, IRAK4 or TRAF6 protein expression. HeLa cells were depleted of HSP27 with HSP27#5 siRNA or transfected with Scramble dsRNA. The cells were stimulated with IL-1 for the times shown and lysed in TAK1 lysis buffer. The samples were separated by SDS-PAGE and analysed by western blot for A. MyD88, HSP27 and α-tubulin, B. IRAK1, IRAK4, HSP27 and α-tubulin and C. TRAF6, HSP27 and α-tubulin. A. was representative of three independent experiments. B. and C. were both representatives of two independent experiments.
In order to determine if IL-1-induced ubiquitination of endogenous TRAF6 could be detected, TRAF6 was immunoprecipitated from the cell lysate and western blots were performed for ubiquitin. Under the conditions tried in eight experiments, endogenous TRAF6 ubiquitination could not be detected. Fig. 4.7 shows two representative experiments. A wide variety of time courses were used based on the times of TRAF6 ubiquitination reported in the literature. Additional time points were added in later experiments to take into account the delayed signalling found in the HeLa cells (Fig 4.7B). The analysis of lysate from one experiment showed the IL-1-induction of an extra band of higher relative molecular mass to the expected band for TRAF6 (data not shown). This band may have been due to ubiquitination of TRAF6 however, it was never reproduced. It may be that upon ubiquitination of the endogenous protein the antibody used for immunoprecipitation is no longer able to recognise the epitope required to bind the protein of interest. Alternatively, ubiquitinated TRAF6 could be of extremely low abundance.

The TAK1 binding proteins TAB2 and TAB3 are reported to mediate the binding of TAK1 to the polyubiquitin chains on TRAF6 after IL-1 stimulation. It was decided to determine if ubiquitination of a protein in the TAK1 complex could be detected. Therefore the membrane from Fig. 4.5 was re-probed with an anti-ubiquitin antibody. No ubiquitination could be detected (data not shown).

In TNF signalling the equivalent protein in the pathway to IRAK1 is Rip1. IKKγ has been reported to interact with polyubiquitin chains on Rip1 after TNF stimulation (Wu et al., 2006a). Therefore it may be possible that IRAK1 in IL-1 signalling is able to act in a similar fashion to Rip1 in TNF signalling. HeLa cells were stimulated with IL-1 and lysed in a buffer containing 2% SDS, iodoacetamide and N-ethylmaleimide. In Fig. 4.8 protein from the lysate was immunoprecipitated with either 1 µg anti-IRAK1 antibody or rabbit IgG. A western blot was performed with anti-ubiquitin to analyse ubiquitination of immunoprecipitated material. A smear of a similar intensity was detected in all immunoprecipitated samples (Fig. 4.8). This would suggest that no significant, specific, IRAK1 ubiquitination occurred. However, there is the possibility that the ubiquitinated IRAK1 is hidden by the general smear with the anti-ubiquitin antibody. A more intense smear was detected in the lysate and the supernatant. This was expected because more proteins would be in these samples and this experiment would also detect other ubiquitin linkages including Lys48-polyubiquitinated proteins that would be targeted to the proteasome. In the experiment shown in Fig 4.8 some cells were preincubated for 1 h with the proteasome inhibitor MG132 (10 µM final concentration) as a positive control for ubiquitination. In the presence of the proteasome inhibitor intense laddering of bands was
Figure 4.7: Ubiquitination of endogenous TRAF6 could not be reproducibly detected. HeLa cells were grown in 10 cm dishes until confluent. HeLa cells were stimulated with IL-1 for 10 min or left unstimulated. Cells were lysed with a highly denaturing buffer which contained 2% SDS and renatured following the protocol in materials and methods. A. Equal protein amounts for the samples were separated by SDS-PAGE and western blot analysis was performed to detect TRAF6 and HSP27. B. Immunoprecipitation from equal amounts of total protein in cell lysates was performed with anti-TRAF6 or rabbit IgG. Samples were separated on SDS-PAGE and western blots performed for TRAF6 and ubiquitin. This figure is representative of seven independent experiments.
Figure 4.8: Ubiquitination of endogenous IRAK1 cannot be detected. HeLa cells were stimulated with IL-1 for the times shown. One point was pre-incubated with 10 µM MG-132 for 1 h prior to lysis. The cells were lysed in a buffer that contained 2 % SDS and the samples were sonicated and renatured. Cell lysates with equivalent amounts of total protein were immunoprecipitated with either 1 µg anti-IRAK1 (IR) antibody or a rabbit IgG (Ig) control. Samples from the lysates (L), supernatant (S) and immunoprecipitate (IP) were analysed by SDS-PAGE and western blot was performed for ubiquitin and IRAK1. This experiment was performed once.
detected on the gel, presumably due to the accumulation of Lys48-polyubiquitinated proteins (Fig. 4.8).

The ubiquitin blot was stripped and re-probed with anti-IRAK1 antibody to determine if the IRAK1 immunoprecipitation had occurred and was specific. IRAK1 was immunoprecipitated with anti-IRAK1 antibody but not rabbit IgG (Fig. 4.8). The band corresponding to IRAK1 was fainter at 15 min post-IL-1 stimulation potentially due to the degradation of IRAK1 reported to occur after IL-1 signalling. There was no noticeable smear indicative of polyubiquitinated IRAK1 in this blot.

4.8 Examination of the interaction between IRAK1 and TRAF6.

The interaction of IRAK1 with TRAF6 has long been reported as pivotal for the propagation of the IL-1 signal. In order to investigate the possibility that HSP27 may inhibit this interaction it was first necessary to try to co-immunoprecipitate the two proteins in untransfected cells. TRAF6 was successfully co-immunoprecipitated (Fig. 4.9) with the anti-IRAK1 antibody but not the rabbit IgG control. However, it was difficult to detect a clear band for TRAF6 because it resolved close to the antibody heavy chain. The antibodies that were used to immunoprecipitate IRAK1 and detect TRAF6 were both raised in rabbits and therefore the heavy chain showed as a strong band in the TRAF6 western blot due to cross-reactivity with the secondary antibodies. The TRAF6 blot was stripped and re-probed for IRAK1. IRAK1 was successfully immunoprecipitated by the anti-IRAK1 antibody and not the rabbit IgG (Fig. 4.9) in agreement with Fig 4.9. The IRAK1 band became fainter in both the lysate and the immunoprecipitate at 20 and 30 min post-IL-1 stimulation, potentially due to degradation as discussed previously. The difficulty in the detection of a clear TRAF6 band in the immunoprecipitates prevented the pursuit of this strategy.

4.9 IRAK1 is rapidly degraded in human dermal fibroblasts upon IL-1 signalling in contrast to HeLa cells

IRAK1 is thought to degrade rapidly upon stimulation by IL-1 (Yamin and Miller, 1997). However, in experiments in HeLa cells it was found that IRAK1 did not degrade over the 20 min IL-1 time course examined (Fig. 4.6B). In order to determine if this result in HeLa cells was an anomaly an IL-1 time course was performed in primary HDFs. It was found that upon stimulation IRAK1 was rapidly degraded in HDFs, in agreement with the literature and there was no detectable IRAK1 band at its unmodified molecular weight by 15 min post IL-1 stimulation (Fig. 4.10).
**Fig. 4.9: IRAK1 interacts with TRAF6.** HeLa cells were stimulated with IL-1 for the times shown. Cells were lysed in TAK1 lysis buffer. Equal amounts of total protein per sample lysate were immunoprecipitated (IP) with either 1 μg rabbit IgG (Ig) or anti-IRAK1 antibody (IR). One tenth of the lysate used for immunoprecipitation and the immunoprecipitated samples were separated by SDS-PAGE and analysed by western blot for IRAK1 and TRAF6. The 60kDa band corresponding to TRAF6 is outlined by a box, in the upper panel. Antibody (Ab) heavy chains are marked. This figure was representative of three independent experiments.
Figure 4.10: IRAK1 protein rapidly degrades in human dermal fibroblasts following IL-1 stimulation. HDFs were stimulated with IL-1 for the times shown and lysed in TAK1 lysis buffer. Samples were analysed by SDS-PAGE and analysed by western blot for IRAK1 and α-tubulin. This experiment was performed once.
4.10 Discussion.

Both Dr. S. Glennie (Fig. 3.10) and I (Fig. 4.1) found an inhibition of IL-1-induced TAK1 activity (and in Fig. 4.1 activation) in HSP27-depleted cells compared to Scramble-transfected cells. However, in the two sets of experiments there was a disparity between the kinetics of the activity. This may have been caused by the difference in stimulation method. In previous work cells were stimulated simultaneously and lysed at different times post-IL-1. However, I stimulated the cells at different times and lysed them together. The late, sustained activity seen in Fig. 4.1 was a surprise because downstream p38 MAPK and JNK activity peaked at 30 min post-IL-1 and returned to basal levels by 60 min (Figs. 3.6 and 3.7). However, sustained TAK1 activity is consistent with the prolonged IL-1-induced activity of MKK7 (Fig. 3.9). This suggests that another form of regulation switches off signalling below the level TAK1, for example involving the MAPK phosphatase, MKP1, or another phosphatase. Dr. S. Glennie found a 30% inhibition of IL-1-induced TAK1 activity in HSP27-depleted HeLa cells (Fig. 3.10). This indicated that HSP27 regulated IL-1 signalling either at or (mainly given the weak inhibition) downstream of TAK1. However, my work to examine the IL-1-induced activation of TAK1 found a strong inhibition of TAK1 phosphorylation in HeLa cells depleted of HSP27 and a corresponding strong inhibition of IL-1-induced TAK1 activity. This may be because I achieved more efficient depletion of HSP27 in these cells than was obtained previously. This strong inhibition following HSP27 depletion is consistent with the blockade of downstream signalling and as a consequence suggests that HSP27 may only act at the level of TAK1 or above.

In order to confirm that HSP27 augmented IL-1 signalling at the level of TAK1 a different approach was taken. IL-1 and TNF signal through different receptors however, their signalling pathways are thought to converge at the level of TAK1 (Fig. 1.1). TNF-induced COX-2 protein was inhibited by HSP27 depletion. It was also found that TNF-induced p38 MAPK activation and JNK activity was inhibited in the absence of HSP27. The regulation of IL-1-induced JNK activity in HSP27-depleted cells found in these experiments was higher than the 64% in Fig. 3.7. The results from Fig. 3.7 are more in line with the TNF results (Fig. 4.3). One reason may be a difference in the IL-1 preparation used between these two sets of experiments, or the HSP27 depletion may have been slightly greater in the set of experiments in section 4.3. Overall these results add further support to the conclusion that the effect of HSP27 depletion on the IL-1 signalling pathway is at the level of TAK1 in HeLa cells. However, it was still possible that HSP27 regulates a common upstream element of the IL-1 and TNF pathways.
When total TAB1 levels were examined, HSP27 appeared to delay the putative phosphorylation of TAB1 that occurs upon IL-1 stimulation in HeLa cells. An alternative approach with the p38 MAPK inhibitor, SB202190, was also used to determine if HSP27 delayed the phosphorylation of TAB1 through p38 MAPK (Cheung et al., 2003). SB202190 did not completely prevent the TAB1 putative phosphorylation mobility shifts. This suggests that either SB202190 was not completely suppressing p38 MAPK activity at the concentration used or an additional kinase could also phosphorylate TAB1. Other kinases reported to phosphorylate TAB1 include JNK1, JNK2 and ERK (Mendoza et al., 2008). HSP27 depletion had an additive effect with the SB202190 at 60 min post-IL-1. Potential explanations include that HSP27 depletion inhibits p38 MAPK activity to a greater extent than SB202190 in this experiment or HSP27 depletion could inhibit another kinase(s) responsible for TAB1 phosphorylation. As IL-1-induced JNK activity was inhibited in HSP27-deleted HeLa cells this would be the most likely candidate. In conclusion these results suggest that HSP27 knockdown inhibits TAB1 phosphorylation, possibly via reduced p38 MAPK activity. However, this mechanism is unlikely to account for the regulation of IL-1-induced TAK1 phosphorylation or activity in HSP27-depleted HeLa cells.

Two potential bands of about the correct relative molecular mass for TAB2 were observed in resting cells. It was unclear if either / both bands were TAB2. The upper band disappeared after 20 min IL-1 in Scramble-transfected cells but was stabilised in HSP27-depleted cells. The upper band may be due to a post-translational modification of TAB2 such as phosphorylation. Alternatively, the upper band could be TAB2 and phosphorylation of TAB2 may shield the epitope with the result that the antibody can no longer detect it. However, TAB2 phosphorylation has previously been shown to occur after IL-1 stimulation (Jiang et al., 2002) rather than be constitutive. Another possibility is that TAB2 is degraded after stimulation, although this has not been reported in the literature. The disappearance of the TAB2 protein corresponds with the time in which TAK1 is phosphorylated on its activation loop and becomes active. If the upper band is TAB2 this result may indicate a potential mechanism whereby HSP27 is involved in the degradation of a form of TAB2 that could regulate TAK1 activity correspondingly. However, the presence of TAB2 for a prolonged time period after stimulation would be expected to prolong the contact of the TAK1 complexes with polyubiquitinated TRAF6 and therefore this result would suggest that TAK1 activity would be prolonged in HSP27-depleted cells. Thus, this result does not explain the inhibition of IL-1-induced TAK1 activation and activity in cells depleted of HSP27.

The results pointed towards TAK1 as the point in the IL-1 pathway that was regulated by HSP27. It was therefore pertinent to investigate whether HSP27 was acting as a scaffold for the
formation and maintenance of the TAK1 complex. However, depletion of HSP27 from HeLa cells appeared to have no effect on the constitutive interaction of TAK1 with its binding partners, TAB1 and TAB2. With the commercial antibodies available TAB3 was unable to be detected and therefore it was not possible to test whether this recently discovered, alternative binding partner to TAB1 was affected. The ability of p38 MAPK to interact with TAB1 was tested because the reports in the literature (Cheung et al., 2003) and the experiment in Fig. 4.4B support the hypothesis that p38 MAPK binds to and phosphorylates TAB1. Surprisingly no interaction was found between p38 MAPK or HSP27 and TAB1. This may because the epitopes on the p38 MAPK and HSP27 recognised by their antibodies were shielded by the interaction with TAB1 or these interactions are transient. Further experiments would be required to examine this interaction.

HSP27 has been reported to act as a chaperone (Jakob et al., 1993; Lelj-Garolla and Mauk, 2006; Rogalla et al., 1999). If HSP27 was acting as a chaperone for a signalling pathway component it would be expected that in the absence of HSP27 they might be misfolded, and could then be targeted to the proteasome for degradation. In order to examine whether HSP27 acts as a chaperone for the upstream signalling components, western blots were performed for TAK1 binding proteins (TAB1 and TAB2), TRAF6, IRAK1 and IRAK4 and MyD88. HSP27 depletion had no effect on the expression on any of these proteins, therefore it was thought that HSP27 was not acting as a chaperone for these proteins. The effect of HSP27 depletion on the expression of the IL-1 receptor was not checked, and defective receptor expression cannot be completely ruled out as a possible explanation. At this point it was discovered that in HeLa cells IRAK1 was not degraded rapidly upon stimulation as reported in the literature, whereas later the expected degradation was shown in HDF cells. This raised the concern that there may be because of an anomaly in protein degradation or signalling in HeLa cells.

The mechanism whereby the signal from the IL-1 receptor reaches TAK1 is still being elucidated, for a current model see Fig. 4.11. During the course of this work a large amount of evidence using overexpression has pointed towards Lys63-linked polyubiquitination of certain signalling pathway molecules as critical for the propagation of the signal. The strongest evidence suggested that polyubiquitination of TRAF6 led to the recruitment and activation of the TAK1 complex through the binding of TAB2 to the polyubiquitinated chains. Experiments to detect the polyubiquitination of endogenous TRAF6 were unsuccessful. Ubiquitinated proteins were only detected when the cells were pre-incubated for 1 hour in the presence of the proteasome inhibitor MG132. The proteasome inhibitor would lead to the accumulation of Lys48-polyubiquitinated proteins that would otherwise have been targeted to the proteasome for degradation.
Figure 4.11: Current proposal for signalling from the IL-1 receptor to the TAK1 and IKK complexes. IL-1 binds to the IL-1R and causes the dimerisation of the IL-1R to the IL-1RAcP. This causes the recruitment of accessory proteins including MyD88. The kinases IRAK1 and IRAK4 are recruited to the receptor complex. IRAK4 phosphorylates and activates IRAK1, which can then dissociate from the complex and recruit TRAF6. TRAF6 possesses E3 ubiquitin ligase activity and can attach Lys63-polyubiquitin chains to itself. IRAK1 and IRAK4 phosphorylate Pellino and Pellino isoforms mediate the formation of Lys63-linked polyubiquitinated IRAK1. The TAK1 complex binds to Lys63-polyubiquitinated TAK1 and autoactivates. The IKK complex can bind to Lys63-polyubiquitinated IRAK1, which leads to the co-localisation of the TAK1 and IKK complexes and the resultant activation of IKKβ by TAK1. This figure is adapted from the website of Sir Philip Cohen’s laboratory.
TAK1 has also been reported to be ubiquitinated (Thiefes et al., 2006) and the membrane from Fig. 4.5 was re-probed for ubiquitin. No ubiquitination was observed. However, in this experiment TAK1 lysis buffer was used, which may not be optimal for the detection of ubiquitin. However, faint smears above the TAK1 band could be observed with long exposures of Fig. 4.5. These smears may be due to polyubiquitination.

A later idea, postulated that polyubiquitination of IRAK1 should be investigated. Rip1 is the equivalent protein to the IRAK proteins in TNF signalling. Polyubiquitination of Rip1 has been reported as important in the recruitment of IKK\(\gamma\) to occupied cytokine receptors and the subsequent activation of the IKK complex (Ea et al., 2006; Wu et al., 2006b). Rip1 has also been reported to recruit the TAK1 complex through TAB2 (Kanayama et al., 2004) (Fig. 1.1), although this is still controversial. Experiments to detect endogenous polyubiquitinated IRAK1 were also unsuccessful. Recent work by Windheim et al., has shown that IRAK1 polyubiquitination is important for the recruitment of the IKK complex (Windheim et al., 2008), which would suggest an equivalent role to Rip1, separate to the activation of TRAF6 and TAK1. No endogenous ubiquitination of IRAK1 in HeLa cells was detected. Only one experiment was tried because the same problems were likely occur in the detection of endogenous TRAF6 and IRAK1 ubiquitination in HeLa cells.

The lack of ability to detect IRAK1 or TRAF6 ubiquitination may be a technical problem for example it could have been a result of the poor sensitivity of the anti-ubiquitin antibody, only a small proportion of the protein may need to be ubiquitinated and this could compound the low sensitivity of the antibody. It may be that the wrong population of complexes were immunoprecipitated by the antibodies, because it is necessary to immunoprecipitate IRAK1, TRAF6, TAK1 that has been bound to the receptor and activated in order to detect ubiquitination. Alternatively, the polyubiquitination may be transient and therefore it could have been missed with the time points of stimulation used. This would seem to be a common problem encountered when examining protein ubiquitination because most papers published in this field use overexpression of ubiquitin and sometimes the protein of interest as well to try to detect Lys63-polyubiquitinated proteins. Overexpression may indeed be necessary in order to study the Lys63-linked ubiquitination of these proteins. This strategy was not pursued because overexpression may generate artefacts and lead to incorrect conclusions. It is also difficult to co-transfect siRNA and DNA together and yield high transfection efficiency to investigate the function of HSP27 depletion in polyubiquitination.

In conclusion work from this chapter has shown that HSP27 enhances IL-1- and TNF-mediated cell signalling as well as gene regulation. HSP27 regulates pro-inflammatory signalling at the
level of TAK1 activation. It is possible that HSP27 regulates IL-1 signalling upstream of TAK1, however this proved difficult to analyse. The magnitude of the regulation of TAK1 by HSP27 is able to account for that of the downstream signalling and potentially the regulation of IL-1-induced pro-inflammatory gene expression. The regulation of p38 MAPK downstream of TAK1 may feedback onto TAB1 and maybe TAB2 and thus back onto TAK1 itself. Concerns over the validity in using HeLa cells to investigate IL-1 signalling arose because IRAK1 did not degrade as expected after IL-1 stimulation, whereas this did occur in the non-transformed HDF cells. Therefore work aimed at analysing upstream signalling was discontinued in HeLa cells. It was important to determine if HSP27 regulated IL-1-signalling in a more physiological cell type in future work.
Chapter 5

Analysis of the function of HSP27 in human fibroblasts, A549 and HeLa cells and with controls for off-target effects
5.1 Introduction

The aim of the work in chapter 5 was to investigate whether HSP27 regulates inflammatory gene expression and signalling in two other cell types; HDFs and A549 cells. This should determine if the upregulation of the IL-1 signalling pathway by HSP27 is a general or HeLa cell-specific mechanism. The concerns raised in chapter 4 about the kinetics of IRAK1 degradation and therefore IL-1 signalling in HeLa cells led to the desire to use a more physiological cell type and HDFs were chosen because they constitutively express large amounts of HSP27. A549 cells were used because these cells had previously been shown to be amenable to RNAi (Alford, 2006) and also constitutively express HSP27. During the course of this work it proved difficult to use HDF cells and therefore A549 cells were focussed on. Work in this chapter highlighted the importance of using thorough controls for RNAi experiments and different approaches were used address potential off-target effects.

The most problematic sequence of siRNAs that cause off-target effects is thought to be the six nucleotide “seed region” at the 5’ end of the guide strand (Jackson et al., 2006b). It is thought that most off-target silencing due to complementarity of alternative mRNAs to the seed region cannot be distinguished from on-target effects by reducing the siRNA concentration. A variety of modifications to siRNA have been suggested to reduce the off-target effects. A new technology that is on offer by Applied Biosystems is termed locked nucleic acids (LNA). Other companies say that they have alternative products available, which reduce off-target effects; for example Dharmacon sell the product ON-TARGET plus. Dharmacon state that the sense strand has been modified to ensure that it is definitely not taken up by the RNA-induced silencing complex (RISC) and the anti-sense strand is also modified, but they do not reveal either modification. Another way to reduce most off-target effects is to perform a 2´-O-methyl ribosyl substitution at position 2 of the guide strand (Fedorov et al., 2006; Jackson et al., 2006a). The logic is that through the modification of the siRNA the RISC-mRNA interaction is weakened or disrupted in the seed region. This should eliminate off-target silencing of mRNA sequences with only partial complementarity to the siRNA guide strand.

The ‘gold standard’ method to verify phenotypes observed in RNAi experiments is to perform a ‘rescue’ experiment (2003). In a ‘rescue’ experiment the protein of interest is depleted and then the protein is reintroduced into the cell. The reintroduced protein may then ‘rescue’ the phenotype caused by the depleted protein. This may be achieved in a variety of ways. One classic experiment is to design the siRNA to target the 3´ untranslated region (UTR) of the mRNA and have an expression plasmid for the gene of interest that does not contain the 3´ UTR and can then be introduced into the cell.
5.2 Regulation of IL-1 signalling by HSP27 in HDFs

HeLa cells are immortal and derived from a human cervical carcinoma and therefore it was preferable to use a more physiological non-transformed cell type, namely HDFs. If inhibition of IL-1 signalling and gene expression was also seen in HSP27-deficient HDFs this would suggest that the mechanism is neither HeLa cell-specific, nor was the regulation observed in chapters 3 and 4 due to a HeLa cell-specific off-target effect. Cell type-specific off-target effects occur because cells have different gene expression patterns and therefore distinct mRNA profiles. The RISC complex may scan and find different mRNAs that have high complementarity to the siRNA sequence of interest and thus different mRNAs may be silenced dependent upon the cell type and condition used.

5.2.1 IL-1-induced COX-2 protein expression is inhibited in HSP27#5-transfected HDFs

It was decided to examine the function of HSP27 in the expression of IL-1-induced COX-2 because it is an important pro-inflammatory mediator expressed in response to IL-1. Also, from previous work IL-1-induced COX-2 protein expression had been shown to be potentially more sensitive than IL-1-induced p38 MAPK activation to HSP27 knockdown in HeLa cells. A former PhD student in the laboratory had limited success with HSP27 siRNA in HDFs and work was stopped in these cells due to difficulties in achieving efficient depletion of HSP27. Since sequential transfection of siRNAs proved successful for the suppression of HSP27 in ATCC HeLa cells, I wanted to see if this improved the suppression of HSP27 expression in fibroblasts. Lipofectamine 2000 was the transfection reagent of choice because the Amaxa nucleofection system requires high concentrations of siRNA, for example, greater than 100 nM to yield strong depletion. High concentrations of siRNA have been shown to increase non-specific concentration dependent effects, including the interferon response (Moss and Taylor, 2003; Persengiev et al., 2004).

