Biochemical and Functional Characterization of Golgi Anti-Apoptotic Proteins (GAAP)

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A THESIS SUBMITTED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY
The data presented in this thesis is the work of the candidate except where clearly indicated. In particular, the artificial bilayer patch clamp recordings were performed by Guia Carrara at Cambridge University. FRET experiments were performed by the candidate at Prof. Colin Taylor lab with the help of Dr. David Prole. Viral GAAP ion channel mutants subcellular localization was done by Dr Benjamin Johnson that together with Dr. David Prole were also directly involved in producing the alignments of GAAP with known ion channels.
Acknowledgements

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Work funded by FCT/POPH
To my parents
Recently a new inhibitor of apoptosis, Golgi anti-apoptotic protein (GAAP), was discovered in camelpox virus and in some vaccinia virus (VACV) strains. GAAP is non-essential for VACV replication but affects virus virulence, is located in the Golgi, inhibits apoptosis and modulates Ca$^{2+}$ fluxes. The VACV GAAP (vGAAP) shows high sequence identity with a previously uncharacterized human protein, named human GAAP (hGAAP). GAAPs belong to the Bax inhibitor-1 family and are highly conserved among eukaryotes, sharing a similar sequence, length and hydrophobicity profile.

A yeast system was used to express and purify vGAAP and *Arabidopsis thaliana* GAAP3 (AtGAAP3). Data from patch clamp experiments using reconstituted purified protein in artificial lipid bilayers showed that vGAAP and AtGAAP3 allow the passage of ions, suggesting that GAAPs might form ion channels or ion exchangers. Amino acid sequence alignments of GAAP with other known ion channels or ion exchangers allowed the identification of amino acid residues in GAAP that might be involved in channel gating or ion selection. These residues were mutated in vGAAP and the mutants screened for anti-apoptotic and Ca$^{2+}$ modulation activity, and purified to confirm and further characterize GAAP’s ion channel-like activity. Using chemical crosslinking, FRET and cysteine mutagenesis it was shown that GAAPs are able to form oligomers in a cysteine-dependent (vGAAP) or cysteine-independent (hGAAP) manner.

Using detachment and attachment assays it was shown that hGAAP overexpression is able to increase cell adhesion and, conversely hGAAP knock down caused decreased adhesion and an elongated cell phenotype. Using a focal adhesion (FA) marker it was observed that the FA turnover after GAAP knockdown is reduced.

Taken together, these results suggest that GAAP modulation of apoptosis and cell adhesion may be via its ion channel activity and subsequent modulation of Ca$^{2+}$ fluxes.
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<th>Description</th>
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<tbody>
<tr>
<td>Ab</td>
<td>Antibody</td>
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<tr>
<td>AIF</td>
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<td>Apoptotic protease-activating factor 1</td>
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<td>ARP</td>
<td>Actin-related proteins</td>
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<td>AtGAAP3</td>
<td><em>Arabidopsis thaliana</em> GAAP3</td>
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<td>ATP</td>
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<td>bp</td>
<td>Base pairs</td>
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<td>Bovine serum albumin</td>
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<td>CaSR</td>
<td>Ca$^{2+}$-sensing receptor</td>
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IP$_3$R  Inositol 1,4,5-Triphosphate Receptor
IRE1  Inositol-requiring protein-1
IRM  Interference reflectance microscopy
KRB  Krebs-Ringer buffer
LB  Luria Broth
LDAO  N,N-dimethyldodecylamine N-oxide
LFG  Lifeguard protein
MEM  Minimum essential medium
MHC  Major histocompatibility complex
MICS1  Mitochondrial morphology and cristae structure 1
min  Minutes
MMP  Mitochondrial membrane permeabilization
ms  Milliseconds
mRNA  Messenger RNA
MRB  Membrane resuspetion buffer
MWCO  Molecular weight cut off
NAADP  Nicotinic acid adenine dinucleotide phosphate
NALCN  Sodium leak channel
NFAT  Nuclear factor of activated T-cells
NF-κB  Nuclear factor κB
NMDAR  N-methyl-D-aspartate receptors
NCX  Na$^+$/Ca$^{2+}$ exchanger
pA  Picoamperes
PBS  Phosphate-buffered saline
PBS-T  Phosphate-buffered saline + 0.01 % (v/v) Tween 20
PCR  Polymerase chain reaction
PEI  Polyethylinimine
p.f.u  Plaque forming unit
PFA  Paraformaldehyde
PI  Propidium iodide
PLC  Phospholipase C
PMCA  Plasma-membrane Ca$^{2+}$-ATPase
P/S  Penicillin/streptomycin
RFU  Relative fluorescence units
RNA  Ribonucleic acid
ROC  Receptor-operated channel
ROI  Region of interest
RT  Room temperature
RT-PCR  Reverse transcription-PCR
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<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate – polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
</tr>
<tr>
<td>Smac</td>
<td>Second activator of caspases</td>
</tr>
<tr>
<td>SMOC</td>
<td>Second-messenger-operated channel</td>
</tr>
<tr>
<td>SOC</td>
<td>Super optimal broth with catabolite repression</td>
</tr>
<tr>
<td>SPCA</td>
<td>Secretory pathway Ca(^{2+}) ATPase</td>
</tr>
<tr>
<td>ssDNA</td>
<td>Single stranded DNA</td>
</tr>
<tr>
<td>STS</td>
<td>Staurosporine</td>
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<tr>
<td>TAE</td>
<td>Tris acetate ethylene diamine tetraacetic acid buffer</td>
</tr>
<tr>
<td>TASK-1</td>
<td>TWIK-related acid-sensitive K(^+) 1</td>
</tr>
<tr>
<td>TEV</td>
<td>Tobacco etch virus</td>
</tr>
<tr>
<td>TIRF</td>
<td>Total internal reflection fluorescence</td>
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<tr>
<td>TNFR1</td>
<td>Tumor necrosis factor receptor</td>
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<tr>
<td>TMBIM</td>
<td>Transmembrane Bax inhibitor containing motif</td>
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<tr>
<td>TMD</td>
<td>Trans membrane domain</td>
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<tr>
<td>Tom</td>
<td>Tandem tomato fluorescent protein</td>
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<tr>
<td>TRPM7</td>
<td>Transient receptor potential M7</td>
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<td>UV</td>
<td>Ultraviolet</td>
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<tr>
<td>UPR</td>
<td>Unfolded protein response</td>
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<td>URA</td>
<td>Uracil</td>
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<td>VACV</td>
<td>Vaccinia virus</td>
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<td>VARV</td>
<td>Variola virus</td>
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<tr>
<td>vGAAP</td>
<td>Viral Golgi anti apoptotic protein</td>
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<tr>
<td>VOC</td>
<td>Voltage-operated channel</td>
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<tr>
<td>VSV-gpG</td>
<td>Vesicular stomatitis virus glycoprotein G</td>
</tr>
<tr>
<td>WCE</td>
<td>Whole cell extract</td>
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<td>WHA</td>
<td>Wound healing assay</td>
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<tr>
<td>YFP</td>
<td>Yellow fluorescent protein</td>
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<tr>
<td>YPG</td>
<td>Yeast peptone glucose medium</td>
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Chapter I

Introduction
1.1 Vaccinia virus (VACV) and the poxvirus family

The most well known member of the Poxviridae family is the causative agent of smallpox, variola virus (VARV). The term smallpox was initially used in the 15th century to differentiate it from the so-called “great pox” or syphilis. Smallpox, the disease caused by VARV, led to an estimated 300-500 million deaths in the 20th century (Fenner, 1993; Mack, 1972).

Edward Jenner discovered in 1796 that inoculation with material from a cowpox lesion led to immunity against smallpox in humans. The immunization process consisted of the inoculation of material from a cowpox virus (CPXV) lesion into humans by scarification. Jenner created the term vaccine from the Latin word for cow (Vacca) from which the material used for immunizing the patients was extracted. In the 20th century it was recognized that the vaccine used to immunize against VARV was a different virus and this was called vaccinia virus. The origin of this virus remains unknown (Baxby, 1981). VACV is best known as the agent used for smallpox vaccination and eradication (Fenner F, 1988). The World Health Organization certified the eradication of the disease in 1979. This was possible due to the absence of an animal reservoir for VARV, the high level of antigenic similarity between VACV and VARV (Macdonald and Downie, 1950), the fact that VARV does not establish latent or persistent infections, and because the vaccine was cheap, abundant and easy to administrate.

Beyond its importance as a smallpox live vaccine, VACV has been used as a vaccine vector mainly due to its high immunogenicity, ability to infect a broad range of cells and to achieve high expression levels of recombinant antigens in the infected cells (Moss et al., 1983; Smith et al., 1983). Successive passages in chicken embryo fibroblasts of the chorioallantois virus Ankara strain of VACV led to the creation of a highly attenuated VACV strain known as modified vaccinia virus Ankara (MVA). The use of this virus as a vaccine vector is currently being tested for diseases such as acquired immune deficiency syndrome (AIDS), malaria and tuberculosis (Gomez et al., 2008). The fact that MVA replication in most mammalian cells is defective makes this a very safe vaccine.

The study of the mechanisms used by VACV to replicate and shut down the host responses to infection has allowed important discoveries in basic cell biology.
and immunology. An example of how the study of VACV contributed to our understanding of not only virus pathogenesis but also basic physiological processes came from the study of the VACV soluble interleukin-1β (IL-1β) receptors, that can block the activity of IL-1β by binding and sequestering this cytokine. VACV prevents the febrile response in infected mice, whereas strains that lack the receptor induce fever (Alcami and Smith, 1996). This was the first example where the suppression of fever by a virus and the molecular mechanism leading to it was described. Another more recent example is the finding and study of the human Golgi anti-apoptotic protein (hGAAP) due to the discovery of a similar gene in camelpox virus (CMLV) (Gubser et al., 2007).