Cells were seeded and 24 h later siRNA was transfected with Lipofectamine 2000. A mock transfection that omitted siRNA oligonucleotides was also performed. The siRNA transfection was repeated 24 h after the siRNA / lipid complexes were added to the cells. After a further 48 h the cells were stimulated with IL-1 for 4 h. A western blot was performed to detect COX-2 protein (Fig. 5.1). A second siRNA, HSP27#2, was used to control for potential off-target effects with HSP27#5. HSP27#2 had been used in the initial experiments (Alford, 2006). Fig. 5.1 shows that HSP27#2 and HSP27#5 efficiently depleted HSP27 from HDFs however HSP27#2, in agreement with previous work, was less effective and residual HSP27 was detected in HSP27#2-transfected cells. IL-1 treatment for 4 h induced COX-2 protein
expression in mock and Scramble-treated fibroblasts (Fig. 5.1). HSP27#5-treated fibroblasts displayed inhibition of COX-2 protein; however HSP27#2-treated fibroblasts did not (Fig. 5.1). In some experiments HSP27#2-transfected cells did show slight inhibition of IL-1-induced COX-2 protein expression. Residual HSP27 protein may be sufficient for the induction of COX-2 protein by IL-1 in HSP27#2-transfected cells. The inhibition of IL-1-induced COX-2 protein in cells depleted of HSP27 with HSP27#5 agrees with the work in HeLa cells (Chapter 3). This gave some confidence that the function of HSP27 in IL-1-induced COX-2 protein expression may be relevant in a more physiological context.

5.2.2 IL-1-induced p38 MAPK activity is inhibited in HSP27#5-transfected HDFs

In order to investigate whether HSP27 depletion also inhibited IL-1 signalling in HDFs IL-1-induced p38 MAPK activation in HDFs was examined. HSP27#2 siRNA was not used because sequential transfection with this siRNA was not sufficient for strong depletion of HSP27 and did not inhibit IL-1-induced COX-2 protein expression (Fig. 5.1). Cells were left untransfected, or were treated with transfection reagent alone (mock), or with Scramble dsRNA or HSP27#5 siRNA with Lipofectamine 2000. 24 h later the transfection was repeated. After 48 h incubation the cells were either left untreated or treated with IL-1 for 30 min before lysis. Western blots were carried out to detect phosphorylated p38 MAPK, total p38 MAPK and HSP27. In two experiments HSP27 was strongly depleted (Fig. 5.2). Phosphorylation of p38 MAPK on Thr180 and Tyr182 was decreased in HSP27#5-transfected cells compared to Scramble-transfected cells (Fig. 5.2). In one experiment p38 MAPK protein expression appeared to be lower in HSP27#5-transfected cells compared to Scramble-transfected cells (Fig. 5.2), although to a lesser extent than phosphorylation of p38 MAPK. In a third experiment inhibition of IL-1-induced p38 MAPK activation did not occur however, there was little depletion of HSP27. Therefore it was hard to determine if the effect on p38 MAPK phosphorylation detected was due to regulation of the activation or expression of p38 MAPK. There were also concerns as to how reproducible the depletion of HSP27 would be using this transfection technique. These difficulties led me to evaluate HSP27 function in a third cell type.
Figure 5.1: HSP27#5 siRNA inhibits IL-1-induced COX-2 protein expression in HDFs. HDFs were subjected to two rounds of siRNA transfection 24 h apart with Scramble dsRNA, HSP27#2 or HSP27#5 using Lipofectamine 2000. A mock transfection with transfection reagent only was also performed. 48 h after the second transfection, the cells were stimulated with 20 ng ml$^{-1}$ IL-1 for 4 h and lysed in whole cell lysis buffer. SDS-PAGE and western blots were performed to detect COX-2, HSP27 and the loading control, α-actin. Fig. 5.1 is one of five experiments.
Figure 5.2: HSP27#5 siRNA inhibited IL-1-induced p38 MAPK activation in HDFs. HDFs were subjected to two rounds of transfection with Scramble (Scr) dsRNA or HSP27#5 siRNA. After 48 h incubation the cells were left unstimulated or stimulated with IL-1 for 30 min and then lysed with kinase lysis buffer. Samples were run on a 10 % SDS-polyacrylamide gel and analysed by western blots for phosphorylation of p38 MAPK, total p38 MAPK and α-actin. This figure was one of three experiments.
5.3 HSP27-dependent regulation of IL-1 signalling in A549 cells

Human alveolar basal epithelial (A549) cells were chosen because these cells elicit strong IL-1 responses and are relatively easy to transfec. It was thought that these cells may provide a system which would help elucidate whether the regulation of IL-1 signalling by HSP27 is a general mechanism.

5.3.1 HSP27 depletion inhibits IL-1-induced COX-2 protein expression in A549 cells

Lipofectamine 2000 is a stronger transfection reagent than Oligofectamine and therefore was chosen for the transfection of A549 cells because they were believed to be more difficult to transfec than HeLa cells. Previous work in the laboratory had also shown that strong suppression of HSP27 expression by HSP27#5 could be achieved with this transfection reagent in A549 cells (Alford, 2006). Lipofection of A549 cells was performed with either Scramble dsRNA or HSP27#5 siRNA to deplete HSP27. 72 hours after transfection the cells were stimulated using IL-1 and analysed by western blot for COX-2 protein expression. HSP27 expression was very efficiently suppressed by a single transfection of HSP27#5 (Fig. 5.3). IL-1-induced COX-2 protein was inhibited following HSP27 depletion in A549 cells (Fig. 5.3). This result would suggest that the augmentation of IL-1-induced COX-2 protein expression by HSP27 is a general phenomenon because it was observed in all the cell types tested.

5.3.2 HSP27 depletion in A549 cells inhibits IL-1-induced p38 MAPK and MKK3 / MKK6 activation

The aim was to use A549 cells to confirm that the general enhancement of IL-1-mediated events was not confined to COX-2 protein expression, especially because difficulties in the transfection of HDF cells made examination of IL-1 signalling difficult. p38 MAPK is known to be important in the stabilisation of pro-inflammatory mediator mRNAs. Fig. 5.4A shows that depletion of HSP27 with HSP27#5 siRNA in these cells inhibited IL-1-induced p38 MAPK activation compared to Scramble-transfected cells. p38 MAPK has slightly reduced expression in HSP27#5-transfected cells, however this was thought unlikely to completely account for the inhibition in the IL-1-induced phosphorylation of p38 MAPK observed in the three independent experiments.
Figure 5.3: HSP27 depletion inhibits IL-1-induced COX-2 protein expression in A549 cells. Lipofection of A549 cells was performed using Lipofectamine 2000 with Scramble (Scr) dsRNA or HSP27#5 siRNA. Cells were stimulated with IL-1 and lysed with whole cell lysis buffer. Proteins were separated by SDS-PAGE and analysed by western blot for COX-2, HSP27 and α-tubulin. This figure is representative of one experiment performed at three different cell confluencies.
It was decided to examine the regulation of the p38 MAPK upstream activators MKK3 and MKK6 in the same samples. Fig. 5.4A shows that in A549 cells HSP27 depletion was found to inhibit IL-1-induced MKK3 and MKK6 phosphorylation at their activatory phosphorylation sites; Ser189 on MKK3 and Ser207 on MKK6. MKK3 (40 kDa) and MKK6 (41 kDa) are highly homologous and only one band was seen on the phospho-western blot. However, when the blot was stripped and reprobed with another antibody which should recognise a shared epitope on both MKK3 and MKK6 two bands were detected of about the correct relative molecular mass. It is possible that these two bands are MKK3 and MKK6 respectively, however it may be that the proteins have not resolved sufficiently and one band is a non-specific band. Alternatively one of the two proteins could be predominantly phosphorylated in the presence of IL-1 and account for the fact that only one band was detected with the anti-phospho MKK3 / MKK6 antibody. The conclusion from these experiments was that the enhancement of signalling by the p38 MAPK pathway via HSP27 was a general phenomenon.

5.3.3 The investigation into the effect of HSP27#5 siRNA on IL-1-induced TAK1 activation

The pivotal point in HeLa cells for regulation by HSP27 of IL-1 signalling appeared to be TAK1 and therefore the regulation of TAK1 in A549 cells was examined. A549 cells were transfected with 10 nM Scramble dsRNA and HSP27#5 siRNA with Lipofectamine 2000. Cells were stimulated with IL-1 and lysed in TAK1 lysis buffer. Surprisingly, upon analysis, phosphorylation of TAK1 at its activation site (Thr187) was found to be prolonged in the A549 cells (Fig. 5.5) compared to Scramble-transfected cells. Peak TAK1 activation also appeared to be slightly increased in HSP27-depleted cells. This is in marked contrast to the inhibition seen with this siRNA in HeLa cells (Fig. 4.1). The same result was seen in three independent experiments. The inhibition of IL-1-induced MKK3 and MKK6 activation made this result even more unexpected. This result may put into doubt whether TAK1 is the MKKK responsible for the activation of MKK3 and MKK6 in A549 cells. Alternatively, this result could suggest that HSP27 is able to regulate several points in the IL-1 signalling pathways.
Figure 5.4: HSP27 depletion inhibits IL-1-induced p38 MAPK, MKK3 and MKK6 activation in A549 cells. Lipofection of A549 cells was performed using Lipofectamine 2000 with Scramble (Scr) dsRNA or HSP27#5 siRNA. Cells were stimulated with IL-1 and lysed with TAK1 lysis buffer. A. A western blot was performed to analyse the phosphorylation of p38 MAPK, and total protein levels of HSP27 and p38 MAPK. B. A western blot was performed to detect MKK3 / MKK6 phosphorylated at the activation sites, HSP27, total MKK3 / MKK6 and α-tubulin. A and B are representative of three independent experiments each.
Figure 5.5: HSP27#5 depletion prolongs IL-1-induced TAK1 activation in A549 cells. A549 cells were transfected with Scramble dsRNA or HSP27#5 siRNA. 72 h after transfection the cells were stimulated with IL-1 for the times shown and lysed in TAK1 lysis buffer. Equal amounts of total protein were separated by SDS-PAGE and analysed by western blot for phosphorylated TAK1 (Thr187), total TAK1, HSP27 and α-tubulin. This figure was representative of three independent experiments.
5.3.4 HSP27 depletion using HSP27#5 siRNA augments IL-1-induced JNK and ATF-2 phosphorylation in A549 cells

TAK1 is thought to activate both the JNK and p38 MAPK pathways. The effects of HSP27 depletion on IL-1-induced TAK1, MKK3, MKK6 and p38 MAPK in A549 cells were confusing. Since MKK3 / MKK6 was inhibited by HSP27 depletion but TAK was not, it was unclear what the effect of HSP27 depletion on IL-1-induced JNK activation might be. To find out whether HSP27 enhanced activation of both p38 MAPK and JNK pathways by TAK1, or p38 MAPK only the IL-1-induced activation of JNK, and its substrate ATF-2 were examined. Fig. 5.6A shows that HSP27#5 enhances the IL-1-induced phosphorylation of JNK. This experiment was repeated twice. In one experiment HSP27#5 appeared to prolong rather than enhance JNK activation. IL-1-induced ATF-2 phosphorylation was both enhanced and prolonged in A549 cells transfected with HSP27 siRNA compared to Scramble-transfected cells (Fig. 5.6B). These results indicate that TAK1 is responsible for the phosphorylation of JNK in A549 cells and is therefore likely to also activate MKK3 / MKK6. Therefore these results do not explain the disparity between the regulation of TAK1 and p38 MAPK in HSP27-depleted A549 or HeLa cells.

5.4 Use of different siRNAs to examine the regulation of IL-1 signalling by HSP27 in A549 cells

HSP27 depletion using HSP27#5 showed different effects on the regulation of TAK1 activation in HeLa cells and A549 cells and a disparity between the affect of HSP27 depletion upon TAK1 and MKK3 / MKK6 activation. One aforementioned possibility was that HSP27#5 siRNA causes cell type-specific off-target effects in one or both of the two cells types tested. The prevalence of off-target effects means that all RNAi experiments need to be interpreted with caution. The most common problem reported is off-target mRNA degradation (Baek et al., 2008). siRNA oligonucleotides are small 21-23 nucleotide sequences that bind to complementary mRNA sequences and target these for degradation by argonaute, a subunit of the RISC complex. However, it is possible that the gene of interest is not the only sequence in the genome with exact or near complementarity to the siRNA sequence. RNAi makes use of a natural pathway of gene regulation and protection from RNA viruses, used by microRNAs (miRNA). miRNAs do not require perfect complementarity with the sequence of interest to silence the target, the most important region is the ‘seed region’ residues 2-7 on the 5’ end (Lewis et al., 2005; Lewis et al., 2003). Therein lies the problem, if perfect complementarity is not required, many more sequences could be silenced and this leads to off-target sequence degradation.
Figure 5.6: Depletion of HSP27 with HSP27#5 caused a slight increase in IL-1-induced JNK activation and ATF-2 phosphorylation in A549 cells. Scramble dsRNA or HSP27#5 siRNA were transfected into cells using Lipofectamine 2000. Cells were stimulated with IL-1 for the times shown and lysed in TAK1 lysis buffer. Equal amounts of total protein were separated by SDS-PAGE and western blot analysis performed for phosphorylated JNK, phosphorylated ATF-2, HSP27 and α-tubulin. Fig. 5.6A was representative of two independent experiments and B of one experiment.
Some of the off-target effects may be concentration dependent (Semizarov et al., 2003). In order to address this problem a relatively low concentration of siRNA, 10 nM, was used in chapters 3, 4 and 5. Strategies that may be employed to validate RNAi results include the confirmation of results in other cell lines or types, use of multiple siRNA sequences, overexpression of the protein of interest and phenotype rescue by the re-introduction of the protein of interest.

Each siRNA sequence may lead to the generation of unique patterns of off-target effects. Therefore the use of multiple siRNAs decreases the chance that a mutual effect observed after transfection would be an off-target effect. The aim of this experiment was to determine if the effects of HSP27 depletion upon IL-1-induced TAK1 and signalling downstream of MKK3 / MKK6 were reproducible with other siRNA sequences. A search through the literature suggested that another siRNA, described by Rocchi et al., named in this work as HSP27#7, was also very efficient at depleting HSP27 (Rocchi et al., 2006). Therefore HSP27#7 was used to deplete cells of HSP27 and examine the effect upon IL-1-induced TAK1 and p38 MAPK activation. Fig. 5.7 shows that both HSP27#5 and HSP27#7 deplete HSP27 very efficiently in A549 cells. Both HSP27#5 and #7 inhibited IL-1-induced p38 MAPK phosphorylation. As observed previously HSP27#5 also appeared to slightly reduce p38 MAPK total protein. HSP27#5 siRNA prolonged TAK1 activation as seen previously however, HSP27#7 siRNA inhibited IL-1-induced TAK1 activation. In Fig. 5.7 TAK1 expression appears to be slightly uneven and there is an edge effect in the untransfected samples. However, this cannot account for the variations in TAK1 phosphorylation observed under the different conditions. The inhibition of IL-1-induced TAK1 phosphorylation in HSP27#7-transfected conditions agrees with the TAK1 data in HeLa cells using HSP27#5 siRNA and suggested that HSP27#5 may exert off-target effects on TAK1 activation in A549 cells. To test this possibility two additional previously reported siRNAs, HSP27#8 (Rocchi et al., 2006) and HSP27#9 (Park et al., 2003) were used. A549 cells were transfected with HSP27#8 and #9 as before. HSP27 depletion by these two siRNAs was suboptimal, especially for HSP27#8 (Fig. 5.8). In agreement with my previous work in HeLa cells (Fig. 3.6) it appeared that only a small amount of HSP27 protein is needed in cells for normal IL-1-induced p38 MAPK activation (Fig. 5.8). The phospho-p38 MAPK membrane was stripped and re-probed for total p38 MAPK. Fig. 5.8 shows a time-dependent reduction in p38 MAPK expression at 10 and 20 min post-IL-1, however this is likely to be a re-probe effect because phosphorylated p38 MAPK runs at the same position as the total protein and would have been removed from the membrane during the stripping procedure. In conclusion despite having different effects on TAK1 both HSP27#5 and HSP27#7 siRNAs inhibited IL-1-induced p38 MAPK activation compared to Scramble-, mock-
Figure 5.7: HSP27#5 and #7 inhibit p38 MAPK activation; however HSP27#7 inhibits rather than prolongs the activation of IL-1-induced TAK1 in A549 cells. A549 cells were transfected with Scramble dsRNA and HSP27#5 and HSP27#7, siRNAs to deplete HSP27, using Lipofectamine 2000. 72 h post transfection the cells were stimulated with IL-1 and lysed with TAK1 lysis buffer. Equal amounts of total protein per sample were separated by SDS-PAGE and western blots were performed to detect phosphorylated p38 MAPK, p38 MAPK, phosphorylated TAK1, TAK1, HSP27 and α-tubulin. This experiment was performed once.
Figure 5.8: Analysis of IL-1 signalling in HSP27-depleted A549 cells using multiple siRNA oligonucleotides. A549 cells were left untransfected (Un), mock-transfected using Lipofectamine 2000 or transfected with Scramble (Scr) dsRNA, 10 nM siRNAs targeting different parts of the HSP27 sequence. HSP27#5 was used along with three siRNAs that had been used in the literature; HSP27#7, #8 and #9. Equal amounts of protein were separated by SDS-PAGE and western blot analysis was performed for phosphorylated p38 MAPK, p38 MAPK, HSP27 and α-tubulin. Fig. 5.8 was representative of two experiments.
transfected or untransfected cells. This is consistent with the results in HeLa cells and still points to a general mechanism of IL-1 signalling upregulation by HSP27.

5.4.2 The employment of modified siRNAs to try to resolve off-target effects in A549 cells

HSP27\#5 and HSP27\#7 siRNAs with the 2′-O-Me modification were synthesised by MWG Eurofin. The RNAi experiment was performed with one round of 10 nM siRNA using Lipofectamine 2000. 72 h post transfection the cells were stimulated with IL-1 and lysed in TAK1 lysis buffer. Western blot analysis was performed to examine the degree of HSP27 depletion by the modified siRNAs and p38 MAPK protein was analysed as a loading control. Modified HSP27\#5 efficiently depleted HSP27 in contrast to modified HSP27\#7 which did not (Fig. 5.9). The modified HSP27\#7 siRNA did not affect the expression levels of HSP27. Therefore, unfortunately, it was not possible to use this modification of the siRNAs to clarify whether both / one siRNA caused an off-target effect on IL-1-induced TAK1 phosphorylation in A549 cells.

5.5 Attempts to rescue the HSP27 RNAi phenotype

5.5.1 Transient transfections of HSP27 expression plasmids in HDFs

Only one siRNA had been used for most of the work in HeLa cells and HDFs. Therefore it was decided to try to rescue the HSP27 RNAi phenotype to find out if these results were caused by RNAi off-target effects. Unfortunately, no appropriate siRNA sequences could be found for the 3′ UTR of HSP27. Therefore an expression plasmid containing the DNA sequence of HSP27 with a silent mutation in the siRNA target region that rendered the mRNA resistant to HSP27\#5 whilst the amino acid sequence of the protein was unchanged (siRNA resistant, sRR) was used. If the ‘rescue’ experiments worked it was intended to use this technique to investigate the function of HSP27 phosphorylation in IL-1 signalling through the transfection of plasmids that encode the non-phosphorylatable and phospho-mimetic versions of the protein.

Previous work in the laboratory with SuperFect suggested that a 40 % transfection efficiency of HeLa cells with DNA could be achieved. It was believed that this would be sufficient to observe a partial restoration of IL-1-induced COX-2 protein expression after HSP27 depletion. Therefore initial work was performed using transient transfections. Rescue experiments were initially attempted in HDFs because these cells were thought to be more physiological model
Figure 5.9: HSP27 depletion with 2’ O-Methyl-modified siRNAs. A549 cells were transfected by Lipofectamine 2000 with either Scramble (S) dsRNA, HSP27#5 (#5), HSP27#7 (#7) or the latter two siRNAs modified by a 2’ O-Me group at position 2 on the antisense strand (#50 or #70). 72 h after transfection the cells were stimulated with IL-1 for the times shown and lysed in TAK1 lysis buffer. Samples were separated by SDS-PAGE and western blot analysis performed for HSP27 and p38 MAPK. This experiment was performed once.
than HeLa cells. IL-1-induced COX-2 protein expression was examined because the HSP27-dependent inhibition of IL-1-induced COX-2 had been more reproducible than p38 MAPK between experiments with HSP27#5 siRNA. Lipofectamine 2000 can be used for the co-transfection of both 10 nM siRNA and 1 μg DNA plasmid simultaneously, therefore this was the transfection reagent of choice. Cells were simultaneously co-transfected with siRNA and DNA using Lipofectamine 2000. 24 h later a second transfection of siRNA was performed. The cells were stimulated with IL-1 42 h after the second transfection. The lysates were analysed by western blot to detect COX-2, HSP27 and α-actin (Fig. 5.10). Transfection with HSP27#5 caused strong depletion of HSP27 protein (Fig. 5.10), however it was weaker than had been observed in previous experiments using HSP27#5 (Fig. 5.1). sRR HSP27 DNA resulted in strong expression of FLAG-HSP27 protein (Fig. 5.10) and the intensity of untagged HSP27 was also immersed. Surprisingly, transfection of cells with wild-type HSP27 DNA (WT) resulted in a similarly strong induction of both tagged and untagged HSP27 expression despite the fact that this plasmid does not confer siRNA resistance (Fig. 5.10). This may be because too much mRNA was produced for all the mRNA to be degraded. A plasmid expressing a FLAG-tagged form of HSP27 was used to try and distinguish between endogenous and exogenous HSP27. The FLAG-tag is an octapeptide that increases the relative molecular mass of HSP27 by about 1 kDa. However, FLAG-HSP27 resolved at a position on the gel corresponds to a protein of 32 kDa, for reasons that are unclear. The WT and sRR DNA also caused the expression of HSP27 that appeared to lack the FLAG tag (Fig. 5.10). This is probably because the expression plasmids contain an internal methionine start codon after the FLAG tag and hence express a mixture of FLAG-tagged and untagged HSP27.

HSP27#5 siRNA did not inhibit IL-1-induced COX-2 protein expression in these experiments (Fig. 5.10). This may be the result of the weaker depletion of HSP27 in this experiment. The difficulties in balancing transfection efficiencies for both siRNA and DNA suggested that rescue experiments would be difficult to perform in HDFs and may be more easily performed in fibroblasts from a HSP25-/- mouse, where it is necessary only to transfect the DNA.

5.5.2 Transient transfection of HSP27 DNA with calcium phosphate in HeLa cells

Since HDFs were found to be unsuitable for ‘rescue’ experiments a similar approach was taken using HeLa cells. To investigate whether the phenotype observed in HSP27-depleted HeLa cells could be rescued initially a calcium phosphate kit was used to transiently transfect the DNA. Previous attempts to co-transfect siRNA and DNA with Lipofectamine 2000 failed in HeLa cells due to toxicity. Therefore the calcium phosphate kit, believed to be a less toxic method of
Figure 5.10: Attempt to rescue inhibition of IL-1-induced COX-2 expression in HSP27#5-transfected HDFs by expression of exogenous HSP27 protein. 150 000 HDFs were seeded into each well 6 well plates, before transfection, 24 h later with either cells were left untransfected, mock-transfected or transfected with 10 nM Scramble dsRNA or HSP27#5 siRNA using Lipofectamine 2000. 24 h afterwards the cells were simultaneously co-transfected with siRNA and DNA. DNA expression plasmids that encode HSP27 (WT) or siRNA resistant HSP27 (sRR) were made up to 1 µg DNA using pCMV FLAG 2 empty vector (EV). 24 h later the cells were stimulated with IL-1 and lysed. Lysates were analysed by western blot to detect COX-2, HSP27 and α-actin. This figure is representative of three experiments.
DNA transfection, was tried. In this transfection procedure a precipitate forms that consists of calcium phosphate and DNA. The precipitate adheres to the cell surface (Graham and van der Eb, 1973) and is believed to be taken up by endocytosis (Loyter et al., 1982).

Cells were transfected with Scramble dsRNA and HSP27#5 siRNA and after 4 h of incubation the medium was replaced. 2 h later the cells were transfected with DNA using a calcium phosphate transfection kit (Sigma Aldrich) with wild-type HSP27 DNA (WT), siRNA-resistant (sRR) HSP27 and pCMV FLAG2 empty vector (EV). Two different amounts of HSP27 DNA were tried: 0.5 µg and 5 µg, to determine the amount of DNA that was needed to rescue HSP27 expression in siRNA-transfected cells to a similar level to the endogenous protein. WT and sRR plasmids expressed similar amounts of HSP27 (Fig. 5.11), as observed in Fig. 5.10. The total DNA transfected was made up to 5 µg per point with empty vector. COX-2 protein was detected in Scramble-transfected cells (Fig. 5.11). In the absence of the expression plasmid HSP27#5-transfected cells were strongly depleted of HSP27 protein (Fig. 5.11). HSP27#5 siRNA inhibited IL-1-induced COX-2 protein expression however this was not rescued by exogenous HSP27 expression (Fig. 5.11). This was surprising because it appeared as if rescue of HSP27 expression had been achieved and it was thought that this would be sufficient to observe a small rescue in COX-2 protein expression. One explanation may be that the transfection efficiency was worse than the predicted 40% with the calcium phosphate kit and the HSP27 seen on the blot is due to extremely high expression in relatively few cells.

5.5.3 Flow cytometry analysis to determine transient transfection efficiencies

To determine whether the failure to rescue IL-1-induced COX-2 protein expression in HeLa cells was due to poor transfection efficiency, flow cytometry was performed. In these experiments cells were transfected with the pmaxGFP plasmid, which encodes green fluorescent protein (GFP), instead of a plasmid that contains DNA to encode HSP27. GFP fluoresces at 509 nm when excited at 488 nm and thus the number of cells that express GFP may be detected by flow cytometry analysis. This method was used to examine the transfection efficiency with the calcium phosphate kit, compared to the commonly used transfection reagent, SuperFect, which others in the laboratory have shown gives a transfection efficiency of approximately 40%. As mentioned above it was thought that 40% transfection efficiency would be sufficient to observe a rescue effect however, it was not known if the calcium phosphate kit transfection efficiency was comparable to that of SuperFect.
Figure 5.11: Attempt to rescue inhibition of IL-1-induced COX-2 expression by re-introduction of exogenous HSP27 protein in HSP27#5-transfected HeLa cells. HeLa cells were transfected with Scramble dsRNA or HSP27#5 siRNA using Oligofectamine. After 4 h incubation the medium was replaced. 2 h later HeLa cells were transiently transfected with either 0.5 µg or 5 µg of a plasmid expressing wild-type HSP27 (WT), siRNA resistant HSP27 (sRR) or empty vector using a calcium phosphate kit or were left untransfected. The DNA was made up to 5 µg per point with empty vector. 72 h after DNA was added the cells were stimulated with IL-1 for 4 h and lysed in whole cell lysis buffer. Lysates were analysed by western blots to detect COX-2, FLAG, HSP27 and α-actin. The FLAG-tag causes a shift in mobility of HSP27. This figure was representative of two experiments.
HeLa cells were left untransfected or transfected with Scramble dsRNA. Cells were transiently transfected with 50 ng of a plasmid expressing GFP (pmaxGFP) made up to 5 µg or 1 µg of DNA using pCMV FLAG 2 empty vector for transfections using calcium phosphate or SuperFect respectively. Forward scatter and side scatter measurements gave information on the cell size and granularity. This allowed live cells only to be ‘gated’ and used for the analysis of GFP expression. A threshold of GFP expression was defined, 10 fluorescence intensity (relative units), and a gate (M1 on Fig 5.12) was used to count the proportion of ‘events’ (cells) that had a higher level of GFP fluorescence than the control untransfected cells in a set number of total cells counted. With this method calcium phosphate transfection was found to result in a greater number of cells with GFP expression (42 %) above the threshold compared to SuperFect (26 %) (Fig. 5.12). The transfection efficiency was not altered by the presence of Scramble dsRNA (data not shown). The histograms show that the calcium phosphate mediated transfection produced a broader spread of expression of GFP compared to SuperFect-transfected cells (Fig. 5.12). With the transfection levels achieved it would be expected that some rescue of IL-1-induced COX-2 protein expression would be observed.

The results from the flow cytometry analysis suggested that transfection efficiency may not account for the lack of rescue observed and therefore alternative rescue techniques may not overcome the difficulties encountered. At this point it was believed that the use of mouse embryonic fibroblasts (MEFs) from the HSP25−/− mouse would provide a more definitive strategy to confirm the results of HSP25/27 on IL-1 signalling.

5.6 Confirmation of the function of HSP27 in IL-1 signalling in HeLa cells through the use of multiple siRNAs

One reason that could account for the failure of the rescue experiments in HeLa cells was if the HSP27-depleted phenotype was an off-target effect. This was thought to be unlikely because initial work by others in the laboratory used six oligonucleotides against different regions of the HSP27 sequence to examine the effects of HSP27 depletion upon IL-1-induced gene regulation in HeLa cells. The three siRNAs (HSP27#1, HSP27#2 and HSP27#5) that efficiently depleted HSP27 all inhibited IL-1-induced COX-2, IL-6 and IL-8 expression (Alford, 2006). The results in the A549 cells confirmed that it is not sufficient to use one siRNA when using RNAi to examine a phenotype. This highlighted the need to confirm data on the enhancement of IL-1 signalling in HeLa cells by HSP27. Whilst waiting for the generation of HSP25−/− mice it was decided to use multiple siRNAs in HeLa cells. HSP27#7 siRNA was used because although it produced contrasting results to HSP27#5 in A549 cells it was the most efficient siRNA of those tried for HSP27 depletion. It was also found that HSP27#2 siRNA was more efficient at
Chapter 5  
HSP27 function in multiple cell types with controls for off-target effects

Figure 5.12: Comparison of the DNA transfection efficiency of HeLa cells using calcium phosphate or SuperFect. HeLa cells were transfected with Scramble dsRNA or left untransfected. 24 h later HeLa cells were transfected with 50 ng pmaxGFP expression plasmid using calcium phosphate or SuperFect. Cells were trypsinised 72 h after the DNA transfection and analysed by flow cytometry. In the histograms (right panel) the Y-axis (counts) represents the number of cells and the X-axis represents the fluorescent intensity detected. A. Scatter plot (left) to show cell population and histogram (right) to show light emission by GFP detected in FL1 by the untransfected cell population. B. Scatter plot and histogram to show GFP transfection efficiency in cells transfected with DNA using calcium phosphate. C. Scatter plot and histogram to show transfection efficiency in cells transfected with DNA using SuperFect. D. Table to compare the transfection efficiency of cells that were untransfected with siRNA and transiently transfected with DNA using the calcium phosphate kit or SuperFect. This figure was representative of two experiments.

<table>
<thead>
<tr>
<th>Sample</th>
<th>siRNA</th>
<th>DNA</th>
<th>DNA transfection reagent</th>
<th>Transfection efficiency (%)</th>
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<td>No</td>
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<td>1</td>
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<td>2</td>
<td>No</td>
<td>Yes</td>
<td>Calcium Phosphate</td>
<td>42</td>
</tr>
<tr>
<td>3</td>
<td>No</td>
<td>Yes</td>
<td>SuperFect</td>
<td>26</td>
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depleting HSP27 from cells when two sequential transfections were performed 24 h apart using 10 nM siRNA. Therefore HSP27#2 and HSP27#7 siRNA oligonucleotides were used to confirm the HeLa cell results previously obtained using HSP27#5.

HeLa cells were transfected with 10 nM HSP27#7 siRNA using Oligofectamine once. 72 h later the cells were stimulated with IL-1 and lysed in whole cell lysis buffer. Fig. 5.13 shows that HSP27 is strongly depleted in HeLa cells using HSP27#7 siRNA. The peak IL-1-induced phosphorylation of p38 MAPK on Thr180 and Tyr182 was inhibited in HSP27#7 compared to Scramble-transfected or untransfected cells (Fig. 5.13).

IL-1-induced JNK phosphorylation was focussed on because there had been a disparity in its regulation in HSP27#5-transfected HeLa and A549 cells. HeLa cells were transfected using Oligofectamine once with HSP27#7 and twice sequentially 24 h apart with HSP27#2 siRNA. With these conditions both HSP27#7 and HSP27#2 siRNAs efficiently depleted HSP27 from HeLa cells (Fig. 5.14). Cells were stimulated with IL-1 and lysed in TAK1 lysis buffer. IL-1-induced JNK phosphorylation was reduced in both HSP27#2 (Fig. 5.14A) and HSP27#7 (Fig. 5.14B) compared to Scramble-transfected cells.

TAK1 was the key point in the IL-1 signalling pathway regulated by HSP27 in HSP27#5 transfected cells. TAK1 kinase assays were performed on HeLa cell lysates from cells transfected with HSP27#2 and HSP27#7. Both siRNA oligonucleotides efficiently depleted HSP27 from the cells and inhibited TAK1 by 50 % or greater (Fig. 5.15, HSP27#7 data not shown). These results are in agreement with those where HSP27 was depleted with HSP27#5 siRNA (Fig. 4.1) and thus give confidence that the results in HeLa cells in chapter 3 / 4 are unlikely to be caused by off-target effects.
Figure 5.13: Depletion of HSP27 with HSP27#7 siRNA confirms the inhibition of IL-1-induced p38 MAPK in HSP27-depleted HeLa cells. HeLa cells were left untransfected (Un) or transfected with Scramble dsRNA or HSP27#7 siRNA using Oligofectamine. 72 h post transfection the cells were stimulated with IL-1 for the times shown and lysed in whole cell lysis buffer. Equal amounts of protein were separated by SDS-PAGE and analysed by western blot for phosphorylated p38 MAPK, p38 MAPK, HSP27 and α-tubulin. This experiment was performed once.
Figure 5.14: HSP27#2 and HSP27#7 inhibit IL-1-induced JNK activation in HeLa cells. HeLa cells were left untransfected (Un) or transfected with Scramble dsRNA, HSP27#2 (A) and HSP27#7 (B) using Oligofectamine. In B the cells are sequentially transfected 24 h apart with HSP27#2. The cells were stimulated with IL-1 and lysed in TAK1 lysis buffer 96 h after the cells were seeded. Equal amounts of total cellular protein were separated by SDS-PAGE and western blot analysis was performed for phosphorylated JNK, HSP27 and α-tubulin. This figure was representative of two experiments with HSP27#2 and three experiments with HSP27#7.
Figure 5.15: Transfection of HSP27#2 inhibits IL-1 induced TAK1 activity in HeLa cells. Lipofection was performed on HeLa cells with Oligofectamine using Scramble dsRNA or HSP27#2 and repeated 24 h later. 96 h after seeding the cells were stimulated with IL-1 for the times shown and lysed in TAK1 lysis buffer. TAK1 kinase assays were performed. The upper panel shows the phosphorimage data of phosphorylated recombinant MKK6. The lower two panels consists of western blots to detect HSP27 and α-tubulin after one tenth of the material used for the kinase assay separated by SDS-PAGE. This figure is representative of two experiments.
5.7 Discussion

Initial work to examine the function of HSP27 signalling was performed in HDF cells because these are a more physiological cell type. This showed that HSP27-depletion inhibited IL-1-induced COX-2 expression in agreement with data obtained using HeLa cells, indicating that this regulation was not HeLa cell specific. Unfortunately, the examination of the function of HSP27 in IL-1-induced p38 MAPK phosphorylation in HDFs was unclear due to inconsistent data on p38 MAPK expression. Therefore the focus of work turned to a third cell type, A549 cells. The kinetics of the IL-1 signalling appeared to be faster in A549 cells rather than HeLa cells. In HeLa cells the peak of TAK1 activity appeared to be at 30 min post-IL-1 stimulation (Fig. 4.1), whereas in A549 cells the peak was at 10 min IL-1, in closer agreement with the reports in the literature (Cheung et al., 2003). This adds confidence that A549 cells may be a more representative cell type than HeLa cells.

The results in A549 cells proved not to be simple because IL-1-induced MKK3 / MKK6 and p38 MAPK phosphorylation was inhibited in HSP27#5-transfected cells, whereas IL-1-induced TAK1 and JNK phosphorylation was enhanced / prolonged. The phosphorylation of ATF-2 was also enhanced by HSP27 depletion, which was unsurprising as it is reported to be a good substrate of JNK (Botteron and Dobbelaere, 1998). This disparity in the regulation of MKK3 / MKK6 downstream signalling compared to TAK1 and JNK was surprising because TAK1 is thought to phosphorylate and activate both MKK3 / MKK6 and MKK4/7. Therefore the effect of HSP27 depletion on the downstream kinases would be expected to be the same as for TAK1. These results may suggest that TAK1 may not be the most important upstream regulator of MKK3 / MKK6 in A549 cells and HSP27 may regulate a different MKKK. Alternatively, HSP27 may act at multiple points in the IL-1 signalling pathway and one (or more) of the regulatory points is different in HeLa cells and A549 cells. For example a regulated protein may be present in only one cell type and not the other. An alternative possibility is that HSP27#5 may cause cell type-specific off-target effects in either HeLa cells or A549 cells at the level of TAK1 and that the specific regulation is directly on MKK3 / MKK6. MKK3 / MKK6 could activate p38 MAPK leading to phosphorylation of the TAK1 binding protein TAB1 and cause negative feedback onto TAK1 (Cheung et al., 2003). If the feedback mechanism is only present in A549 cells but not HeLa cells it is possible that any regulation at the level of TAK1 would be masked in A549 cells but not HeLa cells.

An alternative siRNA was used to deplete HSP27 in an attempt to determine if the results with HSP27#5 were repeatable and not an off-target effect. However, it was found that HSP27
depletion using HSP27#7 resulted in the inhibition of both IL-1-induced p38 MAPK and TAK1 phosphorylations. This result was in agreement with the HeLa cell results (chapters 3 and 4) and therefore one is inclined to think that HSP27#5 causes a cell-type specific off-target effect in A549 cells. However, the possibility that HSP27#7 was instead exerting an off-target effect could not be ruled out. Unfortunately, other siRNAs that target HSP27 did not efficiently deplete HSP27 and therefore the analysis of HSP27 depletion upon IL-1-induced TAK1 phosphorylation could not be conducted with these conditions.

One conclusion that can be drawn from the work in the A549 cells is that it is likely that HSP27 regulates more than one point in the IL-1 signalling pathways. Regardless of which siRNA gave off-target effects, IL-1-induced TAK1 phosphorylation was regulated independently of the regulation of IL-1-induced MKK3 / MKK6 and downstream signalling by HSP27 in these cells. It could be that HSP27 acts as a scaffold protein to bring MKK3 / MKK6 into close proximity to TAK1 and facilitate the activation of MKK3 / MKK6 in this way. HSP27 has previously been reported to interact with MK2 and p38 MAPK (Zheng et al., 2006), therefore it is possible that HSP27 acts as a scaffold protein for MK2, p38 MAPK, MKK3 / MKK6 and TAK1.

The work in A549 cells highlighted the importance of correct controls in RNAi experiments and at this point it was decided to consolidate the work previously performed in HDF cells and HeLa cells. The ‘gold standard’ technique to confirm RNAi results is thought to be ‘rescue’ experiments. These can be performed in a variety of ways. Our approach was to perform RNAi and co-transfect an expression plasmid for the gene of interest with a silent mutation rendering it resistant to the siRNA. Initial work in HDF cells with Lipofectamine 2000 showed a lack of inhibition of IL-1-induced COX-2 in HSP27-depleted cells. In previous experiments where HSP27 knockdown had caused an inhibition in IL-1-induced COX-2 protein expression the depletion of HSP27 had been more complete. The transfections of siRNA alone were performed using 30-50 % confluent cells however the co-transfections of DNA and siRNA were performed using 90 % confluent cells. Co-transfection of siRNA and DNA into 40 % confluent cells caused a large number of cells to detach from the monolayer, which strongly suggests that these conditions were toxic to the cells. This suggested that Lipofectamine 2000 was not suitable for the purpose of siRNA and DNA co-transfection in HDFs. An additional difference that may account for the poor knockdown of HSP27 was that in the rescue experiments, unlike all previous RNAi experiments, the cells were lysed 72 h after the initial transfection compared to 96 h. Protein synthesis is naturally in balance with protein degradation, a process termed protein turnover. HSP27 may have a slow turnover rate and therefore at 72 h post-transfection sufficient HSP27 may still be present in the cell to upregulate IL-1 signalling.
Attempts to rescue the inhibition of IL-1-induced COX-2 protein expression in HeLa cells failed. There are many potential reasons that might account for this problem. The first is that insufficient cells were successfully transfected and could express FLAG-HSP27. However, analysis by flow cytometry suggested that 42% of cells were successfully transfected with the calcium phosphate kit. With this transfection efficiency it would still be expected to observe a partial rescue of the COX-2 expression. As this was not observed it suggests that an alternative problem is involved. One possibility is that the FLAG-tag on the HSP27 may interfere with the natural properties of HSP27. The FLAG–tag contains five aspartic acid residues and would therefore be negatively charged. This may prevent the HSP27 proteins from either forming the homooligomers that have previously been reported to be important for various functions (Rogalla et al.,) or the FLAG-tag may prevent an important interaction with a client protein. The expression of untagged HSP27 would be expected to rescue COX-2 expression. However, even if some untagged HSP27 was expressed it was possible that the FLAG-tagged form or highly elevated expression of untagged HSP27 might have had a dominant negative effect. There is often a delicate stoichiometry between a scaffold protein and its interaction partners. The disruption of this stoichiometry through overexpression of a protein may cause a dominant-negative effect (Kracht, personal communication).

Alternative strategies for rescue experiments include the use of the Penetratin-1 peptide to directly transfect protein into the HSP27-depleted cells. Alternatively an shRNA could be used to generate a stable depleted cell line, into which the protein or expression plasmid could be transfected. A further strategy would be to generate a stable cell line expressing the protein of interest (lacking the 3´ UTR) and use a siRNA against the 3´ UTR of the mRNA to deplete endogenous protein. The most efficient way would be to use a cell type that does not express HSP27 and stably overexpress HSP27 in this line. For example work in the Arrigo laboratory successfully generated stable cell lines that express wild type and mutant HSP25 in the murine fibroblast cell line, L929 (Mehlen et al., 1995). However, before this could be performed it would be necessary to check that the role of HSP25 in inflammation is the same in murine and human cells. It was possible that cells that do not constitutively express HSP27 lack the appropriate cellular components for the regulation of IL-1 signalling to take place. There have been comparatively few successful rescue experiments reported in the literature for reasons that are unclear. At this point it was still thought that MEFs from the HSP25−/− mice would be available by the end of the project and would be a better system to confirm the results.

The focus of work was returned to the verification of the HeLa cell results. Two additional HSP27 siRNAs; HSP27#2 and HSP27#7 were employed to examine HSP27 depletion in HeLa cells. Two sequential transfections of HSP27#2 siRNA 24 h apart improved the depletion of
HSP27 with HSP27#2 siRNA and facilitated the examination of IL-1 signalling regulation. In HeLa cells all three siRNAs used, HSP27#2, #5 and #7, inhibit IL-1 signalling including TAK1 activity, p38 MAPK and JNK activation. An individual siRNA sequence may result in a 1.5-4 fold change in the expression of dozens to hundreds of genes (Birmingham et al., 2006). A large-scale siRNA screen of the human signalling proteome found 5 % of siRNAs caused a change in either direction in transferin uptake and therefore in a worse case scenario 10 % of siRNAs would cause an off-target change on this readout (Galvez et al., 2007). Taken together and assuming that there is a 5 % chance of an siRNA having an off-target effect on any given gene, the probability of three siRNA sequences that are non-overlapping and target a different part of the HSP27 mRNA causing the same off-target effect is $0.05^3 (P = 0.000125)$. These results indicate that it is extremely unlikely that the regulation of the signalling in HeLa cells by siRNAs targeting HSP27 (Chapter 3 and Fig. 4.1) is caused by off-target effects.

In conclusion the results in this chapter emphasise the importance of including controls in RNAi experiments. Although it is difficult to perform rescue experiments, multiple siRNAs that target different parts of the target protein sequence should be used to increase the confidence in the RNAi results. The employment of multiple siRNAs was therefore a future strategy for the rest of the work in this thesis. The use of multiple siRNAs complemented the HSP27#5 siRNA results in HeLa cells and it is highly likely that HSP27 depletion inhibits IL-1-induced TAK1 activation and activity in these cells.
Chapter 6

The function of HSP27 in IL-1 signalling in human dermal fibroblasts
Chapter 6

The function of HSP27 in IL-1 signalling in human dermal fibroblasts

6.1 Introduction

The work in chapter 5 highlighted the importance of using controls for RNAi experiments. The lack of clarity in the function of HSP27 in A549 cells made it more important to find a way of reducing the toxicity of the transfection and improving the depletion of HSP27 in HDF cells, to try to confirm if the regulation of IL-1-induced TAK1 and downstream signalling was a general mechanism. Sequential transfection of siRNAs to deplete HSP27 with Lipofectamine 2000 proved successful but toxic in HDF cells (chapter 5), therefore Oligofectamine was tested as an alternative transfection reagent to see if the toxicity of transfection could be reduced.

6.2 Optimisation of HSP27 depletion in HDFs

The initial work in HDFs described in chapter 5 was performed using Lipofectamine 2000, a transfection reagent that is toxic to HeLa cells, because it had been used with some success in the laboratory previously. However, using the sequential method of transfection with Lipofectamine 2000 there was a variable response and some evidence of toxicity to the cells (chapter 5). HDF cells were left untransfected, treated with transfection reagent alone (mock) or transfected with 10 nM Scramble dsRNA or HSP27#5 twice 24 h apart using either Lipofectamine 2000 or Oligofectamine. The cell viability was determined by performing an MTT assay. Mock-transfection caused a decrease in viability of 25 % with Oligofectamine and 37 % with Lipofectamine 2000. However, the difference between the cell viability of mock-transfected cells compared to Scramble-transfected cells was not great with both transfection reagents. This suggests that it is the transfection reagent rather than the addition of dsRNA, which leads to the loss of cell viability. In the Oligofectamine-transfected cells there was little difference between the mock-, Scramble- and HSP27#5-transfected cells (Fig. 6.1), which suggests that the depletion of HSP27 in these cells does not affect cell viability. However, in the Lipofectamine 2000 transfected samples the transfection of HSP27#5 decreased the cell viability to less than 50 % (Fig. 6.1), which indicates that the combination of HSP27 depletion together with the stress of transfection with Lipofectamine 2000 leads to a decrease in cell viability. These results suggest that transfection with Oligofectamine improves cell viability, in particular when using HSP27#5 siRNA (Fig. 6.1), and therefore two sequential 10 nM siRNA transfections 24 h apart using Oligofectamine were used for work in this chapter.
Figure 6.1: Sequential siRNA transfection of HDF with Lipofectamine 2000 is more toxic than Oligofectamine. HDFs were left untransfected, mock-transfected, or transfected with 10 nM Scramble dsRNA or HSP27#5 siRNAs using either Lipofectamine 2000 or Oligofectamine as the transfection reagent. 24 h later the transfection was repeated. 48 h after the second transfection an MTT assay was performed on the unstimulated cells. This figure is representative of two experiments.
6.3 **HSP27 depletion inhibits IL-1-induced COX-2 and IL-6 protein expression in HDFs**

It was necessary to confirm that the change of transfection reagent from Lipofectamine 2000 to Oligofectamine did not affect the regulation of IL-1-induced COX-2 protein expression by HSP27 in HDFs. As mentioned previously, two different siRNAs were now used to control for off-target effects. Cells were stimulated with IL-1 for 4 h to allow time for the induction of the inflammatory mediators. A western blot was performed to detect COX-2 protein (Fig. 6.2A). IL-1 induced COX-2 protein expression in mock and Scramble-transfected fibroblasts (Fig. 6.2A). The expression of IL-1-induced COX-2 protein was strikingly reduced in HSP27#5- and HSP27#7-transfected fibroblasts (Fig. 6.2). Overall the results with Oligofectamine showed a greater depletion of HSP27 expression and a stronger inhibition of IL-1-induced COX-2 expression compared to the experiments with Lipofectamine 2000 (Fig. 5.1).