1.1.1 Poxvirus classification

VACV is the prototypic member of the *Poxviridae*, and is the most intensely studied virus of this family. Poxviruses are notable for their exclusive cytoplasmic replication, large ovoid virion size and large linear double-stranded (ds) deoxyribonucleic acid (DNA) genomes (130-360 kilobase pairs), with termini that form covalently closed hairpin loops. The *Poxviridae* is divided into two sub-families based on their host range: *Entomopoxvirinae*, infecting insects, and *Chordopoxvirinae*, infecting vertebrates. The sub-family *Chordopoxvirinae* includes eight genera; *Orthopoxvirus*, to which VACV belongs, *Parapoxvirus*, *Avipoxvirus*, *Capripoxvirus*, *Leporipoxvirus*, *Suipoxvirus*, *Molluscipoxvirus* and *Yatapoxvirus*. The *Entomopoxvirinae* sub-family consists of 3 genera termed A, B and C.

The genome organization, gene content and DNA and protein sequences were used to study the phylogenetic relationships of poxviruses (Gubser et al., 2004). Using these parameters it was shown that the central region of the genome of the *Chordopoxvirinae* has a conserved gene content and arrangement. VACV is closely related to CPXV. Also VARV and CMLV clustered together forming a subgroup. Horizontal gene acquisition from host cells is likely to be a very common event within the poxvirus family because many of the viral genes encode proteins with a considerable amino acid similarity and therefore have a similar evolutionary origin to those found in their eukaryotic hosts. This process is enabled by the large
capacity of the virion structure to accommodate variable amounts of DNA (Smith and Moss, 1983).

1.1.2 Poxvirus and human diseases

Other than VARV, the causing agent of smallpox, there are several other poxviruses that can infect and cause symptoms in humans. *Moluscum contagiosum virus* is a human-specific pathogen that spreads through direct contact and can cause benign flesh-coloured, dome-shaped lesions (Hanson and Diven, 2003). Zoonotic transmission of virus such as monkeypox virus and CPXV, which natural host is rodents, despite the names of the virus, to humans can cause much more severe symptoms, especially in immune-compromised individuals (Czerny et al., 1991).

1.1.3 VACV life cycle

VAVC replication leads to the formation of two different infectious particles, the intracellular mature virus (IMV) and the extracellular enveloped virus (EEV), (reviewed in Roberts and Smith, 2008). The IMV particle is surrounded by a single membrane and does not get released from the cell until lysis, whereas the EEV possesses two membranes and is transported and released from the cell prior to cell death. EEV particles localised to the cell surface are called cell-associated enveloped virus (CEV).

The IMV and CEV/EEV forms have different numbers of membranes and they present different viral proteins on their surface, this makes these virion forms structurally, antigenically and functionally different (Boulter and Appleyard, 1973). Both particles play a particular role in VACV dissemination. The CEV and the EEV are important for virus spread within a host. CEV can induce the formation of actin tails from the cell surface that allows the infection of neighbouring cells and EEV, although representing only a small portion of the infectious progeny, are very important in virus long-range dissemination (Payne and Kristensson, 1985; Smith et al., 2002).
1.1.3.1 Entry

The existence of two structurally different types of VACV virus particles (EEV and IMV) makes virus entry complex. The cell receptor for both VACV EEV and IMV particles remains unknown. It is believed that VACV IMV particles fuse their membrane with the host cell plasma membrane in a pH-independent manner (Carter et al., 2005) or with endocytic vesicles after endocytosis that is enhanced by low pH (Townsley and Moss, 2007). One of the mechanisms by which EEV particles get access to the cytosol relies on the EEV outer membrane disruption upon contact with the cell surface. This reveals an IMV particle that can now enter through membrane fusion or endocytosis (Law et al., 2006). It was also shown recently that EEV particles can enter the cell through micropinocytosis followed by an exposure to low pH in endocytic vacuoles that is necessary to disrupt the outer membrane. This results in exposure of the inner membrane that can then fuse with the vacuolar membrane and release the virus core into the cytosol (Schmidt et al., 2011).

1.1.3.2 Morphogenesis

After the initial removal of the IMV or EEV membranes, the virus core, which contains viral structural proteins, viral genome and viral transcription enzymes, is released into the cytosol. This structure is then transported on microtubules close to the perinuclear region in a process that depends on core surface proteins (Carter et al., 2003). Partial uncoating of the genomic DNA allows transcription or early messenger ribonucleic acids (mRNAs) by the virus-associated DNA-dependent RNA polymerase. Viral factories form in the cytosol, where the viral genome is replicated and new virions are assembled.

The VACV genome encodes approximately 200 genes, these are transcribed by the virus-encoded RNA polymerase. Around half of these genes are expressed early during infection and are involved in processes such as viral DNA replication, deviating the cellular machinery to the virus advantage and in escaping the host cell immune response to infection. Some intermediate genes encode regulatory proteins (transcription factors) that trigger late gene transcription. Late genes encode most of the structural virion proteins and enzymes that must be packed in the virion to allow initiation of transcription in the next infected cell (reviewed in Broyles (2003)).
A crescent-shaped lipid structure wraps the new virion cores forming immature virions. After proteolytic cleavage of core proteins the characteristic brick-shaped IMV is formed (Moss and Rosenblum, 1973). A small portion of IMV particles are transported in microtubules away from viral factories (EEV) and wrapped in a double membrane, derived from either endosomal (Tooze et al., 1993) or tran-Golgi cisterna (Hiller and Weber, 1985), forming an intracellular enveloped virus (IEV). Virions are transported to the cell surface in an A36 dependent mechanism (Ward and Moss, 2001). After fusion with the cell membrane a CEV particle is exposed at the cell surface. A36 then triggers actin polymerization (Frischknecht et al., 1999) leading to the formation of an actin tail that pushes the viral particle away from the infected cell. This mechanism relies on A36-mediated recruitment of the non-catalytic region of tyrosine kinase adaptor proteins (NCK) and growth factor receptor-bound protein 2 (GRB2) (Scaplehorn et al., 2002) which in turn can recruit neuronal Wiskott–Aldrich Syndrome protein (N-WASP) and WASP-interacting protein (Moreau et al., 2000) to stimulate actin-related proteins (ARP) 2 and 3-mediated actin polymerization. Recently a novel VACV super-infection inhibition mechanism linked to actin tail formation was described. It was found that the early viral proteins A36 and A33 are expressed early on the surface of infected cells and can repel some incoming EEV virions by triggering the formation of new actin tails (Doceul et al., 2010). Using this strategy the virus is able to “bounce” on the surface of infected cells until it finds a non-infected cell where no actin tail formation will be induced allowing the virus to enter the cell.

1.1.4 Genome structure

Poxviruses have dsDNA genomes that contain tightly packed genes with no introns and little non-coding DNA. The two ends of the genome contain inverted terminal repeats and the two strands of DNA are covalently linked to produce hairpin termini (Baroudy et al., 1982). Genes tend to be organized in blocks that are transcribed in the same direction, possibly to facilitate mRNA synthesis or to prevent formation of double stranded RNA. The overall genome structure of the Chordopoxviridae subfamily members can be divided in two regions, (i) a central portion, where a core of around 90 essential genes involved in replication, transcription and morphogenesis are found and (ii) the end portions of the genome,
where nonessential genes are located, these are involved in controlling the host’s responses to infection (Gubser and Smith, 2002). Although most of the genes located at the terminal regions are classified as nonessential for replication in cell culture, most of them play an important role in host range and/or virulence.

1.1.5 Genome replication

One of the unique features of all poxviruses is the ability to replicate the DNA genome in the host cell cytosol. The first infectious viral particles are produced around 8 h post-infection (Salzman, 1960). In order to do this all poxviruses encode their own DNA polymerase and a series of other enzymes directly or indirectly involved in DNA replication such as thymidine kinase, thymidylate kinase, ribonucleotide reductase, DNA ligase and a DNA topoisomerase (Moss, 2007).

The most generally accepted model for poxvirus DNA replication is the rolling hairpin strand displacement mechanism (Tattersall and Ward, 1976). According to this model, a nick is introduced within the terminal 200 base pairs (bp) of the genome providing the 3’ end to prime the replication complex. The newly synthesised DNA strand folds back on itself allowing the rest of the genome to be replicated.

1.2 Apoptosis

Programmed cell death, also known as apoptosis, is a conserved process used by all multicellular organisms to dispose of unnecessary, old or potentially dangerous cells. It is essential for the maintenance of tissue homeostasis, the shaping of the immune repertoire, termination of immune responses, embryo development and as an innate defence against infections and cancer (reviewed in Hengartner (2000)). To achieve this, mammalian cells use a highly regulated and conserved process that can be triggered by several different mechanisms (Figure 1.1) such as: (i) the intrinsic pathway, also known as the mitochondrial pathway (Cory and Adams, 2002), (ii) the extrinsic pathway, better known as the death
receptor pathway (Boldin et al., 1996; Muzio et al., 1996), (iii) the caspase-2-dependent pathway (Lassus et al., 2002) or (iv) the caspase independent, perforin/Granzyme A (GrA) mediated pathway (Beresford et al., 2001).