IL-6 and IL-8 are two other pro-inflammatory mediators which had been shown by a previous student in the laboratory to be regulated by HSP27 in HeLa cells (Alford, 2006). IL-6 and IL-8 protein in culture medium was measured by ELISA. From two experiments of untransfected or Scramble-transfected cells IL-1 induced an average of 6.2 ng ml\(^{-1}\) IL-6 and 10.6 ng ml\(^{-1}\) IL-8. IL-1-induced IL-6 and IL-8 protein expression was inhibited by 50% in cells depleted of HSP27 with HSP27#7 (Fig. 6.2B/C). IL-1-induced IL-6 protein expression was also inhibited by 50% in HSP27#5-transfected compared to Scramble-transfected or untransfected HDFs (Fig. 6.2B). However, IL-8 expression was not inhibited in HSP27#5-transfected cells (Fig. 6.2C). HSP27 depletion was greater in HSP27#7- rather than HSP27#5-transfected cells (Fig. 6.2A) and this result could imply that a greater depletion of HSP27 is required to inhibit IL-1-induced IL-8 protein expression.

6.4 **HSP27 depletion inhibits IL-1-induced p38 MAPK activation in HDFs.**

Previous experiments using Lipofectamine 2000 in HDFs showed an inhibition of IL-1-induced p38 MAPK phosphorylation in HSP27-depleted cells (Fig. 5.2). However, it was unclear if this was due to an effect on p38 MAPK protein expression. These experiments were repeated with the transfection reagent Oligofectamine. HSP27#2 was used as well as HSP27#5 and HSP27#7 because it was thought preferable to use three siRNAs to control for possible off-target effects. These conditions had improved the transfection efficiencies observed for the other siRNAs and it was thought this may lead to a good depletion of HSP27 with HSP27#2 siRNA. Sequential transfections of either HSP27#5 or HSP27#7 siRNA inhibited IL-1-induced p38 MAPK phosphorylation.
Figure 6.2: The effect of HSP27#5- or HSP27#7-transfection upon IL-1-induced COX-2, IL-6 and IL-8 protein expression in HDFs. HDFs were left untransfected or subjected to two rounds of siRNA transfection 24 h apart with Scramble dsRNA (Scr), HSP27#5 (#5) or HSP27#7 (#7) using Oligofectamine. A mock transfection containing transfection reagent only was also performed. 48 h after the last transfection, the cells were stimulated with IL-1 for 4 h and lysed in whole cell lysis buffer. A. SDS-PAGE and western blots were performed to detect COX-2, HSP27 and the loading control α-tubulin. Fig. 6.3A is representative of two duplicate experiments and a further repeat experiment. B/C. Culture medium was removed from the cells before lysis and used for analysis by ELISA to determine the amount of IL-6 and IL-8 expressed in culture medium from HSP27-depleted, Scramble-transfected or untransfected cells. Fig. 6.3B/C were each representative of two experiments.
phosphorylation compared to Scramble-transfected, mock or untransfected cells in three experiments (two experiments are shown in Fig. 6.3) at both 15 and 30 min post-IL-1. There was no effect of the HSP27 siRNAs on p38 MAPK protein expression (Fig. 6.3A). This suggests that HSP27 regulates IL-1-induced activation of p38 MAPK, rather than its expression. In the second experiment shown the inhibition of IL-1-induced p38 MAPK phosphorylation (Fig 6.3B) was greater than that of the first experiment (Fig. 6.3A) at 15 min post-IL-1. The inhibition of IL-1-induced p38 MAPK phosphorylation in HSP27-depleted HDFs also appeared to be greater at 30 min post-IL-1 in Fig. 6.3A compared with Fig. 6.3B, however because the signals were weak at this time point it was not possible to determine this with accuracy. HSP27#2 depleted HSP27 from the cells to a lesser extent than HSP27#5 or HSP27#7 as seen previously, and IL-1-induced p38 MAPK activation was not inhibited by this siRNA (Fig. 6.3).

6.5 The effect of HSP27 depletion on IL-1-induced JNK activation in HDFs

In HeLa cells depletion of HSP27 resulted in inhibition of IL-1-induced JNK phosphorylation and activity (Fig. 3.7). However, in A549 cells HSP27 depletion with HSP27#5 siRNA enhanced IL-1-induced JNK activation. It was therefore pertinent to examine the role of HSP27 in IL-1-induced JNK activation in HDF cells. Therefore the same samples used to analyse p38 activation (Fig. 6.3A) were used to assess JNK activation. IL-1-induced phosphorylation of JNK was detected by western blot. Depletion of HSP27 with HSP27#2 at both 15 and 30 min post-IL-1 and HSP27#7 at 30 min post-IL-1 had no effect on the IL-1-induced JNK activation (Fig. 6.4). In the case of HSP27#2 this was not unexpected because there had been no detectable effect of HSP27#2 on IL-1-induced p38 MAPK activation in these cells. At 15 min post-IL-1 stimulation HSP27#5 and particularly HSP27#7 appeared to increase p54 (and for HSP27#7 p46) JNK phosphorylation compared to the control conditions (Fig. 6.4). At 30 min post-IL-1 stimulation there was a weak inhibition of JNK phosphorylation in HSP27#5-transfected cells however the signal at the time point was weak in all conditions. Depletion of HSP27 with either HSP27#5 or HSP27#7 inhibited IL-1-induced p38 MAPK phosphorylation in the same samples (Fig. 6.3). In a second experiment HSP27#2 and HSP27#7 siRNAs had no effect on JNK phosphorylation (data not shown). However, depletion of HSP27 with HSP27#5 inhibited p46 JNK phosphorylation at both 15 and 30 min post-IL-1 stimulation. Therefore there was no strong and consistent effect (between siRNAs and experiments) on the IL-1-induced phosphorylation of JNK in HSP27-depleted HDFs. The lack of inhibition of IL-1-induced JNK phosphorylation in HSP27-depleted HDFs was unexpected because TAK1 is believed to be the MKKK responsible for the activation of both p38 MAPK and JNK and it was regulated in HeLa cells.
**Figure 6.3:** HSP27#5 and HSP27#7 siRNAs inhibited IL-1-induced p38 MAPK activation in HDFs. HDFs were subjected to two rounds of transfection. After 48 h incubation the cells were left unstimulated or stimulated with IL-1α for 30 min. The cells were lysed with kinase lysis buffer. Samples were run on a 10 % SDS-polyacrylamide gel and analysed by western blot for phosphorylation of p38 MAPK and HSP27. The membrane was re-probed for total p38 MAPK and α-tubulin. Short and long exposures of HSP27 are shown in A to highlight the difference in knockdown achieved with the three siRNAs in HDFs. A and B are two independent experiments. This experiment was performed three times and A was the most representative experiment.
Figure 6.4: HSP27 depletion has little effect on IL-1-induced JNK phosphorylation in HDFs. HDFs were left untransfected (Un), subjected to two rounds of mock-transfection or transfection of either 10 nM Scramble dsRNA, HSP27#2, HSP27#5 or HSP27#7 siRNAs. Cells were stimulated with IL-1 for the times shown. The cells were then lysed in TAK1 lysis buffer and lysates were separated on a 10% polyacrylamide SDS-PAGE gel and analysed by western blot for phosphorylation of JNK, total p38 MAPK and HSP27.
6.6 HSP27 depletion does not affect IL-1-induced TAK1 activity in HDFs

In HDFs the regulation of IL-1-induced JNK by HSP27 did not appear to be strongly regulated by HSP27, whereas in the same samples HSP27 depletion inhibited IL-1-induced p38 MAPK. Since TAK1 is thought to be the upstream activator of p38 MAPK and JNK in IL-1 signalling it was possible that TAK1 is not regulated by HSP27 in HDFs. Therefore it was important to determine if IL-1-induced TAK1 activity was affected by HSP27 knockdown in HDFs. The same samples used to analyse p38 MAPK activation (Fig. 6.3B and data not shown) were used to investigate the effect of HSP27 depletion on IL-1-induced TAK1 activity. An additional experiment was also carried out. In the three experiments performed IL-1-induced TAK1 activity was unaffected by HSP27 depletion (Fig. 6.5A and B). In two experiments the same samples showed inhibited IL-1-induced p38 MAPK activation upon HSP27 depletion (Fig. 6.3). In the third experiment examination of p38 MAPK phosphorylation was unsuccessful. These results suggest that either HSP27 specifically regulates p38 MAPK, but not JNK or TAK1, or that TAK1 may not be the MKKK responsible for both p38 MAPK and JNK activation in HDFs.

6.7 The effect of TAK1 depletion upon IL-1-induced p38 MAPK and JNK activation

It is not known if TAK1 activates p38 MAPK and JNK in human fibroblasts and studies in MEFs are controversial (Mendoza et al., 2008). It was decided to deplete TAK1 in HDFs to determine if TAK1 was the MKKK responsible for the activation of either / both p38 MAPK and JNK in these cells. TAK1 was depleted in HDFs using a siRNA described previously (Singhirunnusorn et al., 2005). TAK1 siRNA depleted TAK1 protein quite strongly however, the knockdown was not complete (Fig. 6.6). It should be noted that TAK1 protein from IL-1 stimulated cells shifts in mobility on SDS-PAGE relative to that from resting cells. TAK1 protein probably shifts in mobility as a result of an IL-1-inducible phosphorylation. In TAK1-depleted cells the IL-1-induced phosphorylation of p38 MAPK was inhibited at 30 min post-IL-1, with little effect at the peak time of phosphorylation, 15 min (Fig. 6.6). In the case of JNK, the upper band corresponding to phosphorylated 54 kDa JNK was inhibited in TAK1-depleted cells at both 15 and 30 min post-IL-1. This suggests that certain JNK isoforms in the cell are activated downstream of TAK1 in response to IL-1 stimulation. However, the stronger bands for phosphorylated JNK appeared to be barely affected by TAK1-depletion (Fig. 6.6).
Figure 6.5: HSP27 depletion does not inhibit IL-1-induced TAK1 activity in HDFs. HDF cells were left untransfected, mock transfected (transfection reagent alone), transfected with either Scramble dsRNA, HSP27#2, HSP27#5 or HSP27#7 siRNAs twice 24 h apart, stimulated with IL-1 and lysed with TAK1 lysis buffer. TAB1 was immunoprecipitated from the lysates and a TAK1 kinase assay performed. A. shows two of the phosphorimages from the TAK1 kinase assays performed. Western blots were performed to detect phosphorylated p38 MAPK, p38 MAPK, HSP27 and α-tubulin (Fig. 6.3) with 1/10th material used for the material for the kinase assays. These were the same samples used in Fig. 6.3 and the western blots are shown in that figure. B. The radioactive signal for the band that corresponds to MKK3 was measured from the phosphorimage with the AIDA software. The background was measured in the same size area directly above the MKK3 band. The background signal was removed from the signal for each P-MKK3 band. A graph of the results from three experiments is shown. Error bars are standard error of the mean for three experiments. One experiment extended the IL-1 time course to 240 min and because this was only one experiment no error bars are shown for those time points.
This result was confirmed in one experiment with an alternative TAK1 siRNA (data not shown). It is possible that a greater inhibition of JNK and p38 MAPK phosphorylation was not found in TAK1-depleted cells because the knockdown was not complete and the remaining TAK1 present could be sufficient for the activation of the downstream kinases. Alternatively, it may be that there is redundancy with one of the other candidate MKKKs such as ASK1 or the MEKKs. Overall, it appeared that the effects of TAK1 and HSP27 siRNAs on the phosphorylation of p38 MAPK were similar. The effects on phosphorylation of JNK did not correlate as closely but overall phosphorylated JNK was only weakly inhibited by either TAK1 or HSP27 siRNAs.

6.8 Identification of suitable antibodies for MKK3 and MKK6 kinase assays

IL-1-induced TAK1 activity was not inhibited in HSP27-depleted cells and was unlikely to contribute directly to the regulation by HSP27 of IL-1-induced p38 MAPK activation in HDFs. It was possible that the activation of MKK3 or MKK6 by TAK1 or another MKKK was enhanced by HSP27. Therefore it was important to determine if IL-1-induced MKK3 and MKK6 activity was inhibited in HDFs depleted of HSP27. Previous work to investigate MKK3 and MKK6 activation had used an antibody that detects the phosphorylation of the kinases at their active sites. This antibody did not consistently detect two bands corresponding to MKK3 and MKK6 respectively, which may suggest that they are either not expressed to equal amounts in the cell types used or not activated to the same extent. In some experiments it was difficult to detect a band at all. Failure in the use of this antibody led to the desire to design an assay to measure the kinase activity of the two upstream activators as an alternative.

The first difficulty encountered when finding a suitable antibody for this purpose was that MKK3 and MKK6 are highly homologous. There is little amino acid sequence that is specific to either kinase and therefore most antibodies that are commercially available detect both kinases. Previously our laboratory had raised antisera against five different peptide sequences, some of which were specific for MKK3 or MKK6 or were present in both kinases, however they had not been characterised. First the ability of the antisera to immunoprecipitate MKK3 or MKK6 was tested, using the same antiserum to try and detect the kinase from the immunoprecipitate or lysate by western blot. Only one antiserum appeared to successfully immunoprecipitate the kinase and also detect the immunoprecipitate in the lysate (Fig. 6.7A). This antiserum, designated RAW1, was raised against a sequence that is not present in MKK6 and therefore was thought to be specific for MKK3.
Figure 6.6: The effect of TAK1 depletion by RNAi on IL-1-induced p38 MAPK and JNK phosphorylation in HDFs. HDFs were subjected to two rounds of 10 nM siRNA transfection with either Scramble dsRNA or TAK1 siRNA #1 (and in some experiments also #2) or left untransfected. Cells were stimulated with IL-1 for the times shown or left untreated. Cells were lysed in TAK1 lysis buffer (see Materials and Methods). The samples were separated on a 10 % polyacrylamide SDS-PAGE gel and western blots were performed to detect total TAK1, phosphorylated p38 MAPK, phosphorylated JNK and α-tubulin. This figure was representative of three independent experiments. A second siRNA against TAK1 showed the same effect (data not shown).
Millipore sells an antibody which is suggested to be specific for MKK6. Cells were transfected with a DNA construct for MKK6 and the ability of each antibody to immunoprecipitate MKK6 or detect the MKK6 by western blot was analysed. The MKK6 antibody from Millipore successfully immunoprecipitated the exogenous MKK6 and detected this in the lysate (Fig. 6.7B). It was also possible to detect a very faint band that corresponds to endogenous MKK6 immunoprecipitated with the anti-MKK6 antibody (Fig. 6.7B). From this analysis it was also clear that the RAW1 (anti-MKK3) antiserum did not immunoprecipitate recombinant MKK6 or detect it by western blot and therefore was likely to be specific for MKK3 (Fig. 6.7C). The blots were stripped and re-probed with RAW1 to detect MKK3. From these data (not shown) it was clear that no immunoprecipitated MKK3 could be detected in western blot with the anti-MKK6 antibody; also no MKK6 could be detected in the lysate after stripping. This suggested that the antiserum against MKK3 and the anti-MKK6 antibody were specific for their respective kinases and could be used to specifically immunoprecipitate the respective target proteins in kinase assays. When the supernatants were removed from the immunoprecipitate and analysed by western blot it was also apparent that both anti-MKK6 antibody and anti-MKK3 antiserum completely removed all of the respective kinase from the lysate with the conditions used. Therefore these conditions were used as the basis for the MKK3 and MKK6 kinase assays (see Materials and Methods).

6.9 Depletion of HSP27 has no effect on IL-1-induced MKK3 activity

Antibodies/antisera had been characterised that could specifically immunoprecipitate either MKK3 or MKK6, therefore it was possible to examine the effect of HSP27 on their activities (Fig. 6.7). A kinase assay was performed to examine MKK3 activity with GST-p38 MAPK as the substrate. Two bands were observed on the gel corresponding to the addition of a \(^{32}\)P-labelled phosphate group on the GST-p38 MAPK substrate and p38 MAPK which had lost the GST-tag, when compared to the relative molecular mass markers run along side on the gel. However, both substrates showed the same pattern of phosphorylation in the kinase assay, therefore only the band corresponding to the GST-p38 MAPK has been displayed in Fig. 6.8. In three independent experiments it was observed that successful depletion of HSP27 had no effect on IL-1-induced MKK3 activity (Fig. 6.8).
Figure 6.7: Immunodepletion experiments for MKK3 and MKK6. Antibodies were tested for the ability to immunoprecipitate MKK3 (3) or MKK6 (6). A. Five antibodies were tested with the appropriate pre-immune (PI) control for the ability to immunoprecipitate MKK3 / MKK6 from HDF lysate. Lysate, supernatant and immunoprecipitate were separated by SDS-PAGE and western blots were performed for MKK3 / MKK6. B/C. HDF cells were transfected either with empty vector or MKK6 expression plasmid. MKK3 was immunoprecipitated with RAW1 or MKK6 was immunoprecipitated with anti-MKK6 antibody (Millipore). Western blot analysis was performed to detect MKK6 using the same anti-MKK6 antibody used for immunoprecipitation.
Figure 6.8: Depletion of HSP27 has no effect on IL-1-induced MKK3 activity in HDFs. HSP27 was depleted from HDFs using two sequential Oligofectamine-mediated siRNA transfections 24 h apart. The dsRNA, Scramble, was used as a control or the cells were left untransfected. Cells were stimulated with IL-1 for the times shown. MKK3 was immunoprecipitated using RAW1 antiserum overnight and a kinase assay was performed with recombinant GST-p38 MAPK as the substrate. The results were visualised and quantified by phosphorimage (A). 1/10th material used for the kinase assay was separated by SDS-PAGE. Western blots were performed for HSP27 and the loading control, α-tubulin.
6.10 Difficulties encountered with HSP27 depletion in HDFs

At this point the efficiency of HSP27 depletion significantly reduced (Fig. 6.9A). It was unclear why this had occurred because there was no obvious difference between the series of experiments that worked and the one that did not. Initially, the experiment was repeated in case a mistake had been made in the siRNA transfections, however the HSP27 depletion was still poor (Fig. 6.9B) COX-2 protein expression was analysed to determine if HSP27 depletion was able to regulate IL-1 signalling and gene expression because it was thought to be potentially more sensitive to HSP27 depletion than p38 MAPK phosphorylation. There was no regulation of IL-1-induced COX-2 protein expression in the experiment with poor HSP27 depletion (Fig. 6.9B). RNAs, including siRNAs in deprotected form, can degrade over time and the siRNA oligonucleotides in use had been deprotected and stored at -20°C for 6 months. Therefore new siRNAs oligonucleotides were bought from MWG and tested on HDFs. This did not improve the HSP27 depletion in HDFs (Fig. 6.9C) thus siRNA oligonucleotide degradation was not the cause of the loss of transfection efficiency. New batches of HDFs and Oligofectamine were purchased from Lonza and Invitrogen respectively. HSP27 depletion improved (Fig. 6.9D) and the problem of transfection efficiency in HDFs appeared to be solved. The improvement in transfection efficiency appeared sufficient to reproduce the inhibition of IL-1-induced COX-2 protein expression by HSP27 depletion in these cells (Fig. 6.9D).

6.11 Depletion of HSP27 has no effect on IL-1-induced MKK6 activity

It was possible that MKK6, rather than MKK3, is the dominant regulator of p38 MAPK activity in HDF cells therefore a MKK6 kinase assay was performed. In this experiment the HSP27 was strongly depleted by HSP27#5 and HSP27#7 siRNAs (Fig. 6.10). IL-1-induced MKK6 activity was not inhibited in HSP27-depleted cells under conditions where IL-1-induced p38 MAPK activation was weakly inhibited at 30 min post-IL-1 (Fig. 6.10). Although, the HSP27 depletion was better than Fig. 6.9A-C it was not as good as the initial experiments Fig. 6.3. This might account for the weaker inhibition of IL-1-induced p38 MAPK phosphorylation observed compared to Fig. 6.3. These results suggest that HSP27 does not regulate IL-1-induced signalling at the level of MKK3 and MKK6 in HDFs. Therefore it is likely that HSP27 augments IL-1 signalling to COX-2 protein expression in HDF cells through the direct regulation of p38 MAPK activation. The phosphorimage was much weaker for MKK6 than MKK3, suggesting that MKK3 may be the more important activator of p38 MAPK in HDFs.
Figure 6.9: **Difficulties in HSP27 depletion in HDFs.** HSP27 was depleted from HDFs using two sequential siRNA transfections 24 h apart. The controls were leaving the cells untransfected, mock-transfection or transfection of Scramble dsRNA. In C new, recently deprotected, siRNA and Scramble dsRNA was used. In D new cells from Lonza and a new batch of Oligofectamine was used. In four experiments (A-D) cells were lysed in whole cell lysis buffer, separated by SDS-PAGE and analysed by western blot for HSP27 and COX-2. All HSP27 blots shown were 5 min exposures.
Figure 6.10: HSP27 depletion has no effect on IL-1-induced MKK6 activity in HDFs. HSP27 was depleted from HDFs using two sequential Oligofectamine transfections of the siRNAs, HSP27#5 and HSP27#7, 24 h apart. The dsRNA, Scramble, was used as a control or the cells were left untransfected. The cells were stimulated with IL-1 for the times shown. MKK6 from the samples was immunoprecipitated overnight and a kinase assay was performed using recombinant GST-p38 MAPK as the substrate. A. The results were analysed by phosphorimage. 1/10th of the material used for the immunoprecipitation was separated by SDS-PAGE and analysed by western blot to detect phosphorylated p38 MAPK and HSP27. B. Graphical representation of MKK6 activity. This experiment was performed once.
One potential concern with both the MKK3 and MKK6 assays was auto-phosphorylation of the GST-p38 MAPK substrate. Auto-phosphorylation has been reported in the literature (Ge et al., 2002), however it is thought to be a TAB1-dependent process, competitive with the ability of p38 MAPK to bind to MKK3 (Lu et al., 2006). In one experiment the reaction mixture including GST-p38 MAPK was incubated on protein G beads that had never been in contact with lysate. There was no phosphorylation of the substrate observed with unstimulated samples (data not shown). The phosphorylation of the substrate was reduced in immunoprecipitates that had been stimulated with IL-1 for 60 min, which makes it unlikely that autophosphorylation of the substrate was induced following MKK3-mediated phosphorylation. It is unlikely that autophosphorylation of the GST-p38 MAPK substrate is responsible for the lack of regulation by HSP27 observed, although it could possibly mask a weak effect of HSP27 depletion on MKK3 / MKK6 activity.

6.12 MKK3 is a more important activator of p38 MAPK than MKK6 in IL-1 signalling in HDF cells

Two experiments were performed to try to determine if MKK3 or MKK6 is the major activator of p38 MAPK in response to IL-1 in HDFs. This was investigated by performing a kinase assay under conditions where the two antibodies completely immunodeplete their respective kinase from the lysate, thereby ensuring that the total pool of each kinase in the cell was directly compared (Fig. 6.11A). IL-1 stimulation resulted in a much greater induction of MKK3 compared to MKK6 (Fig. 6.11). This result would suggest that MKK3 is likely to be the more important of the two kinases in the activation of p38 MAPK by IL-1 in HDFs.

6.13 p38 MAPK is a negative regulator of TAK1 activity in both HeLa cells and HDFs

The role of p38 MAPK in the negative regulation of TAK1 activity through the phosphorylation of TAB1 has been previously mentioned as a mechanism whereby pro-inflammatory signalling may be switched off (Cheung et al., 2003). It was possible that HSP27 is able to augment IL-1 signalling by activating both p38 MAPK and TAK1 directly. It was hypothesised that the regulation of TAK1 by HSP27 could be disguised in HDF cells by reduced negative feedback on TAK1 by p38 MAPK in HSP27-depleted HDFs. Therefore the apparent difference in TAK1 activity in HDF and HeLa cells following HSP27 depletion may be due to the respective presence or absence of the feedback mechanism respectively. The existence of this feedback mechanism in the two cell types was tested by pre-incubation of HDF or HeLa cells with the p38 MAPK inhibitor, SB202190, before stimulation with IL-1 and analysing signalling downstream of TAK1 (Fig. 6.12). At the low dose used, 1 μM, this inhibitor is thought to be
Figure 6.11: M KK3 rather than M KK6 is the major activator of p38 MAPK in HDFs.

HDFs were stimulated with IL-1 for the times shown. Cells were lysed in whole cell lysis buffer. 80 µg total cellular protein was immunoprecipitated with antibodies against either M KK3 or M KK6 overnight. A kinase assay was performed for M KK3 and M KK6 using recombinant GST-p38 MAPK as the substrate. A. 8 µg total protein was analysed by western blot for either total M KK3 and M KK6 in both the lysate and the supernatant removed after the immunoprecipitation, in order to check the immunodepletion of the antigen during the immunoprecipitation. B. Graphical representation of M KK3 and M KK6 activity. Data shown above are representative of two experiments.
relatively specific for p38 MAPK (Davies et al., 2000). DMSO was used as a vehicle control. In both cell types the pre-incubation with SB202190 caused an increase in the phosphorylation of both p38 MAPK and JNK at 60 min post-IL-1 stimulation (Fig. 6.12). In HDF cells phosphorylation of JNK at 30 min post-IL-1 was also increased (Fig. 6.12). These data are consistent with negative feedback by p38 MAPK on TAK1 occurring in both cell types. These results suggest that the feedback mechanism may be present in both the cell types tested and therefore the presence or absence of the feedback loop cannot account for the differences in signalling in the absence of HSP27 in these cells.

6.14 Examination of the subcellular localisation of p38 MAPK in HDFs

An alternative mechanism whereby HSP27 may enhance the activation of p38 MAPK is through the regulation of p38 MAPK localisation in the cell. The p38 MAPK activators, MKK3 and MKK6 are reported to be present in both the cytoplasm and the nucleus. p38 MAPK is thought to be activated in the nucleus and then transported to the cytoplasm by its substrate MK2 (Ben-Levy et al., 1998). There have been reports that HSP27 can retain p38 MAPK in the cytoplasm (Gorska et al., 2007). If this was the case then in HSP27-depleted cells more p38 MAPK would be expected to be found in the nucleus than in untransfected or scramble-transfected cells. To investigate the hypothesis subcellular fractionation studies were performed.

It was first decided to analyse the nuclear-cytoplasmic distribution of p38 MAPK using a detergent lysis method. In this method a buffer containing 0.5 % IGEPAL CA-350 was used to rupture the cytoplasmic membrane. Once the cytoplasmic fraction was removed, the nuclear pellets were washed and nuclear protein was extracted with a buffer containing a high concentration of salt (0.42 M sodium chloride). The samples were then analysed by western blot for p38 MAPK, MKK3 and MKK6. HSP27 is thought to be predominantly cytoplasmic, except under conditions of heat stress, and therefore could be used as a cytoplasmic marker (McClaren and Isseroff, 1994). Histone H1 was used as a nuclear marker because this is a DNA-binding protein, localised only in the nucleus. From the localisation of the nuclear and cytoplasmic markers the cell fractionation appeared to be effective (Fig. 6.13). MKK3, MKK6 and p38 MAPK (both phosphorylated and total protein) were localised in the cytoplasmic fraction (Fig. 6.13). In another experiment the wash step was omitted in case any material was lost in this step, however this made no difference (data not shown). In further experiments the nuclei were sonicated in either nuclear extraction buffer, as used above, or in whole cell lysis buffer for 10 min to try and shear the DNA and release more proteins in this fraction for analysis however, this made no difference to the localisation of p38 MAPK observed (data not shown).
Figure 6.12: p38 MAPK blockade increases phosphorylation of p38 MAPK and JNK in IL-1-treated HDF and HeLa cells. HeLa cells (A) or HDFs (B) were pre-treated with either 0.01% DMSO, 0.1% DMSO, 1 µM (in 0.01% DMSO) or 10 µM (in 0.1% DMSO) of the p38 MAPK inhibitor, SB202190, for 1 h, or left untreated and then stimulated with IL-1 for the times indicated. Western blots for phosphorylated p38 MAPK, phosphorylated JNK and total p38α MAPK (as a loading control) are shown.
Figure 6.13: Cell fractionation by detergent lysis to determine the localisation of p38 MAPK in HDF cells. HDF cells were cultured on 10 cm dishes until confluent. The cells were stimulated with IL-1 for the times indicated. The cells were initially scraped gently into a cytoplasmic lysis buffer that contains 0.5 % IGEPAL CA-360 to penetrate the cytoplasmic membrane. The cell lysate was centrifuged at 400 × g and the cytoplasmic fraction was removed. The nuclear pellet was washed twice to remove cytoplasmic proteins before being burst in a nuclear lysis buffer that contains a high concentration of sodium chloride (0.42 M). For cytoplasmic fractions equal amounts of total protein were loaded onto a 10 % polyacrylamide SDS-PAGE gel. Cell equivalents of nuclear fractions were also loaded. The samples were separated by SDS-PAGE and analysed by western blot for phosphorylated p38 MAPK, total p38 MAPK, MKK3, MKK6, HSP27 and the nuclear marker, Histone H1.
This result was unexpected because p38 MAPK is reported to be nuclear in unstimulated cells and is believed to shuttle between the cytoplasm and the nucleus upon stimulation with arsenite or anisomycin (Ben-Levy et al., 1998). MKK3 and MKK6 have also been reported to be present in both the nucleus and the cytoplasm. It was possible that the detergent caused the kinases to “leak” out from the nuclei. Therefore an alternative fractionation method was used. The same distribution of p38 MAPK and HSP27 was found between the nuclear and cytoplasmic fractions when cells were lysed by Dounce homogenisation (data not shown). These results could indicate that there are differences in the localisation of these proteins between cell types. Alternatively, it is possible that p38 MAPK leaks from the nucleus into the cytoplasm in both of these cell fractionation techniques because for Dounce homogenisation the cells were not preswollen in an isotonic buffer and this may also have caused proteins to leak out from the nuclei. It would be expected, if this was the case, that there would at least be some residual, detectable p38 MAPK left in the nucleus.

A lot of the information reported on p38 MAPK localisation was obtained by immunofluorescence (Ben-Levy et al., 1998; Gorska et al., 2007). Therefore it was decided to use this technique to determine if the cellular localisation of p38 MAPK in HDFs was the same as that observed using detergent lysis or Dounce homogenisation. This technique should also overcome the difficulties encountered in the examination of HSP27-depleted cells because individual cells can be visualised by confocal microscopy. The aim was to judge the knockdown of HSP27 on a cell-by-cell basis and analyse the results accordingly. The cells were cultured on microscope slides, stimulated with IL-1 and fixed. The same antibodies that were used to detect total p38 MAPK or HSP27 by western blot were used to immunostain the respective proteins because both antibodies only detected one band of the correct relative molecular mass as their target protein by western blot analysis. Propidium iodide was used to stain nuclei. Alexa-fluor-conjugated secondary antibodies raised against the appropriate species were used. In experiments where the detection of multiple proteins within the same cell was required then secondary antibodies conjugated to fluorophores with different excitation and emission wavelengths were used.

Fig. 6.14 shows cells stained with the above-mentioned antibodies. In each strip a greyscale image is shown of a section of cells stained with either anti-p38 MAPK or anti-HSP27 antibodies or propidium iodide. The overlay shows the propidium iodide stain coloured red and the antibody stain in green and where the two stains co-localise the overlaid colour should appear as yellow. HSP27 was found to localise entirely with the cytoplasm (Fig. 6.14) in agreement with the other fractionation techniques (Fig. 6.13). In contrast the anti-p38 MAPK antibody stained both the nucleus and the cytoplasm (Fig. 6.14) and the staining appeared to be
more intense in the nucleus for all cells observed. This was seen in all three confocal experiments performed. In another experiment the secondary antibody alone was incubated on the fixed cells under the same conditions used and very little background staining was observed. The microscope settings were fixed between each condition examined with the same stain to enable the slides to be directly compared. However, it was difficult to eliminate slide-to-slide variation in the efficiency of staining. The nuclear to cytoplasmic localisation of p38 MAPK observed in Fig. 6.14 did not change considerably following IL-1 stimulation. Western blot analysis of lysates obtained from the same experiment confirmed that IL-1 did induce p38 MAPK phosphorylation in this experiment (data not shown). In Fig. 6.14B after IL-1 stimulation it appeared, by visual observation, that there was a slight shift of p38 MAPK from the cytoplasm to the nucleus. In another experiment images were taken of multiple cell sections from each slide and the ImageJ software used to determine the number of pixels of fluorescence in the cytoplasm and nuclei. There was no statistically significant difference found in the nuclear:cytoplasmic ratios of p38 MAPK under any of the conditions examined (data not shown). Staining with the anti-phospho-p38 MAPK antibody did not show any induction of phosphorylation in response to IL-1 stimulation, which indicates that this antibody is not suitable for confocal microscopy under the conditions used (data not shown). An attempt was made to examine HSP27-depleted HDFs (data not shown). However, it was not possible to draw conclusions from this experiment due to problems with the phospho-p38 MAPK staining of the cells, as mentioned above.

6.15 HSP27 depletion enhances MKP1 expression in HDF cells

The previous results indicate that HSP27 enhances IL-1-induced p38 MAPK phosphorylation. One way that this may occur is through the inhibition by HSP27 of a phosphatase that targets p38 MAPK. A candidate phosphatase is MKP1 for which p38 MAPK is a substrate (Franklin and Kraft, 1997). HSP27 was depleted from cells and the lysates were analysed by western blot for MKP1 expression. HSP27 was strongly depleted from the cells transfected with either HSP27#5 or HSP27#7 siRNAs (Fig. 6.15). In Fig. 6.15 peak MKP1 expression occurred at 60 min post-IL-1 and at this time it did not appear to be regulated by the presence or absence of HSP27. In contrast in resting cells and the other times of stimulation examined, except 30 min post-IL-1, depletion of HSP27 appeared to result in a small increase in MKP1 expression. A kinase assay was performed to check IL-1-induced p38 MAPK activity in the same samples that were analysed for MKP1 expression (Fig. 6.15A and B). In this experiment IL-1-induced p38 MAPK activity was inhibited by about 50% in cells transfected with either HSP27#5 or HSP27#7 siRNAs at both 15 min and 30 min.
Figure 6.14: Confocal microscopy analysis of p38 MAPK and HSP27 subcellular localisation in HDFs. HDF cells were grown on microscope slides. The cells were stimulated with IL-1 for the times shown before fixation onto the microscope slide. The cells were permeabilised and incubated in primary antibody against HSP27 or total p38 MAPK. Excess antibody was washed off and the cells were incubated with the appropriate Alexa-Fluor conjugated secondary antibody and the nuclear stain propidium iodide. The alexa-fluor conjugate was excited by a laser at the appropriate wavelength and a camera was used to record the fluorescence emission from the cells. The panels above show the immunofluorescent staining of p38 MAPK and HSP27 from different cell sections at A. 0 min IL-1, B. 15 min IL-1 and C. 30 min IL-1. The right-hand panel shows the overlay of each protein stain with propidium iodide.
post-IL-1 (Fig. 6.15B). p38 MAPK activity returned to basal levels at 60 min, which correlates with the time of peak IL-1-induced MKP1 expression.

This experiment was repeated an additional three times (data not shown). In the second experiment HSP27 depletion was less efficient (residual protein appearing on a 2 min rather than 30 min exposure). In this experiment HSP27 depletion only correlated with a marginal enhancement of MKP1 expression 120 min post-IL-1 with HSP27#5 and 30 min post-IL-1 with HSP27#7. In this experiment HSP27#5 slightly inhibited IL-1-induced p38 MAPK phosphorylation at 30 min post-IL-1 and HSP27#7 had no effect. In a third experiment residual HSP27 was apparent on a 5 min exposure, HSP27#5 had no effect on MKP1 expression, HSP27#7-transfected samples exhibited a marginal increase in MKP1 at 30 and 60 min post-IL-1 and no effect on p38 MAPK phosphorylation. In a fourth experiment neither IL-1-induced MKP1 expression was increased nor was IL-1-induced p38 MAPK activity inhibited when HSP27 was depleted. In experiment four the HSP27 depletion was poor. The reduction in HSP27 depletion in the last three experiments may have affected the regulation of p38 MAPK phosphorylation and possibly MKP1 expression. The reason the variability in HSP27 knockdown was unclear. Possibly it was due to the passage number of the fibroblasts which may cause the depletion of HSP27 to be weaker than observed in early experiments. However, the effect of HSP27 depletion on MKP1 expression and p38 MAPK phosphorylation did, in general, seem to inversely correlate. There also appeared to be a correlation in the strength of effects on p38 MAPK activation and MKP1 expression. Therefore it was possible that HSP27 may be able to regulate MKP1 expression.

6.16 Analysis of the regulation of MKP1 mRNA expression by HSP27

The presence of HSP27 in HDFs appeared to inhibit the expression of MKP1 protein. Therefore it was possible that HSP27 exerted its effects through the regulation of MKP1 transcription; mRNA stability; translation or protein turnover. HSP27 was depleted with sequential transfection of HSP27#7 siRNA. HSP27#7 was used because this was the most effective siRNA for HSP27 depletion. The intention was to confirm any interesting results with HSP27#5 siRNA in the future. Cells were stimulated with IL-1, or left untreated and RNA extracted. Real-time polymerase chain reaction was used to quantify the MKP1 mRNA expression (Fig. 6.16). GAPDH mRNA was measured as a control. Fig. 6.16A is representative of two experiments and shows that MKP1 mRNA expression was not affected by the depletion of HSP27. In order to determine if HSP27 had been successfully depleted in these cells one well of Scramble- and HSP27#7-transfected cells each was stimulated with IL-1 for 30 min, lysed and analysed by western blot. HSP27 was depleted very strongly by HSP27#7 siRNA however, when the
Figure 6.15: Effect of HSP27 depletion upon MKP-1 protein expression in HDFs. HSP27 was depleted from HDFs using two sequential Oligofectamine transfections 24 h apart with either HSP27#5 (5) or HSP27#7 (7), or the control dsRNA, Scramble (S). After 72 h the cells were stimulated with IL-1 for the times shown. A. A p38 MAPK kinase assay was performed as outlined in materials and methods and the phosphorimage shown. The samples were analysed by western blot for MKP1, p38 MAPK and HSP27. B. Graphical representation of the p38 MAPK kinase activity. Weak upregulation of MKP1 was observed in HSP27-depleted cells in three experiments, albeit at different time points.
membrane was analysed for the phosphorylation of p38 MAPK, this was found not to be inhibited in HSP27-depleted cells. Therefore it was impossible to ascertain from this experiment whether HSP27 regulates MKP1 mRNA expression, or if the regulation lies at the level of translation or protein turnover.

At this point it was a concern that the inhibition of IL-1-induced p38 MAPK activation upon HSP27 depletion with either HSP27#5 or HSP27#7 was no longer reproducible. One reason may be that the depletion achieved by the siRNAs was reduced however this was difficult to measure quantitatively because the analysis had been performed by western blot and enhanced chemiluminescence. Alternatively, new HDFs were raised every 10-12 passages and it may be that certain batches of HDFs were less responsive to HSP27 depletion.

### 6.17 Investigation of the interaction between p38 MAPK and MKK3

An alternative mechanism whereby HSP27 could regulate p38 MAPK was also examined at the same time. The indication that HSP27 regulates p38 MAPK activation but not MKK3 activity suggested that in the absence of HSP27, MKK3 may not be able to bind and phosphorylate p38 MAPK: HSP27 may act as a scaffold protein as suggested by Zheng et al., who described a complex containing p38 MAPK, MK2 and HSP27 (Zheng et al., 2006). It was decided to try and co-immunoprecipitate MKK3 and p38 MAPK and investigate whether HSP27 promoted the interaction between the two proteins. SAK7b, the antibody from p38 MAPK kinase assays, was used to immunoprecipitate p38 MAPK from whole cell lysates and RAW1 (anti-MKK3) antiserum was employed to detect co-immunoprecipitated MKK3 by western blot. From two experiments it was determined that p38 MAPK and MKK3 form a constitutive interaction which is not disrupted by stimulation with IL-1 for 30 min (Fig. 6.17). An experiment was then performed in order to determine if this complex was disrupted in the absence of HSP27. Analysis of the supernatants after the immunoprecipitation of p38 MAPK revealed that HSP27 was strongly depleted in HSP27#5 or HSP27#7-transfected cells. The membrane was probed with an antibody against phosphorylated p38 MAPK and there was reduced phosphorylated p38 MAPK detected in the supernatant from samples immunoprecipitated with the MKK3 antiserum compared to the pre-immune anti-serum. This agrees with the data from the immunoprecipitates that suggest that p38 MAPK specifically forms a complex with MKK3 (Fig. 6.17). However, no inhibition of IL-1-induced p38 MAPK activation was observed in HSP27-depleted cells (Fig. 6.17).
Figure 6.16: HSP27 depletion does not significantly effect MKP1 mRNA expression in HDFs. Cells were depleted of HSP27 using two sequential Oligofectamine transfections of the siRNA oligonucleotide HSP27#7 or transfected with the dsRNA control, Scramble. Cells were stimulated with IL-1 for the times shown. A. RNA was isolated and analysed by qRT-PCR for MKP1 and GAPDH. This graph is shows the mean of two experiments. B. Scramble- and HSP27#7-transfected cells that had been cultured at the same time as those for analysis by qRT-PCR were lysed in kinase lysis buffer. Samples were separated by SDS-PAGE and analysed by western blot to detect phosphorylated p38 MAPK, HSP27 and α-tubulin.
Figure 6.17: **MKK3 and p38 MAPK form a constitutive interaction in HDF cells.** HDFs cells were sequentially transfected with Scramble (S) dsRNA, HSP27#5 (#5) or HSP27#7 (#7) siRNAs with Oligofectamine. Cells were stimulated with IL-1 for the times shown and lysed in whole cell lysis buffer. The lysates were incubated with SAK7b antiserum for 2 h to immunoprecipitate p38 MAPK. The supernatant was removed, separated by SDS-PAGE and analysed by western blot for phosphorylated p38 MAPK and HSP27. The immunoprecipitate was washed five times with a high salt concentration lysis buffer to remove non-specific interactions, before separation by SDS-PAGE. Analysis by western blot was performed to detect MKK3 (RAW1 antiserum) and total p38 MAPK (Cell Signalling Technology). This experiment was performed once.
6.18 Phosphorylation of p38 MAPK was not inhibited by HSP27 in the MKK3 kinase assay experiments

The concerns about the lack of reproducibility of the inhibition of IL-1-induced p38 MAPK phosphorylation in HSP27-depleted HDFs in the later work prompted the re-examination of experiments that had been used to determine the effect of HSP27 on MKK3 activity (Fig. 6.8). This is a pivotal result because it was used to underline the importance of p38 MAPK as the potential point in IL-1 signalling regulated by HSP27. The membranes that had been used to check HSP27 depletion and loading were stripped and re-probed for phosphorylated p38 MAPK. From this analysis it appeared that the IL-1-induced phosphorylation of p38 MAPK detected on the re-probed membranes was not inhibited upon HSP27 depletion (Fig. 6.18). In the two experiments successfully re-probed IL-1-induced MKK3 activity was also unaffected.

6.19 Analysis of the role of HSP25 phosphorylation in IL-1 signalling through the use of MK2⁻/⁻ MEFs

The irreproducibility of p38 MAPK regulation by HSP27 in late experiments led to the use of an alternative strategy to examine the function of HSP27 in IL-1 signalling. Initially, it was thought that the role of HSP27 phosphorylation in IL-1 signalling could be examined through the use of rescue experiments or the reintroduction of mutant proteins into HSP25⁻/⁻ MEFs. Unfortunately these techniques had either not been successful or were unavailable during the time of my PhD. It was thought that the phosphorylation of HSP27 could be involved in a positive feedback onto the pathway. Therefore it was decided to try a different approach using MK2⁻/⁻ MEFs. MK2 is thought to be the principal kinase that phosphorylates HSP25 / HSP27 in response to IL-1 stimulation. It was therefore thought that the MK2⁻/⁻ MEFs would facilitate the examination of the function of HSP25 phosphorylation in fibroblasts.

Immortalised MK2⁻/⁻ MEFs that had been stably transfected with wild-type and mutant forms of MK2 were used for these experiments. Three cell lines were analysed; mock-infected with the retro-virus; infected with kinase-dead MK2 or wild-type MK2. These lines were derived from the same parent line of MEFs and allowed for the controlled comparison of phenotypes between cells that either possess or lack the ability to phosphorylate HSP25. MK2⁻/⁻ MEFs displayed reduced p38 MAPK protein expression (Fig. 6.19), in agreement with a previous report suggesting that MK2 stabilises p38 MAPK protein (Kotlyarov et al., 2002). This difference is independent of MK2 kinase activity because total p38 MAPK expression levels in cells that express a kinase-dead or wild-type construct of MK2 were similar (Fig. 6.19), again this is in agreement with Kotlyarov et al (Kotlyarov et al., 2002). Phosphorylation of p38 MAPK and
Figure 6.18: HSP27 depletion has no effect on IL-1-induced p38 MAPK phosphorylation in the M KK3 kinase assay experiments. 1/10th of material used in for the M KK3 kinase assays graphically illustrated in Fig. 6.8B was separated by SDS-PAGE and analysed by western blot for HSP27 and α-tubulin. One of these experiments is shown above. The membrane was stripped and re-probed for phospho-p38 MAPK. The phosphorimage from the M KK3 kinase assay which corresponds to the western blot samples is shown in the upper panel. This figure is representative of two experiments.
JNK peaked at 15 min post-IL-1 stimulation and at this time point was almost unaffected by the expression of MK2, with only slightly elevated p38 MAPK phosphorylation in cells that express the kinase-dead construct (Fig. 6.19). As the total expression of p38 MAPK was reduced in cells lacking MK2 this indicates that p38 MAPK is phosphorylated to a greater extent in the absence of MK2 than in its presence. The total levels of JNK were not measured by western blot and therefore it is not clear if MK2 has a role in the stabilisation of JNK as well. MKP1 was expression was greater in MK2-/- MEFs at all time points of IL-1 stimulation examined, in particular 30 min and 60 min post-IL-1 stimulation.

Analysis of HSP25 phosphorylation in the MEF lines showed that the peak phosphorylation of HSP25 occurred at 15 min post-IL-1 (Fig. 6.19). A non-specific band was observed of relative molecular mass 30 kDa instead of 25 kDa. However, although this phosphorylation was inhibited in MK2-/- cells it was not absent, suggesting that MK2 may be partially redundant in the MEFs. One possible kinase that could account for the residual phosphorylation in the MK2-/- cells is the homologue of MK2, MK3, which has been demonstrated to have similar substrate specificity (Ronkina et al., 2008). Surprisingly, when the membrane was stripped and re-probed for total HSP25 protein it was discovered that the small heat shock protein was expressed at high levels in MK2-/- cells, slightly reduced levels in kinase-dead MK2-mutant expressing cells and at much reduced levels in cells that express wild-type MK2 (Fig. 6.19). The differences in HSP25 expression in the different conditions observed make it more difficult to determine if HSP25 phosphorylation has a role in IL-1-induced signalling in these cells. It also indicates that a much greater proportion of HSP25 is phosphorylated in cells expressing wild-type MK2 than suggested by the phospho-HSP25 blot. In MK2-deficient MEFs there is a high proportion of phosphorylated p38 MAPK at 15 min post-IL-1 relative to total protein levels. These are cells that express the lowest level of phosphorylated HSP25 tested. In the cells that express the wild-type MK2 there is the greatest proportion of phosphorylated HSP25 and proportionately less phosphorylation of p38 MAPK. These results suggest a possible role for phosphorylation of HSP25 in switching off regulation of the pathway by HSP27 / 25.
Figure 6.19: Investigation of the role of HSP25 phosphorylation in IL-1 signalling in MK2⁻/⁻ MEFs. MK2⁻/⁻ MEFs were supplied by Dr. Alexy Kotlyarov. The MEFs provided had been produced following infection of MK2⁻/⁻ MEFs with retroviruses expressing empty vector (mock / M), a kinase-dead mutant of MK2 (KD) or wild-type MK2 (W). Cells were grown until confluent and stimulated with IL-1 for the times shown. Cells were lysed in whole cell lysis buffer. Equal amounts of each lysate were analysed by western blot for phosphorylated HSP25, JNK and p38 MAPK. The blots were also probed for total p38 MAPK, HSP25, MKP1 and α-tubulin. This figure is representative of three experiments however HSP25 expression was only examined once.
6.20 Discussion

The use of sequential transfection 24 h apart with Oligofectamine partially overcame toxicity problems associated with Lipofectamine 2000 and allowed the function of HSP27 in IL-1 signalling to be studied in HDFs. In initial work strong depletion of HSP27 by two siRNAs inhibited IL-1-induced COX-2 protein expression and p38 MAPK activation in HDFs, in agreement with the data from HeLa and A549 cells (chapters 3 and 5). This was confirmed when Oligofectamine was used as the transfection reagent in this chapter. These results suggest that augmentation of IL-1-induced COX-2 expression and p38 MAPK activation is a general mechanism of IL-1 signalling regulation shared in human epithelial cells and fibroblasts.

HSP27 depletion in HDF cells was shown to inhibit IL-6 and IL-8 protein expression in HDF cells, as had been previously observed in HeLa cells (Alford, 2006). However, HSP27#5-transfection, in contrast to HSP27#7-transfection, did not inhibit IL-1-induced IL-8 expression. One possible explanation for the effect of HSP27-depletion on IL-6 and IL-8 protein expression may lie with the p38 MAPK-dependence of the expression of the particular pro-inflammatory protein. Previous work from this laboratory using the p38 MAPK inhibitor SB203580 had found that of the three proteins examined, expression of COX-2 was the most and that for IL-8 the least dependent upon p38 MAPK activity (Ridley et al., 1997).