The death receptor pathway is triggered by death receptors at the cell surface, such as Fas and tumour necrosis factor receptor 1 (TNFR1) binding their ligands TNF and Fas ligand, and initiating a signalling pathway that eventually leads to caspase 8 activation (Boldin et al., 1996; Muzio et al., 1996). The intrinsic pathway is activated in response to internal insults such as DNA damage, endoplasmic reticulum (ER) stress or virus infection. Type one cells can activate pro-caspase 3 directly in a mitochondrion-independent manner. In type two cells, both pathways converge at the mitochondrion often through the activation of a pro-apoptotic member of the B cell lymphoma 2 protein (Bcl-2) family (Cory and Adams, 2002). This leads to recruitment of pro and anti-apoptotic proteins to the mitochondrion outer membrane where they compete to regulate and activate catabolic hydrolases (mainly caspases and nucleases) (Schmidt et al., 2011). If the cell commits to undergo apoptosis, mitochondrial membrane permeabilization (MMP) will occur and cytochrome c (cyt c), second activator of caspases (Smac), pro-caspases and apoptosis inducing-factor (AIF) (Susin et al., 1996) are released from the mitochondria into the cytosol. Cyt c released from the mitochondria associates with the apoptotic protease-activating factor 1 (Apaf 1) and with caspase 9 to form the apoptosome (Li et al., 1997). Pro-caspases and Smac (Du et al., 2000) will activate effector caspases and AIF will activate a DNase located in the nucleus. This process is also regulated by inhibitors of caspases that are themselves negatively regulated by Smac and Omi (Hegde et al., 2002). The caspase-2-dependent pathway is triggered by DNA damage and leads to cyt c release and apoptosome formation, although the mechanism by which this happens is not fully understood (Lassus et al., 2002; Robertson et al., 2002).
Figure 1.1 - Apoptosis pathways in mammalian cells. The diagram represents the main four different signalling pathways that can lead to apoptosis execution in mammalian cells. GAAP anti-apoptotic activity prevents apoptosis at the level of or upstream of pro-caspase 8 activation, protecting from both intrinsic and extrinsic induced apoptosis. Adapted from Orrenius et al. (2003).
All these processes lead to effector pro-caspase 3 activation that, together with caspases 6 and 7, are responsible for most of the death substrate cleavage. These include essential cell components such as cytoskeletal proteins, or act indirectly by activating other enzymes such as nucleases. This leads to the characteristically morphological changes that occur during apoptosis such as cell shrinking and blebbing.

The serine protease GrA mediates a pathway that can trigger apoptosis in a caspase-independent manner (Beresford et al., 2001; Fan et al., 2003). Perforin-mediated pores allow GrA to enter the cell and to induce the cleavage of some elements of the ER SET complex such as SET (a Granzyme A-Activated Deoxyribonuclease (GAAD) inhibitor), HM62 and Ape1. Upon cleavage of these elements GAAD is released, activated and translocated to the nucleus where it can generate single stranded DNA (ssDNA) nicks that are known to lead to an apoptotic cell morphology (Fan et al., 2003).

A good understanding of the molecular mechanisms that regulate apoptosis is essential because it is known to be a central factor in prominent diseases such as cancer, where cell resistant to apoptosis is increased (Hanahan and Weinberg, 2000), and neurodegenerative disorders (Fadeel et al., 1999; Orrenius et al., 2003), in which neurons undergo unwanted apoptosis (Mattson, 2000).

During most viral infections, control of host cell death by the virus is essential to allow the completion of the virus life cycle (Tschopp et al., 1998). In many cases apoptosis is a major host anti-viral response because it is one of the first defence mechanisms used to eliminate infected cells in multicellular organisms (Hengartner, 2000). Infected apoptotic cells display an array of characteristic features that target them for removal by specialised immune effector cells such as local nonprofessional phagocytes or specialized phagocytes such as macrophages (Savill et al., 2002), helping to control infection.

### 1.2.1 The Golgi and ER in apoptosis

Several organelles are able to sense stressful or pathogenic alterations and to either adapt to such alterations or initiate pro-apoptotic signals that may lead to cell death. Although stress sensors have not been found for all organelles it is likely that most will be equipped with such sensing elements that can detect stresses related to
each organelle function and structure (Hicks and Machamer, 2005). The ER is a major site for folding and modification of transmembrane and secreted protein. Accumulation of unfolded or misfolded proteins and depletion of ER luminal Ca\(^{2+}\) can trigger specific stress responses such as the unfolded protein response (UPR) (described in detail in 1.4.4.1) (Ron and Walter, 2007) that lead to either increased ER folding capacity or, if the stress persists for a long period, to cell death by apoptosis.

The Golgi apparatus was discovered over 100 years ago and is a very dynamic structure involved in the sorting, processing (glycosylation, sulphation and phosphorylation) and transport of both transmembrane and secreted proteins and of lipids from the ER to the plasma membrane and other destinations (reviewed in Glick and Nakano (2009)). Although the textbook structure of the Golgi apparatus consists of several polarized (cis and trans sides) stacked flattened cisternae membranes, the Golgi manifests a variety of morphologies in different cell types. The existence of Golgi stress sensors has not been proven but some evidences point to the importance of this organelle in apoptosis. The inhibition of Golgi to ER traffic is known to cause the Golgi to collapse back into the ER and (Dinter and Berger, 1998) in fact the maintenance of the Golgi apparatus structure appears to be dependent on the correct balance between inward and outward membrane traffic (Hicks and Machamer, 2005). Together with the fact that inhibition of protein glycosylation, caused by swainsonine (Goss et al., 1995), is able to trigger cell death, these observations support the hypothesis that the Golgi is a stress sensor. There are several local damage responses from the Golgi that can lead to apoptosis: focal initiator caspase-2 activation can lead to caspase cascade activation (Mancini et al., 2000), the release of Fas receptor from the Golgi membrane to the plasma membrane in response to p53 activation (Bennett et al., 1998) and activation of Ganglioside (GD3) synthase that converts ceramide in GD3, which goes into the mitochondrion and can induce MMP (Rippo et al., 2000).

1.2.2 Poxvirus inhibition of apoptosis

Induction of apoptosis by immune effector cells is one of the most effective mechanisms by which the host controls and clears virus-infected cells (Russell and Ley, 2002). Most viruses have evolved strategies to control the host innate and adaptive immune responses and programmed cell death to insure an efficient viral replication (Benedict et al., 2002; Hay and Kannourakis, 2002).
In response to the selective pressure initiated by the host immune system, many viruses, including poxviruses, developed a large array of strategies to avoid the host anti-viral responses including inhibition of apoptosis (Taylor and Barry, 2006). The repertoire of poxvirus strategies to inhibit apoptosis can be divided into two categories: neutralization of extracellular signals or manipulation of the cell death pathways within the cell.

Examples of strategies used by poxviruses to neutralize extracellular death signals include major histocompatibility complex I (MHC I) downregulation and death receptors decoys. A mechanism used by the host to identify infected cells involves the display of viral antigens associated with the MHC I on the cell surface that are detected by CD8+ cytotoxic T lymphocytes. These can then target the infected cell for apoptosis by the activation of death receptors or by delivery of pro-apoptotic proteases (Edwards et al., 1999). The myxomavirus ER resident leukemia-associated protein is able to downregulate the levels of MHC I at the cell surface of myxoma virus infected tissues (Guerin et al., 2002). Viral production of secreted cytokine receptors or binding proteins is a very recurrent anti-apoptotic strategy in the poxvirus family. The first poxvirus TNF decoy, M-T2, was discovered in myxoma virus (Smith et al., 1990). This protein acts by binding TNF thereby inhibiting its binding to the cellular TNFR, preventing activation of the downstream signalling cascade. VACV protein cytokine response modifier E (CrmE) is also a membrane bound TNFR homolog that can sequester TNF and has been shown to contribute to virus virulence in a murine model (Reading et al., 2002). CPV encodes three TNFRs (CrmB, CrmC and CrmD), the equivalent genes to CrmB and CrmC in VACV (Alcami et al., 1999).

Proteins such as cytokine response modifier A (CrmA), or the VACV equivalent B13, and N1 or F1 act within the cell and are able to inhibit crucial steps in the apoptotic cascade, hence blocking downstream signals that could otherwise lead the cell commitment to apoptosis. CrmA is a serine protease inhibitor (serpin) that is produced early in a CPXV infected cell and has a potent caspase 1 and caspase 8 inhibitory activity. CrmA works by acting as a pseudo substrate for active caspasers, leading to the formation of a stable inhibitory complex (Tewari et al., 1995). VACV encodes a family of proteins that adopt a Bcl-2-like fold despite sharing no significant sequence similarity with cellular Bcl-2 family proteins. Some of these family members are able to prevent apoptosis, F1 and N1 are good examples of such proteins. N1 can prevent apoptosis by binding Bcl-2 homology 3 (BH3) motif peptides of pro-apoptotic
Bcl-2 family members (Cooray et al., 2007) and F1 localizes to the mitochondrion and inhibits loss of MMP and cyt c release induced by a large number of stimuli (Stewart et al., 2005).

The high number of proteins coded by poxviruses to prevent apoptosis attests to the importance of viral inhibition of infection-induced apoptosis in the poxviruses life cycle.

1.3 Golgi anti-apoptotic proteins (GAAPs)

1.3.1 Viral GAAP discovery

The analysis of the CMLV genome sequence revealed that this virus was the closest known relative of VARV and encoded several novel proteins with possible immunomodulatory activity at the genome terminal regions (Gubser and Smith, 2002), one of which was initially referred to as protein 6L encoded by gene 6L.