IL-1-induced TAK1 activity in HDF cells was not inhibited by two different siRNAs that target HSP27, which indicates that the regulation of IL-1 signalling by HSP27 in HDF cells is different to that in HeLa cells. The possibility that TAK1 was not the MKKK responsible for the activation of p38 MAPK in these cells was investigated. p38 MAPK phosphorylation at 30 min post-IL-1 was inhibited by TAK1 depletion, however at 15 min post-IL-1 there was no apparent difference, reminiscent of the results in HSP27-depleted HDFs. One explanation for the incomplete dependence of p38 MAPK phosphorylation on TAK1 may be the presence of residual TAK1 protein. Or alternatively, there is some redundancy between TAK1 and another MKKK. There was only a weak depletion of the p54 JNK isoform by TAK1-depletion in HDFs and JNK phosphorylation was again only weakly inhibited by HSP27 depletion in HDFs. In contrast, work by other members of the laboratory in HeLa cells showed a strong dependence of p38 MAPK and JNK phosphorylation on the presence of TAK1 (S. Glennie, unpublished). Therefore it may be that in HDF cells there is a preferential activation of p38 MAPK over JNK, whereas in HeLa cells both pathways appear to be strongly activated by TAK1.

In the experiment in which IL-1-induced MKK6 activity was examined phosphorylation of p38 MAPK was weakly inhibited in HSP27-depleted cells. In two experiments that examined
MKK3 activity IL-1-induced p38 MAPK phosphorylation was unaffected by HSP27-depletion however, the HSP27 depletion was weaker than the first MKK3 kinase assay for which the anti-phospho-p38 MAPK re-probing of the membrane was unsuccessful. The first experiment analysed for MKK3 kinase assay activity had an equivalent depletion of HSP27 to the MKK6 kinase assay experiment. Therefore, it is likely that IL-1-induced MKK3 and MKK6 activity is unaffected in HSP27-depleted cells. These results suggest that in HDF cells, in contrast to HeLa cells, IL-1-signalling upstream of p38 MAPK may not be inhibited in the absence of the small heat shock protein. This further suggests that the mechanism of regulation of IL-1-induced p38 MAPK activation in HDF cells might be at the level of p38 MAPK itself, or lie between the MKK3 / MKK6 and p38 MAPK. An interaction was found between MKK3 and p38 MAPK in HDFs and HSP27 may be required for the formation or maintenance of this complex. However, the possibility that IL-1-induced MKK3 activity is actually inhibited under certain conditions in HSP27-depleted HDFs cannot be ruled out from my results. If MKK3 activation is regulated by HSP27 in HDFs this could argue for a mechanism in which HSP27 regulates the TAK1 complex in both HeLa cells and HDF cells. For example in HeLa cells this regulation could directly affect TAK1 activation, whereas in HDF cells the activation of MKK3 and MKK6 could be regulated by HSP27.

p38 MAPK activity was inhibited with SB202190 and prolonged IL-1-induced p38 MAPK and JNK activity was observed in both cell types. This was indicative that the mechanism whereby p38 MAPK phosphorylates TAB1 leading to the inactivation of TAK1 is present in both cell types. An alternative explanation is that the expression of a phosphatase, MKP1, which targets both p38 MAPK and JNK is reduced following p38 MAPK blockade. This has been previously reported to occur (Hu et al., 2007). Another possibility for the difference in regulation of IL-1 signalling in the two cell types is the presence of a protein in HeLa cells that mediates the effects of HSP27 onto TAK1, which is not expressed in the protein complement of HDF cells. However, the regulation of p38 MAPK phosphorylation in all three cell types examined suggested that this is likely to be a site of important regulation, potentially linked to the regulation of inflammatory mediator expression including COX-2. Therefore possible mechanisms for the regulation of p38 MAPK by HSP27 were examined.

HSP27 has been previously reported to regulate the localisation of p38 MAPK (Gorska et al., 2007). It was reported that HSP27 retained p38 MAPK in the cytoplasm after IL-1 stimulation (Gorska et al., 2007). This presented as a possible mechanism for HSP27 action in IL-1 signalling because it would be expected that retention of p38 MAPK in the cytoplasm may cause it to co-localise with upstream activators. When p38 MAPK localisation was examined with subcellular fractionation by either detergent lysis or Dounce homogenisation all of the p38
MAPK was localised to the cytoplasm and this did not alter upon IL-1 stimulation. This was surprising given the reports in the literature. Therefore it was decided to analyse the nuclear-cytoplasmic localisation of p38 MAPK by confocal microscopy, a method of choice to examine subcellular localisation. The results from confocal microscopy were in closer agreement with the reports in the literature (Ben-Levy et al., 1998; Gorska et al., 2007) because in these experiments the stain for p38 MAPK was present in both the cytoplasm and the nucleus. The nuclear staining appeared more intense, however HDFs have a large surface area and the volume of cytoplasm was much larger than the nucleus and therefore the staining between the two compartments was similar. However, the concentration of p38 MAPK may more important than the overall amount present within each compartment. Once again the reported shift in p38 MAPK localisation upon stimulation was not observed. HSP27 appeared to be localised to the cytoplasm using confocal microscopy, dounce homogenisation or detergent lysis. There have been some reports that phosphorylated HSP27 can be recruited to nuclear speckles (Bryantsev et al., 2007). Although some weak nuclear staining can be seen with the anti-HSP27 antibody this staining is not punctuate and is likely to be caused by bleed through.

It was not clear why the cell fractionation techniques yielded different results, however each has potential flaws. In the detergent lysis and Dounce homogenisation it is possible that proteins, which are not tightly associated with DNA, leak into the cytoplasm. The potential difficulty, which may account for the observed localisation of p38 MAPK in confocal microscopy, is the presence of non-specific staining. An anti-phospho-p38 MAPK antibody showed a similar pattern of staining to the total p38 MAPK antibody raised in different species, which may add confidence to the confocal microscopy results. However, this phospho-specific antibody did not show induction of p38 MAPK phosphorylation upon IL-1 stimulation, although the western blot from the same experiment did. The lack of clarity in p38 MAPK localisation led to the abandonment of this approach. Unfortunately, it could not be concluded whether HSP27 was able to regulate the subcellular localisation of p38 MAPK in this system. Nevertheless, my results do not concur with and may raise doubts about the validity of observations made by others (Gorska et al., 2007) on the regulation of p38 MAPK localisation by HSP27.

Another potential mechanism is the inhibition of a phosphatase to dephosphorylate p38 MAPK in the presence of HSP27. MKP1 was chosen as the candidate because it is widely reported to dephosphorylate p38 MAPK. MKP1 protein levels were slightly increased in HSP27-depleted cells even in the absence of stimulation in an experiment where IL-1-induced p38 MAPK activity was strongly inhibited. The increase in MKP1 expression appeared to correlate with the degree of inhibition of IL-1-induced p38 MAPK phosphorylation observed. This was slightly surprising because there is strong evidence that MKP1 dephosphorylates JNK as well as p38
MAPK and IL-1-induced JNK activation did not appear to be inhibited to a significant extent following HSP27 depletion. The increase in MKP1 expression levels was small however, MKP1 is a potent phosphatase and this small increase may be sufficient to reduce p38 MAPK phosphorylation. Unfortunately, the regulation of IL-1-induced p38 MAPK by HSP27 became less reproducible and it was therefore not possible to get convincing repeats of the MKP1 protein expression or examine the regulation of MKP1 at the mRNA level.

The regulation of MKP1 or p38 MAPK itself may not be sufficient to explain the regulation of IL-1-induced COX-2 protein expression at 4 h post-IL-1 stimulation because at 60-240 min post-IL-1 stimulation p38 MAPK activity is so low it is difficult to detect in HDFs. Therefore it may be that the early IL-1-induced p38 MAPK activity, augmented by HSP27, activates MK2 and this activity persists in HDFs and is responsible for the stabilisation of COX-2 expression in these cells. However, IL-1-induced COX-2 protein expression may be more sensitive to HSP27 and the regulation of an alternative protein downstream of the peak of p38 MAPK activity may be more important for gene regulation. The regulation of early p38 MAPK activity by HSP27 may actually be the most important point for the enhancement of COX-2 protein expression. The problem of loss of p38 MAPK regulation also hindered the ability to examine if HSP27 depletion disrupted the complex between MKK3 and p38 MAPK, another potential mechanism.

MK2\(^{-/-}\) cells were used to examine the function of HSP25 phosphorylation in IL-1 signalling. The interpretation of these results was hindered by the variation in the total protein expression of p38 MAPK and HSP25 itself. The reduction in HSP25 expression in wild type MEF compared to MK2\(^{-/-}\) or kinase-dead MK2 mutant MEFs may be predicted to inhibit IL-1-induced p38 MAPK phosphorylation based on earlier work in this thesis. However, this was no observed. It is possible that, as seen for previous experiments on HSP27, the expression level of HSP25 in cells that express wild-type MK2 is not low enough to inhibit IL-1-induced p38 MAPK signalling. MK2 had been previously reported to have a role in stabilising of p38 MAPK (Kotlyarov et al., 2002). The higher levels of expression of HSP25 in cells that lack MK2 or MK2 kinase activity (kinase-dead mutant) may be a stress response, although there was no indication of a change in cell viability between the cell types. Upon enquiry with the laboratory that donated the cells it was discovered that HSP25 expression varies considerably between different MEF lines. When the differences in total protein expression were taken into account the results indicate that an increase in HSP25 phosphorylation correlates with a decrease in p38 MAPK phosphorylation. Therefore the phosphorylation of HSP27 may prevent further augmentation of the signalling pathway, acting as a potential switch. However, this role of phosphorylation does not fit with the early work in HeLa cells that suggests that late signalling (MK2) when HSP25 phosphorylation is very low is also regulated by the small heat shock
protein. Although it was not possible to determine if phosphorylation of HSP25 is required for full activation of p38 MAPK the enhanced MKP1 expression in cells lacking MK2 suggests that there may be a connection between MK2 activity and MKP1 protein expression.

An additional conclusion from work in this chapter was that MKK3 is a more important activator of p38 MAPK in HDF cells stimulated with IL-1. There has been a lot of controversy over which MKK is primarily responsible for the activation of p38 MAPK and there does appear to be cell type and stimuli-specific differences. It has been reported that in fibroblast-like synoviocytes dominant-negative mutants of both MKK3 and MKK6 have equivalent and non-redundant roles in p38 MAPK activation in response to IL-1 (Inoue et al., 2005). Targeted gene disruption studies in mice have shown that activation of p38 MAPK by TNF in MEFs requires both MKK3 and MKK6 (Brancho et al., 2003). MKK3−/− fibroblast-like synoviocytes demonstrated a dependence of TNF-induced p38 MAPK on MKK3 however in these cells IL-1β-induced p38 MAPK was MKK3-independent (Inoue et al., 2006). Therefore the relative importance of MKK3, MKK6 and possibly MKK4 (not examined in this study) appears to differ between conditions and cell type. If HSP27 regulation of IL-1 signalling is able to act at this level it is important to have confidence in the dependency of p38 MAPK on the MKKs in the appropriate cell type before further clarification of the mechanism.

The major problem encountered when working on HDFs was the variability in HSP27 depletion. A change to a new batch of Oligofectamine and low passage cells from Lonza appeared to restore the good HSP27 knockdown efficiencies. However, most of the HSP27 knockdowns after this time were not as efficient as the initial Oligofectamine experiments and this may account for the loss and variable strength of the regulation of IL-1-induced p38 MAPK by HSP27. It may be that only a small threshold of HSP27 is required for the regulation of IL-1-induced signalling and gene expression. Unfortunately it is not easy to determine the exact efficiency of the knockdown achieved. Some people use real time PCR to examine the mRNA levels however, this is not necessarily the same as protein expression because of residual protein left that had been translated before the siRNA was transfected into the cells.

One possibility for the reduction in HSP27 depletion efficiency that was not investigated was the change in batch of Biosera foetal calf serum. This could have altered the growth rate of the cells and directly affected the depletion efficiency of the RNAi experiments. Alternatively, this could have altered the signalling in response to IL-1. Another member of the laboratory has recently found that reducing the cell confluency causes a dramatic decrease in the IL-1-induced expression of MKP-1 in HDFs (R. Perelli, unpublished). Thus if the regulation of p38 MAPK by HSP27 was dependent upon MKP-1, this may depend upon the confluency of the cells.
Ways to improve the depletion of HSP27 in HDFs may include the use of three sequential transfections, increasing the dose of siRNA used, or using Amaza technology. None of these techniques are ideal. It may not be practical to do three sequential depletions of HSP27 24 h apart in HDFs. This could also increase the toxicity of transfection because the MTT assay showed that exposure to the transfection reagent was the main factor in the loss of cell viability. Increasing the amount of siRNA used both with Oligofectamine or the Amaza kit would increase the risk of off-target effects and the interferon response (Moss and Taylor, 2003; Persengiev et al., 2004). However, this work suggests that lipofection may not be the best approach for RNAi in HDFs.

Alternative depletion strategies include the use of short hairpin (shRNAs) to produce a stably depleted cell line or pool (Amarzguioui et al., 2005). The advantage with using shRNA to generate either a stable pool or a stable cell line expressing little or no HSP27 over the use of transient siRNA transfection would be that the degree of depletion achieved would be consistent for each experiment with the particular pool or cell line. shRNA RNAi uses RNA polymerase III promoters. The use of a tetracycline-inducible H1 promoter has facilitated the inducible knockdown of a protein from cells with shRNA (Amarzguioui et al., 2005) and allows for a control in the level of depletion achieved. Thus it would be possible to examine the threshold of HSP27 required to regulate IL-1 signalling. Although a shRNA approach would eliminate the difficulty of variable transfection efficiency between experiments, shRNAs are just as susceptible to causing sequence-specific off-target effects as siRNAs and therefore this strategy does not eliminate this significant problem. Multiple depleted cell lines would need to be used in order to ensure that off-target effects were not the cause of the phenotype being examined.

The loss of regulation of IL-1-induced signalling may be a result of difficulties in achieving a sufficient depletion of HSP27 in HDF, as seems likely from Fig. 6.9. This indicates that HSP27 does regulate IL-1-induced p38 MAPK and COX-2 expression and only a small amount of HSP27 is required. However, if the loss of regulation was related to confluence then it is unclear if the regulation would be present and relevant in vivo. It is difficult to determine what the confluence of dermal fibroblasts is in vivo and whether the contact with other cell types or the extracellular matrix would affect any regulation by HSP27. Therefore it would be of greater relevance and interest to pursue the investigation into the function of HSP27 / HSP25 in inflammation in HSP25−/− mice, currently being bred by our laboratory.

In conclusion work from this chapter confirmed that TAK1 has a role in the propagation of the IL-1 signal to p38 MAPK but not the major JNK isoforms in HDF cells. MKK3 was found to be a more important activator of p38 MAPK than MKK6 in HDF cells in response to IL-1. p38
MAPK activation and activity and COX-2 protein expression are inhibited in the absence of HSP27 in all three cell lines examined. The augmentation of p38 MAPK activation by HSP27 is likely to be an important general fine-tuning mechanism in IL-1-mediated signalling. The investigation into a possible mechanism of action of HSP27 suggested that HSP27 may regulate the expression of the phosphatase MKP1, however difficulties in achieving consistently strong depletion of HSP27 made this unclear. Further work is required to dissect this mechanism.
Chapter 7

Discussion
HSP27 has been shown to have many functions in the cell, for example cytoprotection, chaperone function and regulation of cell migration. For almost two decades pro-inflammatory stimuli, such as IL-1, have been known to cause the phosphorylation of HSP27. Changes in HSP27 expression and function have been reported for a large number of diseases, as discussed in the introduction. Many of these diseases involve an inflammatory process. However, whether and in what way HSP27 contributes to inflammation was unclear. Previous work in the laboratory using RNAi found that HSP27 was involved in IL-1-induced COX-2 and IL-6 mRNA stabilisation in HeLa cells (Alford, 2006). I have investigated how HSP27 regulates signalling pathways that are activated by IL-1, which may contribute to the expression of pro-inflammatory mediators such as COX-2.

Work in this thesis has shown a novel role of HSP27 in IL-1 signalling downstream of TAK1 in HeLa cells. The depletion of HSP27 by RNAi inhibited the IL-1-induced activation of TAK1, MKK4/7 (L. Rawlinson), MKK3 / MKK6 (J. Dean), p38 MAPK, JNK and MK2 but not ERK in HeLa cells. Three different siRNAs that target HSP27 were found to inhibit IL-1-induced TAK1 activity and JNK phosphorylation and two HSP27 siRNAs inhibited IL-1-induced p38 MAPK phosphorylation. The agreement of multiple siRNAs increases confidence that the results were not caused by off-target effects. Examination of HSP27 function in IL-1 signalling in HDF cells with two different siRNAs showed that HSP27 is not required for IL-1-induced TAK1 activation but is required for IL-1-induced p38 MAPK activation and COX-2 protein expression. The latter observation is important because HDFs are a more physiological system than HeLa cells. IL-1-induced p38 MAPK phosphorylation and COX-2 expression were also regulated by two HSP27-targeting siRNAs in a third cell type, A549 cells, suggesting that this regulation by HSP27 is a general mechanism.

Work to determine if IL-1-induced TAK1 activation was regulated in A549 cells, proved inconclusive. In HSP27#7-transfected A549 cells both IL-1-induced p38 MAPK and TAK1 phosphorylation were inhibited compared to control cells, in agreement with the HeLa cell data. This would suggest that in both epithelial cell types the regulation of IL-1 signalling was at TAK1 activation or above. However, in HSP27#5-transfected A549 cells IL-1-induced TAK1 phosphorylation was increased whilst MKK3 / MKK6 and p38 MAPK phosphorylation was inhibited. This suggests that HSP27 may target the event whereby TAK1 activates MKK3 / MKK6. The agreement of data obtained in A549 cells with HSP27#7 siRNA and HeLa cell data suggest that HSP27#5 rather than HSP27#7 siRNA causes an off-target effect in A549 cells. Unfortunately, a lack of other efficient siRNAs that target HSP27 prevented any verification of this hypothesis.
Further work would be required to elucidate the mechanism of HSP27 regulation of TAK1 and signalling upstream in epithelial cells. Recent work by Shi et al., suggests that HSP90 may also regulate IL-1 signalling through TAK1 (Shi et al., 2008). In this paper HSP90 inhibition is found to inhibit IL-1-induced MAPK activation, NF-κB activation and COX-2 protein expression through the destabilisation of TAK1. HSP90 has well-characterised chaperone functions and it is proposed that this chaperone activity is required for the stability of TAK1. HSP27 has also been proposed to have chaperone activity (Rogalla et al., 1999) however, there have been far fewer substrates identified for HSP27. Work in this thesis suggests that the chaperone function of HSP27 is not required for the regulation of IL-1-induced TAK1 activation because no effect was found on the protein expression of TAK1 upon HSP27 depletion in HeLa cells. HSP27 depletion also has no effect on the expression levels of TRAF6, IRAK1, IRAK4 or MyD88. This is not an exhaustive list of the upstream IL-1 signalling pathway components and thus further work would be required to determine if HSP27 can act as a chaperone for the IL-1R, TOLLIP, Ubc13-Uev1A.

Work in this thesis was unable to detect endogenous ubiquitination of either TRAF6 or IRAK1 and therefore it was not possible to determine if HSP27 was able to regulate these important points in signal transduction. Most reports in the literature use overexpression of ubiquitin or to a lesser extent the protein of interest. This approach was not used in this work because overexpression can generate artefacts and future work to examine the function of HSP27 in the process would require co-transfection of DNA and siRNA. It is difficult to optimise the co-transfection of DNA and siRNA as demonstrated by the attempts to rescue the COX-2 phenotype in HDF cells (chapter 5).

It is possible that the difficulty in the detection of endogenous ubiquitination was a result of technical difficulties, for example the immunoprecipitation of the wrong complex. It is important to immunoprecipitate proteins that had been bound to the receptor. If, in the experiments in this study, the immunoprecipitated proteins had not been involved in the initial signalling event from the receptor it is possible that endogenous ubiquitination may not have been observed. HSP27 is reported to interact with polyubiquitin chains and the 26S proteasome and degrade IκBα (Parcellier et al., 2003). The interaction between ubiquitin and HSP27 was shown in this report with lysates of U937 cells that overexpress HSP27 incubated with recombinant GST-ubiquitin. The same group also showed that HSP27 favours the ubiquitination and degradation of the cyclin-dependent kinase inhibitor, p27Kip1 (Parcellier et al., 2006). Gln31 and Gln190 of HSP27 have been shown to cross-link to Lys29 and Lys48 of ubiquitin in Alzheimer’s patients (Nemes et al., 2004) and therefore it was proposed that HSP27 could link
two polyubiquitin chains. If HSP27 could cross-link the polyubiquitin chains of TRAF6 and IRAK1 (or possibly TAK1 itself) this could be a potential way in which HSP27 could regulate IL-1 signalling, at least in terms of NF-κB activation. It would be interesting to determine if HSP27 could act in this way. The structure of the lysine 48 and lysine 63-linked polyubiquitin chains are thought to be very different (D. Komander, personal communication) and therefore it is unlikely that HSP27 would be able to recognise both linkages. In a recent report, overexpression of HSP27, TRAF6 and ubiquitin uncovered an interaction between HSP27 and TRAF6 (Wu et al., 2008). This interaction enhanced the ubiquitination of TRAF6 and is disrupted by the phosphorylation of HSP27 at Ser78 and Ser82. Although, I did not see an interaction between TRAF6 and HSP27, this would be a plausible mechanism to explain my results in epithelial cells. Further work is required to examine the possibility that HSP27 is acting as a scaffold in this way.

The data in this thesis may all suggest that HSP27 impinges on a signalling complex, involving MKK3 / MKK6, p38 MAPK, TAK1 and possibly TRAF6. For reasons outlined in chapter 6 it was not possible to be certain as to whether MKK3 was regulated by HSP27 in HDFs. However, MKK6, which was shown to be the minor activator of p38 MAPK in HDFs, was unaffected by HSP27 depletion. MKK3 was found to be in a complex with p38 MAPK in HDFs. It would be of interest to determine if TAK1 was also in this complex and if the complex was disrupted in HSP27-depleted HDFs. If such a complex also contained TAK1 the presence of HSP27 in HeLa cells may facilitate the binding of TAK1 from this complex to TRAF6 polyubiquitinated chains and thus lead to the activation of TAK1. In HDFs and A549 cells, HSP27 may be required for the interaction of MKK3 with either p38 MAPK or TAK1, leading to a loss of signalling in its absence.

The loss of regulation of IL-1 signalling and gene expression in HDFs prevented the thorough investigation of the regulation of IL-1-induced MKK3 activity by HSP27. This was likely to be a result of reduced efficiency of HSP27 deletion by RNAi, as discussed in chapter 6. My results suggest that there is a small threshold of HSP27 required for the enhancement of IL-1 signalling and therefore a near complete knockdown of HSP27 protein expression is required to observe the phenotype. HDFs are more difficult to transfect than either HeLa or A549 cells and therefore it was more difficult to achieve the required transfection efficiencies. Future work should either use three sequential transfections or an alternative technique such as Amaxa technology to overcome this difficulty. The regulation of MKK3 / MKK6 by HSP27 is a critical point and therefore it would be important, once the conditions of p38 MAPK regulation by HSP27 depletion have been re-established, to verify whether this occurs in HDF cells. Further work would need to be done to determine if the general mechanism of enhancement of IL-1 signalling
by HSP27 occurs at the activation of p38 MAPK or at the level of MKK3 / MKK6 / TAK1 activation.

The potential disparity between the regulation of IL-1 signalling in HeLa and HDF cells could indicate that HSP27 regulates multiple points in the signalling pathways activated by IL-1 in HeLa cells. HSP27 may enhance signalling at the level of TAK1 or above in HeLa cells and regulate p38 MAPK activation (or MKK3 activity / activation) in both cell types. This situation could arise due to the variation in the expression of proteins found in different cell types. For example, a protein that interacts with HSP27 to regulate signalling at or above the level of TAK1 may only be expressed in HeLa / epithelial cells and not HDF cells, leading to the apparent difference in the point of regulation by HSP27. IL-1-induced p38 MAPK was inhibited by multiple siRNAs in A549 cells suggesting that the enhancement of p38 MAPK activation is a general mechanism, because it was observed in three cell types.