The CMLV 6L gene was shown to encode an early protein of 237-amino acid that migrates at 23 kilodaltons (kDa) in sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS-PAGE) although it has a predicted size of 26.5 kDa. Using an antibody (Ab) raised to the N-terminus and a haemagglutinin (HA)-tagged version of 6L, the protein was shown to be localized in the Golgi during infection and had a broad spectrum of anti-apoptotic activity (Gubser et al., 2007). In view of these properties the protein was called viral Golgi anti-apoptotic protein (vGAAP). The gene was found to be absent in most orthopoxviruses but present in three VACV strains, Lister, USSR and Evans. These VACV GAAPs were very similar (94-95% identity) to CMLV 6L (Gubser et al., 2007).

To determine the importance of vGAAP for virulence the gene was deleted from VACV strain Evans (ΔvGAAP) and re-inserted with or without a C-terminal HA tag (vGAAP revertant or vGAAP-HA revertant). Experiments done with these viruses showed that the protein was mainly localized to the Golgi complex during infection, was expressed early during viral life cycle and was not essential for virus replication but influenced virus virulence in vivo (Gubser et al., 2007).
Furthermore, stable cell lines overexpressing VACV strain Evans and CMLV vGAAP displayed significant resistance to apoptosis triggered by a wide range of intrinsic and extrinsic signals used (Gubser et al., 2007). The same results were obtained in apoptosis assays where transient overexpression of GAAPs was used.

1.3.2 GAAP subfamily (TMBIM4)

Viral GAAP is very closely related (73 % aa identity) to a human protein (called z-protein, CG1-119 and S1R in different databases) identified from the human genome-sequencing project and expressed in all human tissues tested (Gubser et al., 2007). This protein was named human GAAP (hGAAP) because it localized to the Golgi apparatus and also exhibited anti-apoptotic properties, protecting the cells from intrinsic and extrinsic apoptotic signals at a similar level as vGAAP, either at the level of or upstream of pro-caspase 8 activation. hGAAP is essential for cell survival because siRNA-mediated knockdown of hGAAP led to cell death by apoptosis (Gubser et al., 2007). It was also identified as a possible housekeeping gene using statistical analysis of microarrays (Lee et al., 2007). hGAAP messenger RNA (mRNA) levels were found to be upregulated in some breast tumours in humans making it a putative oncogene (van 't Veer et al., 2002). In an attempt to investigate this possible link with tumorigenesis, a member of our laboratory has demonstrated that hGAAP overexpression contributed to transformation of primary rat embryo fibroblasts in cooperation with a constitutively active Harvey ras (Hras) (Benjamin Johnson, unpublished data). It was found that co-expression of Hras and hGAAP increased the number of transformed cells by three fold when compared to Hras transfection alone.

GAAPs are highly conserved among eukaryotes and closely related proteins are predicted from the genome sequences of most eukaryotes including fungi, plants, where four different putative GAAPs have been identified, insects, fish, amphibians and mammals. All these GAAPs, from evolutionary diverse sources, share a similar sequence length and hydrophobicity profile that points to an important and evolutionarily conserved function. In fact, human and viral GAAP are so similar in terms of function that cells in which hGAAP was knocked down using siRNA can be rescued from cell death by the expression of vGAAP (Gubser et al., 2007).

Different bioinformatic prediction programs used to determine GAAP topology gave either six or seven transmembrane domains (TMD), with the seventh TMD being
the inconsistent TMD (Carrara et al, submitted). Using digitonin to selectively permeabilize the plasma membrane before performing IF with an Ab specific for tags strategically located at either the ends of GAAP or the inter TMD loops, it was shown that vGAAP, hGAAP and Bax inhibitor one (BI-1) have only six TMDs and that both the N terminus and the C terminus of these proteins are located in the cytosol (Figure 1.2).

hGAAP and its counterparts from other species are included in the transmembrane Bcl-2-associated X protein (Bax) inhibitor containing motif (TMBIM) family and represent number four (TMBIM4) of this family.

1.3.3 The Bax inhibitor-1 family (TMBIM)

Bioinformatic analysis assigned vGAAP and hGAAP to the protein family UPF0005, also known as TMBIM. These proteins are characterized by having a similar secondary protein structure of multiple (6 or 7) transmembrane spanning segments with short interconnecting loops, a charged C terminus (Reimers et al., 2008) and a conserved motif in the beginning of the third to the middle of the fourth predicted transmembrane domain. GAAP (TMBIM4) is one of the seven members described so far for this family. The TMBIM family includes other anti-apoptotic proteins like Bax inhibitor 1 (described in more detail in 1.3.3.1), lifeguard protein (LFG), and mitochondrial morphology and cristae structure 1 (MICS1).

LFG, also known as TMBIM2, is a plasma membrane associated protein that is abundant in neurons, although there are contradictory data about its expression in other tissues. It can protect cells from Fas, but not from TNFα, -induced apoptosis and binds to the Fas receptor (Somia et al., 1999).

MICS1, also known as TMBIM5 localizes to the mitochondrial inner membrane and is required for mitochondrial cristae organization. MICS1 knockdown by siRNA stimulates the release of cyt c from the mitochondrion whereas its overexpression partially inhibits cyt c release (Oka et al., 2008).

More recently it was proposed that GAAPs are very widely distributed and very ancient within eukaryotes. Phylogenetic analysis supports the expansion of some of the members of the BI-1 family from a GAAP ancestor about 2,000 million years ago (Hu et al., 2009). This and the fact that a set of conserved sequence motifs was found for a subset of the members of the BI-1 and N-methyl D-aspartate-associated protein 1
(NMDARA1) families led to the proposed creation of a new gene family, the LFG family (Hu et al., 2009).

**Figure 1.2 - GAAP (TMBIM4) protein topology.** GAAPs topology as described by Carrara et al, (submitted). The N- and C-terminal regions of both vGAAP and hGAAP were shown to be located at the cytosolic side of the Golgi membrane. There is still some uncertainty about the C-terminal region organization as there are still two possible situations, (i) the C-terminal region is free in the cytosol or (ii) it is a re-entry loop that partially enters the membrane without crossing it.

### 1.3.3.1 Bax inhibitor-1

The most studied member of the TMBIM family, BI-1, was identified in a screening of mammalian proteins that rescued yeast from Bax-induced apoptosis. Like GAAP, BI-1 is a widely expressed and evolutionarily conserved integral membrane protein that protects cells against many different intrinsic and extrinsic death stimuli (Xu and Reed, 1998). Cells from BI-1 knockout mice display an increased hypersensitivity to apoptosis induced by ER stress agents (Chae et al., 2004). This phenotype was later shown to be related to the ability of BI-1 to bind the ER stress sensor Ser/Thr protein kinase and endoribonuclease IRE1α and reduce its endoribonuclease activity, leading to a reduction in levels of X-box-binding protein-1 and hence downregulating the UPR target gene expression (Lisbona et al., 2009). Bax inhibitor-1 primarily localizes to the
ER membrane where it increases Ca\(^{2+}\) leakage in a pH-dependent manner, reducing the ER Ca\(^{2+}\) concentration and consequently the amounts of Ca\(^{2+}\) that can be released upon stimulation (Xu et al., 2008). Deletion of the charged C terminus of the protein abrogated its ability to modulate ER Ca\(^{2+}\) content. Acidic conditions induced both Bi-1 oligomerization and Ca\(^{2+}\) leakage from the ER (Kim et al., 2008). Work done with pure Bi-1 protein reconstituted in liposomes suggests that Bi-1 has a Ca\(^{2+}/H^+\) anti-porter-like activity because it allowed Ca\(^{2+}\) entrapped in liposomes to be released (Ahn et al., 2009). The anti-porter activity is regulated by the lipid composition of the membrane that Bi-1 is reconstituted in (Ahn et al., 2010). Moreover, recent data from Bultynck et al. (2011) showed that a peptide containing 20 amino acid residues from Bi-1 C terminus was sufficient to create a pore that can cause the release of Ca\(^{2+}\) from the ER. Two aspartic acid (D) residues (D209 and D213) proved to be essential for Ca\(^{2+}\) flux. This could explain the mechanism by which Bi-1 regulates the ER Ca\(^{2+}\) filling state, presenting a possible mechanism for its anti-apoptotic function. The physiological relevance of Bi-1 ability to prevent cell death is highlighted by the fact that its activity is manipulated by the enteropathogenic _Escherichia coli_ in order to prevent apoptosis in infected cells by expressing the NheH protein that binds to Bi-1 and modulates its Ca\(^{2+}\) release activity (Hemrajani et al., 2010).

Increased actin polymerization is observed when Bi-1 is overexpressed, and this is dependent on the Ca\(^{2+}\) release activity of Bi-1 and leads to increased cell adhesion (Lee et al., 2010a). Expression levels of Bi-1 are upregulated in some human breast cancer cells (Igney and Krammer, 2002) and prostate cancer (Grzmil et al., 2006) and indeed Bi-1 can contribute to cancer development and drug resistance (Grzmil et al., 2006; Igney and Krammer, 2002). Moreover Bi-1 was shown to increase metastasis by increasing cell motility and invasiveness and by altering glucose metabolism (Lee et al., 2010b). Recently, Bi-1 has been identified as a good putative target for anti-cancer therapy (Yun et al., 2011).