The potential mechanisms of regulation of IL-1-induced MKK3/MKK6 or p38 MAPK by HSP27 could be similar. As mentioned before, HSP27 may act as a scaffold protein. HSP27 has been reported to act as a scaffold protein for p38 MAPK, MK2 and Akt (Zheng et al., 2006). Work in our laboratory was unable to find any inhibition of Akt following HSP27 depletion (S. Glennie, unpublished). This complex would be unlikely to explain an effect on either p38 MAPK or MKK3 / MKK6 activation. However, in HDFs I was able to detect a constitutive interaction between p38 MAPK and MKK3. In order to be sure that this was a bone fide interaction it would be important to check that using the complementary antibodies for immunoprecipitation and western blots could also result in a detectable interaction. One possible mechanism is that HSP27 promotes an interaction and facilitates the phosphorylation of p38 MAPK by MKK3.

If HSP27 sequesters a phosphatase, this could give rise to increased phosphorylation of either p38 MAPK or MKK3 / MKK6. Work in HDFs indicated that HSP27 depletion may cause a small increase in the expression of the dual specificity phosphatase, MKP1. The expression of MKP1 was strongly induced 30-60 min post IL-1 stimulation. However, a small amount of MKP1 was detected in resting cells and the basal expression appeared to be increased upon HSP27 depletion. One way that HSP27 could regulate MKP1 expression is through stabilisation of MKP1 mRNA. The MKP1 3’UTR contains an AU-rich element and is highly unstable. A recent report provides evidence that HSP27 can regulate and bind directly to the TNF ARE (Sinsimer et al., 2008). It is possible that MKP1 mRNA could be regulated in a similar manner. Further work is required to determine if the increase in MKP1 expression in HSP27-depleted cells is sufficient to account for the inhibition of p38 MAPK activity observed following HSP27
depletion. Regulation of MKP1 by HSP27 would provide a link between HSP27 and glucocorticoid action. MKP1 is also thought to dephosphorylate JNK. It is therefore surprising that IL-1-induced JNK phosphorylation was not inhibited by HSP27 depletion. The lack of regulation of TAK1 in HDFs argues that in these cells there must be a p38 MAPK pathway-specific mechanism instead. Alternatively, in HDFs, HSP27 could regulate another phosphatase that targets p38 MAPK / MKK3/ MKK6, but not JNK.

The importance of the phosphorylation of HSP27 in the enhancement of IL-1 signalling is unclear. Lasa et al. found that phosphomimetic mutants of HSP27, in which the serine phosphorylation sites are mutated to glutamate, cause stabilisation of COX-2 ARE reporter mRNA (Lasa et al., 2000). This stabilisation was weak when compared with overexpressed MKK6 or MK2. In this thesis MK2^{-/-} MEFs were used to address this question because MK2 is believed to be the principal kinase responsible for HSP25 / 27 phosphorylation. However, one difficulty which complicated the interpretation of the role of phosphorylation was the much increased expression of HSP25 in MK2^{-/-} MEFs compared to kinase-dead mutant MK2-expressing cells or wild-type MEFs. Further complicating the interpretation of the results was the requirement of MK2 for p38 MAPK protein stability, as previously reported (Kotlyarov et al., 2002). Taking into consideration the differences in total protein expression there is the greatest proportion of phosphorylated HSP25 and proportionally less p38 MAPK phosphorylation in the wild type MEFs. These results would suggest, in contrast to Lasa et al., that the phosphorylation of HSP25 / 27 may inhibit IL-1 signalling. Therefore one could postulate that unphosphorylated HSP27 (large oligomers) can enhance IL-1 signalling. However, the subsequent phosphorylation of HSP27 (dimers) by MK2 in response to the activation of the pathway, can act to switch off the regulation. Further work would be required to evaluate this hypothesis.

The findings of this thesis, summarised in Fig. 7.1, strongly indicate that HSP27 has a role in pro-inflammatory IL-1 signalling. Therefore HSP27 could be involved in inflammatory disease phenotypes and be a potential drug target for chronic and destructive inflammatory conditions. However, there have been several recent papers that claim HSP27 is an anti-inflammatory agent rather than a pro-inflammatory agent.
Fig. 7.1: Regulation of IL-1 signalling by HSP27. HSP27 enhances IL-1-induced p38 MAPK phosphorylation and activation in all cell types tested. HSP27 also enhances IL-1-induced TAK1 phosphorylation and activity in HeLa cells and possibly A549 cells. This positive regulation leads to the regulation of the gene expression and mRNA stability of a variety of IL-1-induced inflammatory mediators including COX-2 and IL-6.
Park et al., found that HSP27 associated with the IKKα and IKKβ complex in HeLa cells (Park et al., 2003). The high abundance of HSP27 can cause difficulties in the detection of specific interactions due to non-specific protein binding however, the association between HSP27 and IKKβ was increased by TNF-induced phosphorylation of HSP27, suggesting that this interaction is specific and induced by TNF. In this study depletion of HSP27 using HSP27#9 (in this thesis) increased TNF-induced IKK activity. The opposite result was found in our laboratory for IL-1 induced IKK activity in HSP27#5-transfected HeLa cells (Alford et al., 2007). In my experience HSP27#9 siRNA is very poor at depleting HSP27 from cells and had no effect on IL-1-induced p38 MAPK phosphorylation. Therefore these results do not fit with the pattern expected from work performed using HSP27#5 and HSP27#7 siRNAs in this thesis. However, the result was supported by an experiment overexpressing HSP27 and an NF-κB reporter construct. Overexpression of FLAG-tagged constructs of HSP27 which were mutated to prevent phosphorylation of HSP27 indicated that phosphorylation of HSP27 downregulated TNF-induced NF-κB and its association with IKKβ.

Sur et al. examine the role of HSP27 in the regulation of TNF signalling in keratinocytes (Sur et al., 2007). When HSP27 is depleted they find increased prostaglandin E2 in both resting and TNF-stimulated cells. Downregulation of HSP27 expression with the same siRNA used by Park et al. (Park et al., 2003) caused an increase in TNF-induced IL-8 protein expression that was mediated by an increase in NF-κB activity. They found an association between HSP27 and the IKK complex and postulated that this may be the mechanism for regulation. Sur et al. use 100 nM siRNA against HSP27, which is quite a high concentration and may cause off-target effects. This may have been necessary due to the weak depletion caused by the siRNA they used (HSP27#9), as seen in this thesis. Only one siRNA has been used and no attempt made to rule out off-target effects. Another potential reason for the difference in HSP27 function in inflammatory signalling compared to the work in this thesis may be the use of different cell types. As has been seen from this thesis, HSP27 appears to have a different function in the regulation of signalling upstream of p38 MAPK in HeLa cells and HDFs.

Su et al., reported that depletion of HSP27 from human lung microvascular endothelial cells did not have any effect on TNF-induced ICAM-1 or IL-8 expression (Su et al., 2008). In this study cells were transfected with a 60 nM pool of three siRNAs targeting different parts of the HSP27 sequence. This is not ideal because in the pool there are three sets of sequences which can cause off-target silencing. It is very difficult to directly compare knockdown efficiencies by western blot analysis, however the knockdown they find is not complete and from the experience of the
work in this thesis a residual level of HSP27 is often sufficient for the regulation of signalling. This has directly been shown in this thesis for IL-8 expression in HDFs.

Wu et al., overexpressed HSP27 in HeLa cells and found that it enhanced IL-1 stimulated phosphorylation of IKKα / IKKβ but not IL-1- or TNF-induced p38 MAPK or JNK phosphorylation (Wu et al., 2008). HSP27 is very abundant in HeLa cells and work in this thesis shows that only a small amount of the small heat shock protein is required to enhance IL-1-induced p38 MAPK signalling. Therefore swamping the system with yet more HSP27 would not be expected to have any effect on the MAPK signalling.

High levels (2 \( \mu \) g ml\(^{-1}\)) of exogenous HSP27 have been reported to stimulate the p38 MAPK-mediated production of IL-10 and to a lesser extent TNF (De et al., 2000). However, it is not clear that HSP27 would ever reach these extracellular levels in a physiological context. Exogenous HSP27 has also recently been reported to bind to the Scavenger Receptor-A and prevent acLDL uptake (Rayner et al., 2008). However, no one particular receptor has been proposed to act as a HSP27 receptor and it is possible that exogenous HSP27 is taken into the cell by pinocytosis and thereby acts in the same way as endogenous HSP27 in the enhancement of p38 MAPK signalling. Alternatively, it is possible that endogenous HSP27 is able to be secreted from the cell in response to IL-1 stimulation and act as an autocrine stimulus to increase p38 MAPK signalling, through an unknown receptor. However, it is unlikely that this mechanism would be sufficiently fast to account for the regulation of peak IL-1-induced signalling in my experiments. HSP27 secretion was detected in the medium of macrophages 1 h after the addition of 17\( \beta \)-estradiol but peaked after 24 h (Rayner et al., 2008). Further work would be required to elucidate exogenous and endogenous HSP27 act in the same way.

Epithelial cells are the first line of defence against invading microorganisms and these cells together with endothelial cells and fibroblasts have important roles in the repair response to injury and the defence against infection. HSP27 is expressed at high levels in all of these cells. Work in this thesis has highlighted an important role of HSP27 in enhancing the production of pro-inflammatory mediators in response to IL-1 and TNF. Previous work in the laboratory (Alford, 2006) and the experiments in this thesis demonstrate the importance of HSP27 in the expression of COX-2, IL-6 and IL-8. COX-2 is important in chronic inflammation (Mitchell et al., 1995) and rat adjuvant arthritis (Anderson et al., 1996). COX-2 catalyses the conversion of arachidonic acid into prostaglandin H\(_2\), the precursor of prostaglandins and thromboxane. Prostaglandins in turn mediate vasodilation. IL-6 is particularly important in the acute phase response and the T cell-dependent antibody response against certain viruses including vesicular stomatitis (Kopf et al., 1994). IL-8 is an important chemokine for neutrophils and therefore has
an important role in acute inflammation. The regulation of p38 MAPK (and JNK in HeLa cells) by HSP27 suggests an involvement of HSP27 in the expression of all the inflammatory mediators downstream of these MAPKs. This includes the regulation of adhesion molecules on the vascular endothelium, important in selecting leukocytes to migrate to the site of infection, through binding to leukocytes and initiating leukocyte rolling before extravasation. Another laboratory found a decrease in the expression of vascular cell adhesion molecule (VCAM)-1 in HUVECs depleted of HSP27 with RNAi (Gorska et al., 2007). VCAM-1 selectively recruits T cells and eosinophils and thus adds further evidence for a role of HSP27 in chronic inflammation. HSP27 may also have a role in the expression of matrix metalloproteinases, the expression of several of which has been shown to be p38 MAPK-dependent (Ridley et al., 1997; Underwood et al., 2000). One report of suggests that HSP27 overexpression up-regulates the expression and activity of MMP-9 (Hansen et al., 2001). MMPs have been reported to have many functions in inflammation (Parks et al., 2004). MMPs can degrade the extracellular matrix, however recently MMPs have also been implicated in the modulation of cytokine and chemokine activity and establishing chemokine gradients (Parks et al., 2004). Therefore the function of HSP27 in the regulation of IL-1-induced signalling and gene expression may be important in both acute and chronic inflammation. An involvement in chronic inflammation would suggest a potential role of HSP27 in diseases such as RA and Crohn’s disease, although further work is needed to determine if this hypothesis is correct.

Important work to resolve whether HSP27 is important in inflammation in vivo is the generation of HSP25−/− mice. Unfortunately, although these mice were in the process of being made during the course of this work, they were not generated in time to be used. Recently a group produced HSP25−/− mice, which were viable, fertile and without any morphological abnormalities (Huang et al., 2007). In this study ~0.6 kb of genomic sequence downstream of the start codon, including the first exon and 0.3 kb of the first intron were replaced with the lacZ reporter gene. This disruption allowed the examination of tissue distribution of HSP25 expression during mouse development, whilst eliminating HSP25 protein. Under heat challenge HSP25 was found to act synergistically with HSP70 in the development of thermotolerance and protected cells from apoptosis (Huang et al., 2007). Surprisingly, no significant changes were found in HSP25−/− MEFs compared to wild type when challenged with the apoptosis-inducing ionising radiation, etoposide, serum starvation or menadione (Huang et al., 2007). HSP27 had previously been reported to protect against apoptosis induced by these agents (Huot et al., 1996; Paul et al., 2002). However, this group have yet to report whether inflammation is dysregulated in the HSP25−/− mice. It would be exciting to investigate the function of HSP25 in inflammation in vivo in the future.
HSP25<sup>−/−</sup> mice in which all three exons have been removed are in the final stages of development in our laboratory. Specific cell types could be removed from the mice and challenged with IL-1, TNF and LPS. The use of cells from the HSP25<sup>−/−</sup> mice will be instrumental for future studies through providing a more definitive analysis of which genes are actually regulated by HSP25 / HSP27 and the elucidation of the signalling mechanism. In particular, use of HSP25<sup>−/−</sup> cells will overcome the hurdles imposed when RNAi was attempted in human dermal fibroblasts. Future work could include the use of inflammatory models to determine if the absence of HSP25 ameliorates inflammation. Infiltration of cells into inflamed tissue could be examined with the air-pouch model using IL-1 as a stimulus. The ability of the knockout mice to clear infections owing to defects in the innate immune response could be studied after the introduction of bacteria such as *Listeria Monocytogenes*. It would also be of great interest to examine the role of HSP25 in collagen-induced arthritis.

Future work *in vitro* to further elucidate the mechanism of HSP27 action in IL-1 signalling could include expanding the confocal microscopy studies. Different stimuli could be used to activate p38 MAPK, such as anisomycin or heat shock to determine if the nuclear / cytoplasmic ratio of p38 MAPK is altered. The use of additional stimuli may help reveal the mechanism of action. HSP27 has been shown to form a complex with p38 MAPK, MK2 and Akt (Rane et al., 2001; Zheng et al., 2006) and potentially with upstream activators such as TRAF6 (Wu et al., 2008). Therefore it would also be interesting to use confocal microscopy to determine if HSP27 depletion alters the nuclear-cytoplasmic ratio or localisation of any upstream activators. An alternative strategy to investigate this could include gel filtration of nuclear and cytoplasmic fractions to examine potential complexes. Immunophilin FKBP12 could be fused to HSP27 and used to investigate protein-protein interactions and the effect of oligomerisation of HSP27 on IL-1 signalling, in a similar way to previous studies of TRAF2 and TRAF6 (Baud et al., 1999). It would also be of interest to determine if HSP27 regulates IL-1 signalling in cell types particularly relevant for chronic inflammatory conditions, such as RA. These could include synovial fibroblasts or capillary endothelial cells.
Chapter 8

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Publication:


NB: This work was published under my maiden name of Turrell.
Heat Shock Protein 27 Functions in Inflammatory Gene Expression and Transforming Growth Factor-β-activated Kinase-1 (TAK1)-mediated Signaling*5

Received for publication, November 29, 2006, and in revised form, December 21, 2006. Published, JBC Papers in Press, January 3, 2007, DOI 10.1074/jbc.M610987200

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Heat shock protein (HSP) 27 has long been known to be a component of the p38 mitogen-activated protein kinase (MAPK) signaling pathway. p38 MAPK has important functions in the inflammatory response, but the role of HSP27 in inflammation has remained unknown. We have used small interfering RNAs to suppress HSP27 expression in HeLa cells and fibroblasts and found that it is required for pro-inflammatory cell signaling and the expression of pro-inflammatory genes. HSP27 is needed for the activation by interleukin (IL)-1 of TAK1 and downstream signaling by p38 MAPK, JNK, and their activators (MKK-3, -4, -6, -7) and IKKβ. IL-1-induced ERK activation appears to be independent of HSP27. HSP27 is required for both IL-1 and TNF-induced signaling pathways for which the most upstream common signaling protein is TAK1. HSP27 is also required for IL-1-induced expression of the pro-inflammatory mediators, cyclooxygenase-2, IL-6, and IL-8. HSP27 functions to drive cyclooxygenase-2 and IL-6 expression by augmenting the activation of the kinase downstream of p38 MAPK, MK2, resulting in stabilization of cyclooxygenase-2 and IL-6 mRNAs. The mechanism may not occur in cells of myeloid lineage because HSP27 protein was undetectable in human monocytes and murine macrophages.

Heat shock proteins (HSP)4 are constitutively expressed in certain cell types and are also induced upon exposure of cells to elevated temperatures and other cell stresses (1). HSP27 (or HSPB1) is a widely expressed 27-kDa protein and one of ten members of the small HSP family. These proteins include the lens proteins αA- and αB crystallin, and they all share a conserved C-terminal motif, the α-crystallin domain (2). The function of HSP27 is poorly understood, but recently missense mutations in the gene encoding it have been associated with the human neurodegenerative disorders, Charcot-Marie-Tooth disease, and distal hereditary motor neuropathy (3). However, the role of the HSP27 in these diseases is unclear.

HSP27 is phosphorylated in response to heat shock, cell stresses, pro-inflammatory stimuli, and various agonists including phorbol 12-myristoyl 13-acetate (PMA) (4–6). In fact HSP27 was found to be a major protein phosphorylated upon interleukin (IL)-1 treatment of fibroblasts (7). Phosphorylation of the human protein occurs at three serine residues Ser-15, Ser-78, and Ser-82 (6) and is performed by mitogen-activated protein kinase (MAPK)-activated protein kinase-2 (MK2) (8). In vitro phosphorylation of HSP27 by a kinase in cell lysates allowed the purification of an IL-1-activated protein kinase cascade now known as the p38 MAPK pathway (9). It consists of upstream activators (later identified as MAPK kinase (MKK3 and MKK6 and Refs. 10 and 11), p38 MAPK, the downstream kinase, MK2, and HSP27 (9, 12). Phosphorylation of the protein by MK2 has been suggested to modulate different functional properties of HSP27, including oligomerization; the unphosphorylated protein exists as large ~700-kDa oligomers (e.g. 24-mers), which dissociate to form dimers upon phosphorylation (13–15).

Various cellular functions have been proposed for HSP27. It is cytoprotective against heat shock (16) and apoptotic agents including tumor necrosis factor (TNF) (17), Fas/APO-1, staurosporine (18), H2O2 (19), and anticancer drugs (20). HSP27 possesses chaperone-like activity and refolds denatured proteins, preventing their aggregation (21). Reduced glutathione-dependent chaperone activity against misfolded or oxidized proteins has been suggested to be responsible for the HSP27 protective role (22). Several other anti-apoptotic functions have also been proposed (23); however, at present, there is no common mechanism that explains the cytoprotective function of HSP27. HSP27 may also regulate cytoskeletal dynamics.

* This work was supported in part by the Medical Research Council and the Arthritis Research Campaign. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

+ The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1 and S2.

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4 The abbreviations used are: HSP, heat shock protein; MAPK, mitogen-activated protein kinase; MKK, MAPK kinase; IL, interleukin; ELISA, enzyme-linked immunosorbent assay; MTI, 3-4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; JNK, c-Jun N-terminal kinase; ARE, AU-rich element; TNF, tumor necrosis factor; ERK, extracellular signal-regulated kinase; UTR, untranslated region; COX, cyclooxygenase; RNAI, RNA interference; siRNA, short interfering RNA; IKKβ, IκB kinase; TAK, transforming growth factor-β-activated kinase; dsRNA, double-stranded RNA; MK2, MAPK-activated protein kinase-2; PKB, protein kinase B; GST, glutathione S-transferase; TAB, TAK1-binding protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
expression of HSP27 stabilizes actin filaments, and this process appears to require phosphorylation (24, 25).

The p38 MAPK pathway, in addition to phosphorylating HSP27, plays a key role in the induction of many genes of the inflammatory response. An important function is to stabilize otherwise unstable mRNAs, thereby increasing their expression (26). The mechanism involves AU-rich elements (ARE) present in the 3′-untranslated regions (UTR) of mRNAs that direct instability but also allow for stabilization by p38 MAPK (27, 28). The process requires the downstream kinase, MK2 (27–29) and is thought to involve proteins that interact with the ARE and thereby regulate the decay of mRNA (26).

The fact that HSP27 is a substrate of MK2 makes it a candidate for involvement in the inflammatory response. Although its phosphorylation following IL-1 treatment of cells was first described some 20 years ago (30), its function in inflammation remains unknown. To examine the role of HSP27 in the inflammatory response we suppressed the expression of the protein in HeLa cells and human fibroblasts by RNA interference (RNAi). We found that HSP27 is needed for the induction by IL-1 of COX-2 and IL-6 protein and mRNA. In HSP27-depleted cells, the inhibition of COX-2 and IL-6 mRNA was a result of instability of these mRNAs, which was caused by reduced MK2 activation. These findings led us to uncover an unexpected function for the small heat shock protein in pro-inflammatory cell signaling.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—HeLa cells and human dermal fibroblasts were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and 100 units ml⁻¹ penicillin and 100 μg ml⁻¹ streptomycin. Cells were maintained at 37 °C in a humidified atmosphere containing 5% CO₂.

**Oligonucleotides**—21-nucleotide double-stranded siRNA oligonucleotides with 3′-dTdT overhangs were designed according to Tuschi and co-workers (31). They were synthesized in 2′-ACE protected form (Dharmacon) and were deprotected according to the manufacturer’s instructions. dsRNAs used were as follows: Scramble 2 (Scr): 5′-CAGUCGGCUUU-GCCACTGdtdTdT-3′; HSP27#1: 5′-GGAUGGCGUUGGAGAUCdtdTdT-3′; HSP27#2: 5′-CGAGAUCACAAUCCAGUCdtdTdT-3′; HSP27#3: 5′-GUGUUAGGCGCAAGCCGdtdTdT-3′; HSP27#4: 5′-GUAUGCACACAGUCCGdtdTdT-3′; HSP27#5: 5′-UGGCCAAGCUGCCACACGdtdTdT-3′; HSP27#6: 5′-UCGCCAGAGACUGCCGdtdTdT-3′; TAK1 siRNA: 5′-UGGCUUAUCUUACUGCCGdtdTdT-3′.

**siRNA Transfection**—HeLa cells were seeded in 6-well plates (5 × 10⁴ cells per well) and cultured for 24 h. Cells were transfected with siRNAs at a final concentration of 10 nm using lipofectamine 2000 (Invitrogen) according to the instructions supplied. Cells were re-transfected 24 h later in the same way. Cells were stimulated and harvested for the times indicated at 72 h after the initial transfection.

**Western Blotting**—Cells were lysed in lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Igepal CA-630, 1 mM phenylmethylsulfonil fluoride, 3 μg ml⁻¹ aprotinin, 10 μM E64, 2 μg ml⁻¹ pepstatin, 10 mM NaF, 5 mM sodium orthovanadate, 1 μM microcystin, 2 mM dithiothreitol), and protein was separated by 10% SDS-polyacylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes. The membranes were stained with rabbit anti-COX-2 antibody (Alexis), a mouse anti-α-tubulin antibody DM-1A (Sigma-Aldrich), a mouse monoclonal antibody against actin (Sigma), a rabbit antiserum raised against MKK3/6 (32), a rabbit antiserum to human HSP27 (7), a rabbit antiserum that recognizes both human HSP27 and murine HSP25 (Stressgen), a rabbit antiserum to the C-terminal peptide of p38α MAPK (33), a rabbit antiserum against TAK1 (sc-7162; Santa Cruz Biotechnology), and a rabbit antiserum against IκBα (sc-203; Santa Cruz Biotechnology). Rabbit anti-β-actin-ERK1/2, rabbit anti-phospho-p38 MAPK, rabbit anti-phospho-MKK3/6 antibodies, and mouse anti-phospho-JNK1/2 monoclonal antibody were from Cell Signaling, and secondary antibodies coupled to horseradish peroxidase were from Dako. Protein detection was carried out by enhanced chemiluminescence (GE Healthcare).

**Enzyme-linked Immunosorbent Assay (ELISA)**—IL-6 and IL-8 concentrations in medium were determined by ELISA using a kit (BD Biosciences) according to the manufacturer’s instructions. A prostaglandin E₂ (PGE₂) ELISA kit was from R&D Systems and was used according to the instructions supplied. All samples were developed using the TMB peroxidase substrate system (Kirkegaard & Perry Laboratories), and the reactions were stopped using 1 M H₂SO₄. Absorbance was read on an ELISA Multiskan Biochromic plate reader (Labsystems). Data were analyzed using Ascent software (Labsystems).