### 1.3.4 hGAAP and Ca\(^{2+}\) modulation

The only insight into the possible molecular mechanism by which GAAPs modulate apoptosis came from de Mattia et al. (2009). Using both hGAAP overexpression and knockdown it was shown that hGAAP is capable of reducing the cytosolic and mitochondrial Ca\(^{2+}\) raises observed upon stimulation with an apoptotic
stimuli (staurosporine (STS)). The same result was observed for histamine-induced rises in cytosolic and mitochondrial Ca\(^{2+}\). This is very important because it is known that changes in Ca\(^{2+}\) homeostasis play a central role in determining cell susceptibility to an apoptotic stimuli and hence to apoptosis (detailed in section 1.4). Moreover, this mechanism pointed to hGAAPs ability to reduce the Ca\(^{2+}\) filling state of the intracellular Ca\(^{2+}\) stores, the Golgi and the ER. The findings that hGAAP could co-immunoprecipitate with type 3 IP\(_3\)R and was able to alter the IP\(_3\)-induced Ca\(^{2+}\) release from intracellular stores were consistent with the hypothesis that hGAAP Ca\(^{2+}\) modulation was occurring via interaction and modulation of IP\(_3\)R activity instead of by acting as an ion channel itself (de Mattia et al., 2009).

1.4 Ca\(^{2+}\) homeostasis

Ca\(^{2+}\) signalling is involved in controlling many different cellular functions such as migration, contraction, gene transcription and cell proliferation (Carafoli et al., 2001). Variations in duration, localization, frequency and amplitude of Ca\(^{2+}\) transient rises makes Ca\(^{2+}\) one of the most versatile cellular signal elements (Berridge et al., 2000). Cytosolic Ca\(^{2+}\) levels are tightly regulated and are the result of the balance between a number of reactions that introduce Ca\(^{2+}\) in the cytosol (“on” reactions) and reactions that remove Ca\(^{2+}\) from the cytosol (“off” reactions) (Figure 1.3) (Berridge et al., 2003) and it is this tight control that allows the large variety of Ca\(^{2+}\) signals. Ca\(^{2+}\) is released into the cytosol from either the extracellular medium or from intracellular Ca\(^{2+}\) stores such as the ER or the Golgi. The result is a transient increase in cytosolic Ca\(^{2+}\) concentration and because most of this Ca\(^{2+}\) is quickly chelated by buffers or pumped out of the cytosol into the ER, the mitochondrion, the Golgi and the extracellular medium (Berridge et al., 2003), only a very small portion will be available to bind effectors and lead to the stimulation of Ca\(^{2+}\)-dependent processes.

To control and manipulate Ca\(^{2+}\) signalling, different cells express different repertoires of Ca\(^{2+}\)-modulating molecules such as Ca\(^{2+}\) receptors, transducers, channels, buffers, effectors, pumps and exchangers. Using this large tool kit, cells can generate a huge variety of Ca\(^{2+}\) pulses by manipulating the “on” and the “off” reactions (Berridge et al., 2003).
Figure 1.3 - Ca²⁺ signalling and homeostasis. The group of reactions that remove free Ca²⁺ from the cytosol are generically called “off” reactions while the series of reactions that introduce Ca²⁺, from either the intracellular Ca²⁺ stores or from the extracellular medium, are known as “on” reactions. The balance between the “on” and the “off” reactions determines the levels of active Ca²⁺ (free to bind effectors) in the cytosol at any given time. This activated Ca²⁺ can trigger very different cellular processes depending on the intracellular localization, intensity and duration of the Ca²⁺ release. Adapted from Berridge et al. (2003).
### 1.4.1 Ca²⁺ flow across the plasma membrane

Ca²⁺ flow into the cytosol coming from the extracellular medium can be triggered by a variety of stimuli such as membrane depolarization, stretch, extracellular agonists, intracellular messengers or by depletion of intracellular stores. These stimuli can also lead to the formation of second messengers that can trigger the release of Ca²⁺ from intracellular stores (reviewed in Bootman et al. (2002)). This release is mostly controlled by Ca²⁺ itself and by messengers such as inositol-1,4,5-triphosphate (IP₃) or cyclic ADP ribose and nicotinic acid adenine dinucleotide phosphate (Lee, 1997). Many stimuli work by phospholipase C (PLC) to generate IP₃. Different PLC isoforms are activated by different stimuli and this leads to the generation of different IP₃ production levels and for different periods (Kelley et al., 2001; van der Wal et al., 2001). Entry of Ca²⁺ from outside is mostly driven by the electrostatic gradient that exists through the plasma membrane (Berridge et al., 2003). Different channels in the plasma membrane open in response to different stimuli allowing the generation of different Ca²⁺ signals that will lead to different outcomes. These channels at the plasma membrane can respond to voltage (voltage-operated channels - VOCs) (Dolphin, 2006), to the binding of a ligand (receptor-operated channels - ROCs), to second messengers (second-messenger-operated channels - SMOCs) such as the arachidonic-acid-sensitive channel (Mignen and Shuttleworth, 2000), to intracellular store Ca²⁺ content (store-operated channels - SOCs) (Parekh and Putney, 2005) and to many other stimuli such as temperature or stretch. VOCs are usually found in excitable cells and are able to generate fast Ca²⁺ fluxes to control fast cellular processes such as muscle contraction. A good example of a ROC is the N-methyl-D-aspartate receptors NMDAR that allow a Ca²⁺ flow in response to glutamate (Masu et al., 1993). SOCs respond mainly to ER lumen Ca²⁺ depletion. This response allows the entry of external Ca²⁺ that can mediate longer cytosolic Ca²⁺ signals and also contribute to the replenishment of intracellular Ca²⁺ stores.

After an increase in cytosolic Ca²⁺ levels a number of pumps located at either the plasma membrane or at cellular organelles will actively remove Ca²⁺ from the cytosol to re-establish its normal resting Ca²⁺ concentration of approximately 100 nM (Carafoli, 1987). The plasma-membrane Ca²⁺-ATPase (PMCA) (Carafoli, 1994) and the Na⁺/Ca²⁺ exchanger (NCX) (Blaustein and Lederer, 1999) are the main pumps that actively remove Ca²⁺ from the cytosol into the extracellular medium. Different pumps present different transport rates and affinities to Ca²⁺ and different pumps will respond
to different Ca\(^{2+}\) concentrations allowing the cell to control cytosolic Ca\(^{2+}\) levels quickly, precisely and efficiently after a Ca\(^{2+}\) rise. PMCA and the sarco(endo)plasmic reticulum Ca\(^{2+}\) ATPase (SERCA) (Vangheluwe et al., 2005) respond to small Ca\(^{2+}\) elevations but with a low transport rate. These pumps are essential in setting the basal cytosolic Ca\(^{2+}\) levels. In contrast, NCX and the mitochondrial uniporter have a much higher transport rate allowing them to limit Ca\(^{2+}\) rises within a bigger range (Collins et al., 2001).

### 1.4.2 Ca\(^{2+}\) intracellular stores

The ER has a central role as the main cellular Ca\(^{2+}\) store although other organelles such as the Golgi or lysosomes are also able to store Ca\(^{2+}\) and release it in response to stimuli. High Ca\(^{2+}\) levels in the ER lumen (100-500 µM) are maintained by the action of the SERCA pump that actively transports Ca\(^{2+}\) from the cytosol into the ER lumen (Vangheluwe et al., 2005). Release of Ca\(^{2+}\) from the ER is mainly regulated by the activity of the IP\(_3\) receptor (IP\(_3\)R) (Thrower et al., 2001) and the ryanodine receptor (RYR) (Bezprozvanny et al., 1991). Both these channels are sensitive to Ca\(^{2+}\) and can lead to Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR), a process involved in generating rapid Ca\(^{2+}\) rises (Endo, 2009). There are three isotopes of the IP\(_3\)R and they can form homo or hetero-tetramers. IP\(_3\)R is also regulated by, among others factors, IP\(_3\) which induces a conformational change in the channel (Thrower et al., 2001) that can increase the channel sensitivity to Ca\(^{2+}\) at low Ca\(^{2+}\) concentrations but has an inhibitory effect at high Ca\(^{2+}\) concentrations.

The Golgi apparatus is involved in processing and sorting membrane bound and secreted proteins and occupies a perinuclear position in most cell types. Some of its functions are regulated by Ca\(^{2+}\) concentration changes within the Golgi lumen or in the adjacent cytosol. The Golgi’s ability to accumulate Ca\(^{2+}\) relies on the activity of the two main Ca\(^{2+}\) transport pumps located on the Golgi membrane, the secretory pathway Ca\(^{2+}\) ATPase (SPCA) (Wuytack et al., 2003) and the SERCA pump. While these two pumps are responsible for Ca\(^{2+}\) uptake that lead to an accumulation of Ca\(^{2+}\) in the Golgi lumen, IP\(_3\)R localized in the Golgi membrane is able to release Ca\(^{2+}\) in to the cytosol. Most IP\(_3\)Rs are expressed in the ER membrane but it has been reported that IP\(_3\) is able to stimulate Ca\(^{2+}\) release from the nuclear envelope or the Golgi (Pinton et al., 1998).
1.4.3 Ca\textsuperscript{2+} spatial and temporal organization

Temporal and spatial control of Ca\textsuperscript{2+} signals contributes to the high versatility of Ca\textsuperscript{2+} signals. Spatial control of Ca\textsuperscript{2+} release is relevant for rapid cellular processes, such as contraction, but appears to be less important in slower processes, such as cell proliferation (Lipskaia and Lompre, 2004), where Ca\textsuperscript{2+} signals are usually present as repetitive Ca\textsuperscript{2+} waves. Ca\textsuperscript{2+} binding proteins or buffers, such as D-28, calretinin, or parvalbumin are important for both the establishment of spatial and temporal regulated Ca\textsuperscript{2+} signals because Ca\textsuperscript{2+} binding and releasing dynamics of these molecules are able to fine-tune Ca\textsuperscript{2+} concentrations (John et al., 2001). A temporal control of Ca\textsuperscript{2+} signals is essential because different length Ca\textsuperscript{2+} signals will trigger different cellular responses (Figure 1.3). The longer a Ca\textsuperscript{2+} rise lasts for the more likely it is that it will spread as a wave that spreads through the cell or locally. When subjected to a prolonged stimulation these Ca\textsuperscript{2+} signals can form regular Ca\textsuperscript{2+} oscillations caused by several rounds of Ca\textsuperscript{2+} release and diffusion from IP\textsubscript{3}R or RYP clusters (Niggli, 1999). The Ca\textsuperscript{2+} oscillation frequency is crucial to determine what cellular process is activated and Ca\textsuperscript{2+} oscillations are known to regulate processes such as cell growth, migration (Gomez et al., 1995) or disassembly of focal adhesions during cell migration (Giannone et al., 2002, Taufiq Ur et al., 2009).

1.4.4 Ca\textsuperscript{2+} role in apoptosis

The role of Ca\textsuperscript{2+} in apoptosis has been compared to the “conductor of an orchestra” (Orrenius et al., 2003) regulating different portions of the cell death program. Ca\textsuperscript{2+} overload is known since the late 1960s to be highly toxic and to cause activation of proteases and phospholipases. Although this effect has been associated with necrosis, more recent data point to an important role in other types of cell death such as apoptosis. Indeed different studies detected an increase in Ca\textsuperscript{2+} cytosolic concentration in early and late stages of apoptosis (Lynch et al., 2000). It is now a common view that an increase in cytosolic Ca\textsuperscript{2+} concentration can occur when the cell is subjected to insults, promoting apoptosis.

There are two main mechanisms by which Ca\textsuperscript{2+} can contribute to the induction of cell death; (i) by increasing mitochondrial matrix Ca\textsuperscript{2+} concentration leading to cyt c
release or (ii) by activating effectors such as calcineurin or calpain2 that can lead to a downstream activation of an apoptotic pathway.

(i) Mitochondrial matrix Ca$^{2+}$ plays a central role in regulating ATP production by activating the citric acid enzymes (Rizzuto et al., 2004) but a Ca$^{2+}$ overload results in a mitochondrial depolarization that leads to the release of mitochondrial matrix proteins, such as cyt c. This activates apoptotic pathways leading to activation of caspases and nucleases that finalize the apoptotic process (Orrenius et al., 2003). The mitochondrial matrix Ca$^{2+}$ levels can rise in response to a generalized increase in cytosolic Ca$^{2+}$ levels, originated for instance by PMCA cleavage by caspases, preventing it from pumping Ca$^{2+}$ out of the cytosol (Schwab et al., 2002), or, as a result of Ca$^{2+}$ influx, through the plasma membrane. IP$_3$R is involved in apoptosis because cells lacking or knocked down for some types of IP$_3$R show an increased resistance to apoptotic stimuli (Jayaraman and Marks, 1997; Khan et al., 1996). IP$_3$R itself is a caspase-3 substrate and its cleavage creates a leaky IP$_3$R channel that can allow Ca$^{2+}$ to flow out of the ER and possibly the Golgi (Nakayama et al., 2004). More recently a series of molecules involved in apoptosis such as cyt c, Bcl-2 and B-cell lymphoma-extra large (Bcl-xl) were also found to be able to control IP$_3$R activity. Cyt c release from mitochondria adjacent to the ER was found to bind ER IP$_3$Rs, increasing Ca$^{2+}$ release from the ER and resulting in a positive feedback loop of cyt c release from the mitochondrion (Boehning et al., 2003). This was proposed as the mechanism by which a cell can synchronize cyt c release from all mitochondria. Bcl-2 is also able to bind IP$_3$R preventing Ca$^{2+}$ release (Rong et al., 2008). Bcl-xl binding to IP$_3$R increases its sensitivity to Ca$^{2+}$ and IP$_3$ reducing the Ca$^{2+}$ filling state of the ER and by doing so, reducing the amount of Ca$^{2+}$ release upon an apoptotic stimuli (White et al., 2005).

(ii) IP$_3$R Ca$^{2+}$ release can lead to calcineurin activation and this enzyme will dephosphorylate Bad leading to its translocation from the cytosol to the mitochondrion and induction of cyt c release (Jayaraman and Marks, 1997). The Ca$^{2+}$-activated cysteine proteases calpain family includes several tissue-specific isoforms and two ubiquitous isoforms calpain1 (also known as µ-calpain) and calpain2 (also known as m-calpain). Many of these proteins are responsible for cleaving several cellular proteins during apoptosis (Vanags et al., 1996). Calpain is able to cleave pro-caspase-12 thereby activating it and leading to pro-caspase 3 and 7 activation (Nakagawa et al., 2000). Calpain activity is controlled, among other factors, by its inhibitor calpastatin.
This protein can be cleaved by caspase-3 during apoptosis, thus activating calpain activity (Porn-Ares et al., 1998).

### 1.4.4.1 ER stress responses

The ER is responsible for folding, modifying and sorting of newly formed transmembrane and secreted proteins and to store Ca\(^{2+}\). The disruption of any of these functions can lead to ER stress. The ER can respond to the accumulation of unfolded proteins by activating two responses that can increase the folding capacity of the ER and remove incorrectly folded proteins, the UPR (reviewed in Ron and Walter (2007)) or the ER overload response (EOR). The three main ER stress transducers are the inositol-requiring protein-1 (IRE1), the activating transcription factor-6 and the protein kinase RNA-like ER kinase. In all three cases an integral membrane protein senses the protein-folding status of the ER lumen and transmits that information to the cytosol (Schroder and Kaufman, 2005). The UPR leads to the activation of expression of ER chaperons (Kozutsumi et al., 1988) and to the inhibition of translation. Prolonged ER stress can also lead to apoptosis by activation of calpain that cleaves pro-caspase 12 in the ER membrane and activates effector caspases (Nakagawa et al., 2000). The EOR leads to the production of cytokines and interferons through activation of the nuclear factor κB (NF-κB). Changes in ER Ca\(^{2+}\) concentration can lead to both reactive oxygen species production and calpain2 activation.

### 1.4.5 Viral manipulation of Ca\(^{2+}\) homeostasis

Viruses are known for their abilities to subvert the cellular machinery to their advantage allowing them to generate the conditions needed to complete their life cycle. As the most common and versatile cellular messenger it is to be expected that viruses have developed strategies to modulate cellular Ca\(^{2+}\) signals to their benefit (Zhou et al., 2009).

Most viral infections tend to increase the host-cell cytosolic Ca\(^{2+}\) levels in an attempt to facilitate the virus life cycle. Increases in cytosolic Ca\(^{2+}\) concentration can activate and accelerate several enzymatic processes and can activate several transcription factors. These include nuclear factor of activated T-cells (NFAT), which
can be important for promoting viral replication or to establish a persistent infection (Ding et al., 2002; Kinoshita et al., 1997; Lee et al., 2005). Ca\textsuperscript{2+} released from the ER can be taken up by mitochondria, and in some circumstances this can activate adenosine triphosphate (ATP) production that is essential to meet the high levels of energy required for virus replication (Li et al., 2007). Some viruses can reduce the ER and Golgi Ca\textsuperscript{2+} levels and inhibit protein traffic to the plasma membrane, thus preventing some of the host-cell anti-viral responses to be initiated (Doedens and Kirkegaard, 1995). The accumulation of ER and Golgi-derived vesicles might also be important for some viruses that require the microenvironment created by these vesicles to replicate their RNA (van Kuppeveld et al., 2005). By promoting or preventing Ca\textsuperscript{2+} trafficking from the ER to the mitochondrion viruses can also either prevent or induce apoptosis.

To achieve all these effects viruses can encode proteins that directly or indirectly manipulate the host-cell Ca\textsuperscript{2+} homeostasis by either altering membrane permeability or by subverting Ca\textsuperscript{2+} signalling pathways. Some of those viral proteins can form pores and alter membrane permeability by allowing the flow of ions, and these proteins are generically called viroporins. Examples of these proteins that are partially or totally selective for Ca\textsuperscript{2+} include the ER located proteins 2B from coxsakievirus (Nieva et al., 2003), p7 protein from hepatitis C virus (HCV) (Griffin et al., 2003), and protein p13" from the human T-lymphotropic virus-1 (HTLV-1) located at the mitochondrial inner membrane (D'Agostino et al., 2005).

### 1.5 Ion channels

An ion channel is typically defined as a protein that can form a pore in a lipid bilayer, allowing the passive flow of ions, such as Na\textsuperscript{+}, K\textsuperscript{+}, Ca\textsuperscript{2+}, Mg\textsuperscript{2+}, Cl\textsuperscript{-} and H\textsuperscript{+}, along electrochemical gradients across the membrane, which is otherwise impermeable to ions (Darnell et al., 1990). Ion channels can create changes in membrane potential or alter ion concentrations in the cell. By doing this ion channels are essential for electrical excitability, maintenance of membrane potential, hormone/neurotransmitters release, regulation of cell volume, apoptosis, activation of secondary messengers and signalling cascades, among others.
Because ion channels are involved in controlling so many cellular events and functions, it is not surprising that ion channel malfunctions are responsible for many human pathologies such as cystic fibrosis (Boucher, 2002). Due to this fact they have revealed to be important targets for drug development and so far there are approximately 180 drugs approved by the food and drug administration (FDA) that exert their effect through ion channels, and these include peptides, toxins and small organic molecules. These drugs act by either blocking (Catterall, 2000; Voets et al., 2002) or by modifying the gating properties of ion channels (Swartz and MacKinnon, 1995).