**RNA Isolation and Northern Blotting**—RNA was isolated from cells using an RNA Blood Mini kit (Qiagen) according to the manufacturer’s instructions. 8 μg of HeLa cell RNA was electrophoresed on denaturing formaldehyde/1% agarose gels with 0.41 M formaldehyde. RNA was capillary-transferred onto Hybond XL membranes (GE Healthcare) and fixed by UV cross-linking. For cDNA probes, membranes were prehybridized for 3 h and then hybridized overnight with [α-³²P]dCTP-labeled cDNA probe at 42 °C in Ultrahyb (Ambion). Blots were then washed three times for 1 h at 52 °C with 2 × SSC/0.1% SDS, 1 × SSC/0.1% SDS, and 0.1 × SSC/0.1% SDS. For GAPDH riboprobe pre-hybridization, hybridization, and washes were carried out at 65 °C. RNA was visualized and quantified using an FLA-2000 phosphorimager (Fuji, Tokyo, Japan). The GAPDH riboprobe template was from BD Biosciences and prepared according to Ref. 34. The HSP27 probe was prepared using a fragment of pCMVFLAGHSP27 plasmid (28) obtained by BamHI digestion. IL-6 and COX-2 probes have been described previously (35, 36).
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Cell Viability Assay—3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (0.5 mg ml\(^{-1}\) final concentration) was added to 1.5 ml of cell culture medium, and cells were incubated for 4 h. 1.5 ml of 10% SDS in 10 mM HCl was then added, and cells were incubated at 37°C overnight. Aliquots were transferred to a 96-well plate, and absorbance at 620 nm was measured using an ELISA Multiskan Biochromic plate reader (Labsystems).

Kinase Assays—For MK2 and p38 MAPK, these were performed as described previously (36). JNK was immunoprecipitated with an antibody against HSP27 (HSP27#1–6) or a control scrambled double-stranded oligonucleotide (Scr) as a control or were left untransfected. Cells were incubated for 72 h to allow for suppression of HSP27 protein. They were then incubated for a further 48 h. Cells were treated with IL-1 for 4 h, harvested, and lysed. Lysates were blotted for COX-2, HSP27, and \(\alpha\)-tubulin (as a loading control). The blot shown is representative of six different experiments.

RESULTS

HSP27 Is Required for the Induction of COX-2, IL-6, and IL-8 Proteins by IL-1—To investigate whether HSP27 is required for the regulation of inflammatory response genes, its expression was suppressed in HeLa cells by RNAi, and the induction of IL-1 of COX-2, IL-6, and IL-8 was measured. Cells were transiently transfected separately with six different siRNA targeted against HSP27 (HSP27#1–6) or a scrambled double-stranded oligonucleotide (Scr) as a control or were left untransfected. Cells were incubated for 72 h to allow for suppression of HSP27 protein. They were then incubated for a further 4 h with or without IL-1. Culture medium was then removed, and cells were harvested, lysed, and HSP27, COX-2, and \(\alpha\)-tubulin (as a loading control) were detected by Western blotting (Fig. 1A).

All siRNAs, except for HSP27#4, suppressed HSP27 protein expression, with HSP27#2 and HSP27#5 as the most effective (Fig. 1A). IL-1 induced COX-2 protein to a similar extent in
untransfected cells and in cells transfected with the scrambled oligonucleotide (Fig. 1A). The induction of COX-2 protein by IL-1 was inhibited by four different siRNAs but not by HSP27#3 or HSP27#4 (Fig. 1A). It is possible that HSP27#3 exerts off-target effects that block the inhibition of COX-2 upon HSP27 depletion. Because HSP27#2 and HSP27#5 siRNAs were the most effective at depleting HSP27 protein, they were selected for subsequent experiments. The effect of depleting HSP27 on IL-1-induced PGE\(_2\) secretion was also examined. The amount of PGE\(_2\) secreted following a 4-h IL-1 treatment was inhibited by 47 and 60% by HSP27#2 and HSP27#5 siRNAs compared with untransfected cells (data not shown).

The amounts of IL-6 and IL-8 secreted into the culture medium in response to IL-1 were also measured (Fig. 1, B and C). These were reduced in cells in which HSP27 expression was suppressed: siRNAs HSP27#2 and #5 inhibited IL-6 secretion by 72\%/\(\pm\)8% and 81\%/\(\pm\)7%, respectively (Fig. 1B) and reduced IL-8 secretion by 65\%/\(\pm\)4% and 68\%/\(\pm\)9%, respectively (Fig. 1C).

**FIGURE 2.** HSP27 regulates IL-1-induced COX-2 and IL-6 mRNA levels and stabilization. A, HeLa cells were transfected with HSP27 siRNAs, Scr, or left untransfected (as in Fig. 1) and either left untreated or treated with IL-1 for 2 h. Cells were then harvested, lysed, and RNA was isolated. Northern blots for COX-2, IL-6, HSP27, and GAPDH mRNA (as a loading control) are shown. Data are representative of two independent experiments. B, cells were transfected (as above), treated with IL-1, and incubated for 1.5 h before addition of actinomycin D (final concentration 5 μg ml\(^{-1}\)) to block transcription. Cells were harvested at the times shown, and RNA was isolated and Northern-blotted as above. Graphs show COX-2 or IL-6 mRNA levels normalized to GAPDH mRNA levels for representative actinomycin D chase experiments. Data shown in each panel are representative of at least two independent experiments.

**FIGURE 3.** HSP27 is needed for IL-1-induced activation of MK2. Cells were transfected as before and treated with IL-1 for the times shown. The cells were harvested and lysed. Lysates were immunoprecipitated with an antiserum against MK2 and assayed for MK2 activity using recombinant HSP27 protein. A portion of the lysates was Western-blotted for HSP27 and α-tubulin. The plot shows MK2 activity in Scr- and HSP27#5-transfected cells as a percentage of peak activity in Scr-transfected cells. MK2 activity was inhibited to a similar extent in another three comparable independent experiments.

HSP27 is Required for Induction by IL-1 of COX-2 Protein in Human Fibroblasts—To investigate whether HSP27 functions in inflammatory gene expression in cells other than HeLa cells, HSP27 expression was examined in different cell types. HSP27 was undetectable in human monocytes, human T cells, murine bone marrow-derived macrophages, and the RAW 264.7 murine macrophage-like cell line (Fig. 1D). HSP27 could not be detected in lipopolysaccharide-treated monocytes or macrophages (data not shown). It was expressed to a similar degree in HeLa cells and human fibroblasts and was also expressed in the 293 cell line (Fig. 1D). HSP27 was also detected in human endothelial cells and human chondrocytes (data not shown).
RNAi was used to test whether HSP27 functions in inflammatory gene expression in human fibroblasts. A single round of transfection by lipofection was found to be insufficient for strong depletion of HSP27 protein (data not shown). The cells were therefore transfected twice by lipofection, with an interval of 24 h between transfections, to improve depletion of HSP27. The suppression achieved by HSP27#2 after sequential transfection of the fibroblasts was still not as complete as in HeLa cells (compare Fig. 1, A and E) and was not sufficient to inhibit COX-2 expression (Fig. 1E). However in fibroblasts, HSP27#5 strongly suppressed HSP27 expression and, also inhibited the induction of COX-2 protein by IL-1 (Fig. 1E). Thus the pro-inflammatory function of HSP27 is not limited to HeLa cells but also occurs in human fibroblasts.

HSP27 Is Required for the Induction of COX-2 and IL-6 mRNA by IL-1—Suppression of HSP27 expression might inhibit the translation of COX-2 and IL-6 or reduce their mRNA levels. To test this, cells were treated with the siRNAs as before and were left unstimulated or stimulated with IL-1. The cells were harvested after 2 h, and RNA was isolated and examined by Northern blotting. IL-1 treatment induced COX-2 mRNA in untransfected and Scr-transfected cells to a similar extent (Fig. 2A). HSP27#2 and HSP27#5 inhibited COX-2 mRNA by 50 and 56%, respectively, compared with untransfected cells (Fig. 2A). Stronger inhibition was seen for IL-6 mRNA (68% inhibition for both siRNAs) (Fig. 2A). The inhibition of IL-1-induced IL-6 mRNA (Fig. 2A) reflected the reduction in IL-6 protein (Fig. 1B). Therefore, HSP27 does not appear to regulate IL-6 translation. These results indicate that HSP27 regulates either the transcription or the stability of these inflammatory transcripts. Northern blots were also performed to measure the degree of suppression of HSP27 mRNA (Fig. 2A). Both siRNAs suppressed HSP72#2 mRNA (HSP27#2: 65 ± 8%; HSP27#5: 71 ± 7%).

HSP27 has previously been reported to have a cytoprotective role. It was possible that the inhibition of expression of inflammatory proteins in response to IL-1 was caused by the death of cells in which HSP27 expression was suppressed. To check this, the viability of oligonucleotide-treated cells was measured by the MTT assay. In three experiments, Scr and HSP27#2 had no effect on cell viability, and only a small reduction was seen with HSP27#5 (14 ± 3%). Decrease in viability did not account for the differences in COX-2, IL-6, and IL-8 expression.

HSP27 Is Needed for Stabilization of IL-1-induced COX-2 and IL-6 mRNAs—Because HSP27 is a component of the p38 MAPK signaling cascade and this pathway has an important post-transcriptional role in inflammatory gene expression, we...
examined the stability of COX-2 and IL-6 mRNAs in HSP27-depleted cells. Cells were transfected with dsRNA as before, then stimulated with IL-1 for 1.5 h. Actinomycin D was added to block transcription, and cells were then incubated further and harvested at different times. RNA was isolated and examined by Northern blotting to measure mRNA decay rates. After 1.5 h of IL-1 treatment, COX-2 mRNA in Scr-transfected cells was stable; however, in HSP27-suppressed cells, it was reduced in amount and decayed rapidly (Fig. 2). IL-6 mRNA behaved similarly (Fig. 2). The stability of various other mRNAs (Bcl-x, GADD45, c-Fos, p21, Bax, Mcl-1, and L32) was unaffected by HSP27 depletion (supplemental Fig. S1) showing that of the mRNAs tested, the effect is not general and may be specific to COX-2 and IL-6 mRNAs.

Overexpression of HSP27 has previously been shown to protect against the cytotoxic effects of actinomycin D (39). To check whether actinomycin D affected the viability of cells in which HSP27 protein was suppressed, MTT assays were performed. Scr- and HSP27#2-transfected cells were left untreated, treated with IL-1 for 2 h, treated with actinomycin D for 1.5 h, or treated with IL-1 for 2 h followed by actinomycin D for 1.5 h. In two separate experiments, cell viability was unaffected by any of these treatments (data not shown). Thus the instability of COX-2 and IL-6 mRNAs in HSP27-suppressed cells was not caused by death of the cells, which might have been caused by actinomycin D.

HSP27 Is Required for Activation of MK2 by IL-1—It is possible that suppression of HSP27 protein (which is phosphorylated by MK2) was affecting upstream signaling by the p38 MAPK pathway. To examine this possibility, Scr or HSP27#5-transfected cells were stimulated with IL-1 for different times. Activation of MK2 was measured in lysates by immunoprecipitating it and assaying for its ability to phosphorylate recombinant HSP27 in vitro (Fig. 3). MK2 activity peaked at 30 min after IL-1 treatment and was inhibited by 56% by HSP27#5 siRNA (Fig. 3). Because MK2 activity is required for stabilization of COX-2 and IL-6 mRNAs, the reduced stability of these transcripts in HSP27-depleted cells is entirely consistent with inhibition of MK2 following HSP27 depletion.

HSP27 Is Needed for Full Activation by IL-1 of p38 MAPK and JNK, but Not ERK—Because HSP27 regulates MK2, the activation by IL-1 of its upstream activator, p38 MAPK, was measured in Scr and HSP27#5-transfected cells. p38 MAPK activity was maximal at 30 min after IL-1 stimulation and was inhibited by 59 ± 14% following HSP27 depletion (Fig. 4A). The reduction in p38 MAPK activity in HSP27 knockdown cells was not a result of reduced p38 MAPK expression as this was the same in Scr- and HSP27#5-transfected cells.

JNK is a MAPK, which, like p38 MAPK, is strongly activated by inflammatory stimuli including IL-1. ERK, the first discovered MAPK, is typically activated by mitogens, but is also activated by IL-1 in many cells. To find out whether the effect of HSP27 depletion on signaling is limited to the p38 MAPK pathway or affects the other MAPK pathways, the activation of JNK and ERK was examined. JNK was immunoprecipitated from dsRNA-transfected cell lysates and assayed using recombinant ATF-2 protein as a substrate (Fig. 4B). JNK activity was strongly activated by IL-1 in Scr-transfected cells, peaking at 30 min IL-1 stimulation and was inhibited by 59 ± 14% following HSP27 depletion (Fig. 4B). The role of HSP27 in regulating signaling is not restricted to the p38 MAPK pathway.
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ERK activation was assessed by Western blotting lysates of recombinant JNK protein. This mutant kinase (JNK K55R) was not detected in Western blotting with phosphospecific antibodies. For p38 MAPK and JNK, but not ERK, this was inhibited in cells transfected with both HSP27#2 and HSP27#5 (Fig. 4D). The inhibition of p38 MAPK and JNK is not an off-target effect associated with a single siRNA.

HSP27 Is Required for Activation of MKK3/MKK6 and MKK4/MKK7—It was possible that HSP27 directly targets p38 MAPK and JNK or, alternatively, it could regulate upstream signaling. To test the latter possibility, the activation of the upstream activators of p38 MAPK and JNK was analyzed. Activation of MKK3 and MKK6, the kinases directly upstream of p38 MAPK, was detected by Western blotting with a phosphospecific antibody that recognizes activated forms of both kinases (Fig. 5A). As for MK2 and p38 MAPK, activation of MKK3 and MKK6 peaked at 30 min IL-1 and was inhibited upon suppression of HSP27 expression (Fig. 5A). The reduced signal for phospho-MKK3 and -MKK6 in HSP27 knockdown cells was not caused by a decrease in expression of these kinases, as this was normal in the HSP27-depleted cells (Fig. 5A). These results prompted us to examine the activation of the kinases responsible for JNK activation, MKK4 and MKK7.

These two kinases were immunoprecipitated and assayed for their ability to phosphorylate a kinase-dead mutant form of recombinant JNK protein. This mutant kinase (JNK K55R) does not undergo autophosphorylation during the assay, allowing MKK activity to be measured. IL-1 induced MKK4 activity, which peaked at 30 min post-IL-1 (Fig. 5B). Unlike the activation of the other IL-1-regulated kinases examined, which declined rapidly after their peak at 30 min, MKK7 activity was more sustained, with similar activity detected at 30 min and 1 h IL-1 (Fig. 5C). Peak MKK4 and MKK7 activity was lower in HSP27 knockdown cells by 65 and 60%, respectively (Fig. 5, B and C). The inhibition of MKK4 and MKK7 upon deleting HSP27 fully accounts for the inhibition of JNK. The amount of MKK4 and MKK7 protein in cells was found to be unchanged following HSP27 depletion (data not shown).

HSP27 Regulates IKKβ Activation but Not IκBα Degradation—The p38 MAPK and JNK pathways are both strongly activated by pro-inflammatory stimuli, and the degree of activation depended upon HSP27. Another pathway that plays a pivotal role in the expression of inflammatory mediator genes is that of activating the nuclear factor (NF)-κB. It is possible that this is also regulated by HSP27. The transcription factor NF-κB is held in the cytoplasm of resting cells by an interaction with an inhibitor of NF-κB-α, the major form of which is IκBα. Upon cell activation, IKKβ phosphorylates IκBα, triggering its degradation and releasing NF-κB, which translocates to the nucleus to activate transcription. IKKβ activity and IκBα degradation were analyzed in HSP27-depleted cells.

IKKβ exists in a complex with IκKα and IκKγ (41). Activation of IKKβ was measured by immunoprecipitating IKKγ and assaying phosphorylation of recombinant IκBα (Fig. 6A). IKKβ was activated by IL-1, with activity peaking at 15 min post-IL-1 (Fig. 6A). HSP27 depletion inhibited IKKβ activity by 51% at 15 min IL-1 (Fig. 6A). Thus HSP27 regulates the activity of three IL-1-activated protein kinase cascades, namely the p38 MAPK, JNK, and IKKβ pathways, to similar extents. Despite inhibition...
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FIGURE 7. HSP27 is needed for full activation of TAK1 by IL-1 and activation of JNK by TNF. A, cells were transfected and treated with IL-1 for the times shown. TAK1 was immunoprecipitated from lysates of Scr- or HSP27 siRNA-transfected cells using an antibody against TAB1. TAK1 activity was detected using MBP-MKK6 as substrate. A portion of the lysates used in the kinase assay was Western-blotted for the proteins indicated. Graph shows mean TAK1 activity (± S.E.) for four independent experiments as a percentage of that in Scr-transfected cells treated with IL-1 for 10 min. B, cells were transfected as before and treated with TNF (20 ng ml\(^{-1}\)) or IL-1 (20 ng ml\(^{-1}\)) for the times shown. JNK was immunoprecipitated from lysates and assayed using GST-ATF-2 as substrate. Graph shows JNK activity as a percentage of that in Scr-transfected cells treated with TNF for 30 min. Similar results were obtained in two separate experiments.

HSP27 Regulates TAK1 Function—IL-1 activates the p38 MAPK, JNK, and NF-κB pathways via a common activator, transforming growth factor-β-activated kinase-1 (TAK1) (42). In cells, TAK1 exists in a complex with its binding partners, transforming growth factor-β-activated kinase-1-binding protein (TAB)1, TAB2, and TAB3. The complex was immunoprecipitated from lysates with an antibody against TAB1 and assayed for its ability to phosphorylate recombinant M KK6 (Fig. 7). In resting cells, significant TAK1 activity could be detected, and this was not regulated by HSP27 depletion (Fig. 7). TAK1 activity peaked at 10 min post-IL-1 in Scr- and HSP27 siRNA-transfected cells (Fig. 7). IL-1-induced TAK1 activity was inhibited by 53 ± 4% and 30 ± 7% at 5 and 10 min IL-1, respectively (Fig. 7). HSP27 depletion did not affect the amount of TAK1 protein in the cells (Fig. 7). Thus HSP27 activates pro-inflammatory signaling pathways by augmenting the function of TAK1.

It is generally accepted that TAK1 regulates the JNK, p38 MAPK, and NF-κB pathways. It was therefore surprising that IκBα degradation was unaffected by HSP27 depletion (Fig. 6B). To investigate whether TAK1 regulates IκBα degradation in HeLa cells, TAK1 expression was suppressed by RNAi (supplemental Fig. S2). TAK1 siRNA suppressed TAK1 protein levels and strongly inhibited IL-1-induced IκBα activity, but had little effect on IκBα degradation (supplemental Fig. S2).

HSP27 regulates a signaling event common to TNF and IL-1—TNF and IL-1 both activate the MAPK and NF-κB pathways via activation of TAK1 (42). However, these two cytokines activate TAK1 by different upstream mechanisms. IL-1 signaling involves MyD88, IRAK1, IRAK4, and TRAF6 (41), whereas IL-8. In the case of COX-2 and IL-6, we found that HSP27 regulated expression of the mRNAs. This was, at least in part, because of regulation of the stability of these mRNAs in IL-1-treated cells. HSP27 depletion had no effect on cell viability. Its effect on mRNA stability was not general and may be specific to mRNAs of inflammatory mediators such as COX-2 and IL-6. It is possible that IL-8 transcripts are regulated in a similar way.

COX-2 and IL-6 mRNAs are known to be stabilized upon p38 MAPK activation (36, 43). Reporter mRNAs bearing either the COX-2 (28), IL-6, or IL-8 (27) AREs are stabilized by co-expression of an active mutant of MK2 in a HeLa cell line. IL-6 mRNA is also unstable in lipopolysaccharide-treated macrophages from MK-2-null mice (29). We found that HSP27 is needed for activation of both p38 MAPK and MK2. Thus the regulation of p38 MAPK/MK2-mediated mRNA stabilization represents a mechanism whereby HSP27 contributes to the expression of pro-inflammatory mediators.

Regulation of pro-inflammatory cell signaling by HSP27 is not limited to the p38 MAPK pathway. HSP27 also functions in the activation by IL-1 of the JNK and NF-κB pathways. Reduced activity in these pathways in HSP27 knockdown cells was due to a defect in the function of the upstream activator, TAK1. TAK1 was originally thought to be involved in transforming growth factor-β signaling. It is now known to play an important role in signaling induced by a range of pro-inflammatory stimuli in fibroblasts (42), B cells (42), and T cells (44–46). TAK1 knock-out is lethal (42). Embryonic fibroblasts from TAK1-null mice display impaired activation by IL-1 and TNF of the p38 MAPK, JNK, and NF-κB pathways (42). Depletion of TAK1 by RNAi in HeLa cells also inhibits IL-1- and TNF-induced TNF signaling requires TRADD, TRAF2, and TRAF5 (41). To investigate whether HSP27 regulates signaling that is restricted to IL-1, or is common to both IL-1 and TNF, the effect of HSP27 depletion on TNF-induced JNK activation was examined (Fig. 7B). As seen for IL-1, TNF-induced JNK activity was also inhibited following HSP27 depletion (Fig. 7B). Thus TAK1 is the most upstream component common to both pathways that is targeted by HSP27.

DISCUSSION

We found that HSP27 functions in IL-1-induced cell signaling and pro-inflammatory gene expression. HSP27 is needed for full activation by IL-1 of TAK1 and signaling by the p38 MAPK, JNK, and IκBα pathways. It is also required for the induction by IL-1 of three inflammatory mediators whose expression is known to be dependent on these pathways, namely COX-2, IL-6, and IL-8. The role of HSP27 in modulating pro-inflammatory cell signaling and gene expression appears critical in the regulation of pro-inflammatory gene expression.
TRAF2 and TRAF5. TRAF6 is a ubiquitin ligase, and TRAF2 receptor interacts with TRAF6, whereas that for TNF binds HSP27. Another difference between IL-1 and TNF signaling is that the IL-1 response to TNF, which is also regulated by HSP27. Another of these proteins, because they do not participate in signaling in the future.

HSP27 regulates p38 MAPK, JNK, and IKKβ. It is thus possible that in addition to regulating mRNA stability, the small heat shock protein also activates transcription. In cells, IKK phosphorylates and activates the AP-1 transcription factors c-Jun and ATF-2 (51, 52). It is possible that transcription driven by AP-1 binding is regulated by HSP27. Despite regulation of IKKβ activity upon HSP27 depletion, the degradation of IkBα induced by IL-1 was not regulated. TAK1 depletion also inhibited IKKβ activity with almost no effect on IkBα degradation. Cells from TAK1−/− mice do show an impairment in NF-κB-driven transcription (42). The lack of an effect of either HSP27 or TAK1 siRNA on IkBα degradation is likely to be a result of residual IKKβ activity. Thus it is possible that HSP27-null cells would also show a defect in NF-κB-regulated transcription.

Phosphorylation causes dissociation of HSP27 multimers and may regulate the proposed function of HSP27 as a chaperone. It is unclear whether phosphorylation of HSP27 is required for it to promote signaling. The kinetics of HSP27 phosphorylation following IL-1 stimulation are similar to those for the activation of MK2 and p38 MAPK. TAK1 activity is regulated by HSP27 at 5 min following treatment of cells with IL-1. It is unclear to what extent HSP27 is phosphorylated at this time, because MK2 and p38 MAPK activity peak later (30-min post-IL-1).

HSP27 is known to have a cytoprotective role, and may also be involved in modeling of the actin cytoskeleton. Both of these processes could be regulated by HSP27 as a consequence of its function in cell signaling and gene expression. Cellular stresses, including heat shock, activate the p38 MAPK pathway, and HSP27 could contribute to signaling induced by these stimuli. Modeling of the actin cytoskeleton necessary for cell migration is regulated by LIM kinase, a kinase activated by MK2 (53). Thus the function of HSP27 may not be limited to pro-inflammatory signaling and the expression of only pro-inflammatory genes.

We have shown that HSP27 is required for pro-inflammatory gene expression in HeLa cells and human fibroblasts. The function of HSP27 may be to sensitize these cells to pro-inflammatory stimuli by augmenting pro-inflammatory signaling. It is likely that HSP27 plays a similar role in other cell types. It does not appear to be constitutively expressed in leukocytes, nor is it induced by lipopolysaccharide treatment of human monocytes or macrophages. It is perhaps rather surprising that these cells do not require HSP27 for pro-inflammatory signaling. The absence of HSP27 in hemopoietic cells suggests that it may function in innate, but not adaptive immunity.

Acknowledgments—We thank Andy Clark for valuable discussions. We are grateful to Mark Windheim and Michael Karin for generous provision of reagents.

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