The development of the “patch-clamp” technique that enabled the recording of electrical activity in small areas of the membrane was essential for ion channel characterization and understanding. The use of this technique allowed Neher and Sakmann (1976) to resolve single channel events and formally demonstrate the presence of ion channels. This technique remains the gold standard approach to ion channel functional analysis. Another technique that has proven essential in revealing the mechanisms behind ion channel properties is protein structure determination by X-ray crystallography. In 1998 the structure of the first ion channel, K⁺ bacterial channel KcsA, was published, (Doyle et al., 1998) and in 2005 the first mammalian ion channel structure was solved (Long et al., 2005). These findings helped answer many questions regarding channel assembly, permeability and gating.

Most known ion channels are composed of a single repeat-structure protein or by multiple “subunits” arranged to form a water-filled pore across the lipid bilayer. Hydrophobic segments of these units span the membrane as coiled “α-helices” typically 20-27 amino acids long or as β-barrel sheets with 9-13 amino acids and are both referred to as transmembrane domains (TMD) (Kew and Davies, 2010). These TMDs form the core region of the channel and include the pore-forming region.

In most cells the cytosolic side of the plasma membrane is negatively charged due to the accumulation of negatively charged organic acids and proteins. This gradient is maintained by the action of pumps that actively force ions across the membrane against electrochemical gradients (Darnell et al., 1990). One of the most important pumps that maintains electrochemical gradients in the membrane is the Na⁺/K⁺ ATPase, and similar ion transporters exist for Cl⁻ and Ca²⁺. The accumulation of negative charges on the inside attracts positive charges to the outside face of the membrane creating a potential difference across the membrane, better known as
cellular membrane potential. The movement of ions across the membrane, down concentration gradients, into or out of cells, is offset by this electrical gradient (Hille, 1992).

### 1.5.1 Ion channels versus ion pumps

Pumps can also allow passage of ions across the lipid bilayer but at a rate more than 1000 times slower than ion channels. The main difference between ion channels and ion pumps is that the first can only allow ions to flow if a favourable electrochemical gradient exists, in contrast, the second can use energy to actively push ions across the membrane against a gradient at a much slower rate than observed for ion channels (reviewed in Gadsby (2009)). Ion channel activity will allow the generation of electrical currents while ion pumps can create and/or maintain ion gradients. Pumps can use two main sources of energy: primary pumps use energy from ATP hydrolysis; while secondary pumps use energy stored in electrochemical gradients by coupling thermodynamically downhill movements of ions to drive uphill transport of another substrate. These are also called co-transporters or exchangers (Jardetzky, 1966). The main structural difference between pumps and channels relates to the number of gates required for function. A gate is the part of the protein that prevents ions from moving across the channel in its closed conformation but allows its passage in the open conformation. While ion channels only need one gate that will open and allow ions to flow, an ion pump needs to have two gates that are never open at the same time (Gadsby, 2004). Pump gates work by opening alternately, creating a space in which the ion is kept before being release to the other side of the membrane against a gradient. But although ion channels only need one gate, many actually have two gates (Hille, 1992) and if both gates of an ion pump get open at the same time it will behave as an ion channel. These observations highlight the fact that ion channels and ion transporters are actually not that different in practice although they have conceptually two very distinct functions.
1.5.2 Ion channel permeability and ion selectivity

Different ion channels have different single channel conductance values (expressed in Siemens (S)) and these values can range between fS up to several hundreds pS. Conductance represents the rate at which a given ion channel allows ions to flow and in a constant voltage situation (“clamped”) is directly related to current and can be calculated as the slope of a linear regression between the voltage applied to the membrane and the current of ions obtained in “patch-clamp” experiments (Kew and Davies, 2010). Some channels can exhibit what is known as rectifications, where the current-voltage relationship is not linear (Matsuda et al., 1987).

Several aspects contribute to the fact that different ions flow more or less readily through different ion channels. The relative permeability of a given ion channel is defined by a series of factors such as the hydrated state, the shape and charge of the ion in question, and also by the pore structure that includes the selectivity filter. This filter, that works as a sieve, is usually made up by a series of charged amino acid residues and is usually 3-7 angstroms in diameter (Kew and Davies, 2010). The ion selectivity will depend on the balance between the energy required to remove water molecules from the ion and the energy of allowing the partially dehydrated ion to interact with the charged amino acid residues in the filter (Hille, 1992).

Some channels show mixed selectivity, usually for either cations or anions. Other factors such as ion relative abundance and concentration, can also influence the ions that eventually flow through the channel. For example in the ER where Ca$^{2+}$ is very abundant, some Ca$^{2+}$ ion channels that allow Ca$^{2+}$ out of the ER lumen, such as the IP$_3$R, don’t need to have a very strong or specific ion selectivity filter because Ca$^{2+}$ is the major cation with an appreciable electrochemical gradient across the ER membrane (Foskett et al., 2007; Williams et al., 2001).

1.5.3 Ion channel gating

Most channels have different conformational states, each with a different conductance, and gating describes the process by which a channel changes between these different states. The transition between the closed state, at which the channel is impermeable to ions, and the open state, that allows ions to flow through the pore, is
called activation (Ulbricht, 2005). Classically, ion channels are classified accordingly to their gating mechanism and they can be included in two main classes: voltage-gated or ligand-gated. Ligand-gated ion channels are activated by ligands such as Ca\(^{2+}\), nucleotides or neurotransmitters, while voltage-gated channels are activated by changes in membrane potential (Kew and Davies, 2010). Although useful this is certainly a very simplistic classification because each class is subdivided into many subclasses. Some ion channels can be included in both classes because they are regulated by both voltage and ligands or they can be activated by other stimuli such as light or mechanical stress (Kew and Davies, 2010). The overall activity and selectivity of an ion channel is controlled not only by its gating mechanism but also by factors such as phosphorylation, oligomerization (homo or hetero), association with regulatory proteins, subcellular localization and tissue specificity.

### 1.5.3.1 Ligand versus voltage-gated channels: focus on Ca\(^{2+}\) channels

Ion channels that allow the flow of Ca\(^{2+}\) selectively can be included in the two classes of ion channels, the ligand-gated and the voltage-gated ion channels. The first class includes the IP\(_3\)R, RYR and store-operated Ca\(^{2+}\) channels among others; some of these were already referred in section 1.4. This group of ion channels is typically constituted by three domains: in the case of a plasma membrane channel these are the: extracellular domain involved in binding the ligand, transmembrane cylinder that constitutes the pore, and the intracellular region that might be involved in subcellular localization and regulation by cytosolic secondary messengers (Striggow and Ehrlich, 1996).

The voltage-gated channels class includes channels that require depolarization of the membrane to be activated. They can be divided into subclasses according to the levels of membrane depolarization that they require for activation (Ertel et al., 2000). L, P and N-type channels are examples of Ca\(^{2+}\) channels that require large membrane depolarization for activation. L-type channels are found in cardiac muscle and are essential for muscle contraction, P and N-type are mainly found in the brain and are involved in neurotransmitter release. T-type channels respond to relatively small levels of plasma membrane depolarization but show a very rapid voltage-dependent activation. These channels can be found in cells with “pacemaker” activity and are involved in sinus rhythm regulation.
The selectivity filter domain for voltage-gated Na\(^+\) channels (Nav) usually contains the signature amino acid sequence DEKA (Heinemann et al., 1992) while TVGYG is frequently found in voltage-gated K\(^+\) channels (Kv) (Jiang et al., 2002). For voltage-gated Ca\(^{2+}\) channels (Cav) EEEE is found commonly (Sather and McCleskey, 2003), although both aspartic acid (D) and glutamic acid (E) can contribute to the four COO\(^-\) groups usually present in Ca\(^{2+}\) channels selectivity filters. Ca\(^{2+}\) ion channel selectivity filters are highly negatively charged so they can attract cations, balancing the charges and minimizing free energy. This will force cations such as Na\(^+\) and Ca\(^{2+}\) to compete for the space in the filter. The charge/space mechanism competition model implies that Ca\(^{2+}\) can more efficiently balance the charges of the filter because it provides twice as much charge per ion than Na\(^+\) while occupying a similar space. This model implies that the main determinant of Ca\(^{2+}\) versus Na\(^+\) selectivity is the filter volume (Malasics et al., 2009).

### 1.5.4 Ion channel basic structure

The basic structure of the K\(^+\) voltage gated ion channels (Kv) consists of a tetramer of 6 TMDs (named S1-S6) with a short re-entrant P-loop between S5 and S6 (Figure 1.4). Na\(^+\) and Ca\(^{2+}\) voltage gated channels, Nav and Cav, respectively, have a similar structure to the Kv but whereas the Kv is composed of four subunits with 6 TMDs each called homologous repeat domains, Nav and Cav are usually composed of a single protein that contains all 24 TMDs (Kew and Davies, 2010). S4 typically contains the voltage sensor while S5, the P-loop and S6 contain the selectivity filter (Noda et al., 1984).

The non-voltage-gated inward rectifying K\(^+\) channels constitute the most basic structure of an ion channel. These are tetramers of subunits that contain two TMDs (M1 and M2), which are analogous in function and structure to the S5 and S6 TMDs of Kv channels (Figure 1.4). This implies that a minimum of eight TMDs is required to form an ion channel, although it is possible to form membrane pores with much smaller peptides (Choe, 2002).
1.5.5 Viral ion channels

Some viruses encode ion channels and these are generically called viroporins. Viral ion channels are usually smaller than cellular ion channels and they seem to represent a miniaturized and simplified channel system. The first viral ion channel was found in influenza virus and is called M2. This is a small protein composed of 97 amino acid residues that can form tetramers (Sugrue and Hay, 1991) to create a pore permeable to H⁺ (Schroeder et al., 1994). This protein is important during virus entry.
because it promotes virus uncoating within the endosomes by lowering the pH of the virion interior (Mould et al., 2000). M2 can also affect glycoprotein processing and trafficking (Henkel and Weisz, 1998).

Another example of a viroporin is the human immunodeficiency virus-1 (HIV-1) viral protein u (Vpu). This protein plays a role in CD4 degradation (Schubert et al., 1994) and is important for viral particle release (Klimkait et al., 1990). The N-terminal region of Vpu was found to have ion channel activity and to interact with TWIK-related acid-sensitive K+ 1 (TASK-1), a mammalian ion channel, forming a dysfunctional heteromultimer that may reduce TASK-1 activity (Hsu et al., 2004). The ion channel activity of Vpu has not yet been connected to its role in viral particle release.

Some other examples of viral Ca\(^{2+}\) channels were already referred in section 1.4.5.

The study of these proteins may prove relevant for the development of new antiviral drugs that can modulate viroporins activity. Examples of drugs that target viral ion channels include the influenza A M2 protein inhibitor amantadine that is clinically used (Oxford and Galbraith, 1980).

### 1.6 Cell adhesion and migration

A major role of Ca\(^{2+}\) is in cell migration. Cell migration involves a series of sequential events starting with protrusion of the cell membrane followed by formation of new adhesions at the cell front, that establish the physical connection between the substratum and the actin cytoskeleton, leading to the generation of traction forces that ultimately make the cell move forward. Movement requires the disassembly of adhesions at the rear of the migrating cell.

Cell migration can be triggered by several stimuli such as the presence of growth factor or chemokine gradients, extracellular matrix (ECM) proteins, mechanical forces or electrochemical gradients (Petrie et al., 2009). The polarization and extension of protrusions of the cell membrane towards the stimuli initiate the migration process. Protrusions are driven by the polymerization of actin filaments and comprise large
broad lamellipodia and spike-like lamellipodia (Pollard and Borisy, 2003). These structures are stabilized by adhesions that link the intracellular actin cytoskeleton to the ECM proteins and allow, together with actomyosin contractions and the generation of the traction force. Contractibility will also allow the cell to move forward by allowing the disassembly of adhesions at the rear of the cell (Ridley et al., 2003). Signals generated by both newly formed and more stable adhesions, cytoskeleton reorganization and actin polymerization can influence each other and ultimately coordinate the directional cell movement.

Several cell adhesion receptors such as integrins, syndecans and cadherins have been identified. Integrins were first discovered about 25 years ago (Tamkun et al., 1986). Members of this large family of heterodimeric (subunit α and subunit β) glycoproteins (Ruoslahti and Pierschbacher, 1987) are expressed in most cells and are directly involved in cell migration due to their direct involvement in cell-substrate adhesion. Integrins work as receptors, their extracellular domain can bind ECM proteins such as fibronectin (Tamkun et al., 1986), vitronectin (Pytela et al., 1985) or collagen (White et al., 2004) and generate intracellular signals (Hynes, 2002). These receptors are important targets for drug development as their deregulation or malfunction is associated with many disorders such as cancer, thrombosis and autoimmune disease (Cox et al., 2010).

1.6.1 Cell adhesions

Integrins can directly link the actin cytoskeleton with the extracellular substratum by suffering a conformational change upon binding to ECM proteins that allows them to interact with intracellular proteins such as talin (Campbell and Ginsberg, 2004), vinculin (Ziegler et al., 2006), or the actin cross-linking protein α-actinin (Otey and Carpen, 2004). These can all bind filamentous-actin (F-actin) and, by doing so, establishing a link with the actin cytoskeleton (Figure 1.5). Integrin activation also induces the recruitment of scaffolding and signalling proteins such as paxillin (Brown and Turner, 2004) and focal adhesion kinase (FAK) (Mitra et al., 2005). These scaffolding proteins are rich in phospho tyrosines (Choi et al., 2008) which indicate that these are active signalling complexes. These proteins will in turn associate with molecules that are involved in regulating the activity of the Rho-family of guanosine triphosphate (GTP) hydrolases (GTPases) (which includes Rac, Rho and CDC42) by
recruiting guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs) to the adhesion complexes (Cote and Vuori, 2007; Premont et al., 2004; Tomar et al., 2009). Rho GTPases act as a central node that regulates adhesion assembly and disassembly by promoting contraction and actin polymerization.

Podosomes and invadipodia are other types of cell adhesions, both work as sites for localized protease release that leads to ECM proteins degradation (Albiges-Rizo et al., 2009) and contributes to cell invasiveness (Linder, 2007). Podosomes are small, circular, highly dynamic adhesions that contain a central actin core with integrins and other adhesion-associated proteins arranged in a ring-like shape (Linder, 2007). These structures are mostly found in leukocytes of the monocytic lineage, endothelial and smooth muscle cells. Invadipodia are only found in tumour cells and are structurally similar to podosomes but are much more stable structures (Linder, 2009).

Actin polymerization in the lamellipodium and in the lamellum drives the formation of protrusions at the leading edge of the migrating cell. In the lamellipodium actin polymerizes into a branched structure, and this is driven by the ARP 2 and 3 complex (Nicholson-Dykstra et al., 2005) that is itself regulated by Rac and CDC42. Branched actin is reorganized in bundles, also called stress fibres, in the region where the lamellipodium and the lamellum meet (Shemesh et al., 2009). The contraction of the actin fibres is controlled by myosin-II (Parsons et al., 2010). This protein binds actin stress fibres and can move the actin fibres in an anti-parallel way. Myosin-II can also bind together several actin filaments in bundles (Vicente-Manzanares et al., 2008; Vicente-Manzanares et al., 2009). This movement is responsible for providing the force necessary to rearrange the actin cytoskeleton.

Adhesions act mainly as traction points. They provide the necessary resistance to the forces generated by the rearward flow of actin filaments and transfer this force into the substrate allowing the formation of protrusions (Petrie et al., 2009).
Figure 1.5 - Molecular organization of a typical focal adhesion. The link between the extracellular and intracellular components of this structure is provided by integrins that bind both the ECM proteins and some of the intracellular components of the focal adhesion. Several proteins, namely talin, FAK, vinculin and paxillin, serve as scaffolding and signalling molecules to establish the stable linkage of the adhesion to the actin cytoskeleton by inducing the connection with actin stress fibres. It is important to point out that this is a highly dynamic structure and what is represented here is a snapshot of a focal adhesion that tries to include the typical elements that regulate and compose this complex molecular structure. Adapted from Mitra et al. (2005).
### 1.6.2 Adhesion dynamics

The assembly or nucleation, maturation and disassembly of adhesions represent a continuous dynamic process that is modulated by actin polymerization and actomyosin contraction. At any given time in a typical migrating cell several different adhesion structures can be present. These represent different stages of a continuous process rather than different classes of adhesions. Just behind the cell leading edge, in the lamellipodium, we can find small and short-lived nascent adhesions that can either disassemble or mature into bigger and more stable structures called focal complexes that localize to the transition zone between the lamellipodium and the lamellum (Parsons et al., 2010). These structures can finish the maturation process leading to the formation of focal adhesions that are typically located at the ends of large actin stress fibres (Zimerman et al., 2004).

There are currently two not mutually exclusive models that try to explain the mechanisms by which adhesion sites assemble. The first model assumes that it is the binding of integrins to ECM proteins that triggers the clustering of all adhesion intracellular components and that these will assemble on the cytosolic domains of integrin clusters (Miyamoto et al., 1995). The second model considers that assembly of adhesion proteins is triggered by actin polymerization, using branched actin as the template for adhesion complexes to nucleate (Choi et al., 2008).

The tension generated by myosin activity is the main determinant in the balance between adhesion disassembly and maturation. Inhibition of myosin-II with blebbistatin prevents adhesion maturation and increases the number of nascent adhesions (Puklin-Faucher and Sheetz, 2009). Myosin has two main functions that contribute to stabilize adhesions it can generate the tension necessary to induce conformational changes in the adhesion complex proteins that will trigger maturation (del Rio et al., 2009) and it can also cross-link actin filaments creating actin bundles.

There is adhesion disassembly at both the front and at the rear of the cell (Broussard et al., 2008). At the front of the cell this process happens mostly at the lamellipodium-lamellum interface and its major driving force comes from actin depolymerisation and reorganization. At the rear of the migrating cell where retraction happens, the adhesions appear to slide, in a Rho GTPase and myosin-II dependent process (Ballestrem et al., 2001; Digman et al., 2008), just before the complete adhesion structure disassembles. The fact that integrins are often found in the
substratum behind a migrating cell indicates an abrupt cut with the intracellular components of the adhesion complex (Palecek et al., 1998). Calpains are Ca\(^{2+}\)-activated proteases that play an important role in adhesion complexes disassembly in retracting areas, because they are responsible for the proteolytic cleavage of several adhesion molecules such as FAK, talin and paxillin, among others (Franco et al., 2004a) (introduced in more detail in section 1.6.3).

It is ultimately the balance between actin-mediated protrusion formation and myosin-II mediated contraction that regulates adhesion dynamics. The family of Rho GTPases, including Rac, Rho and CD42, control cell adhesion dynamics by directly controlling actin-mediated protrusion formation and myosin-II mediated contraction (Nobes and Hall, 1995). Rac and CDC42 are preferentially active at the front of the cell and they have partially overlapping functions in promoting actin-rich protrusions formation at the leading edge (Kurokawa et al., 2005). Rho is active in both the front and the rear of the cell. At the rear edge Rho activates myosin-II by activating the Rho-associated protein kinase, which can inactivate the myosin phosphatase, allowing the sustainable phosphorylation of myosin-II by regulatory light chains (Machacek et al., 2009). At the front edge it can complex with the formin mDia1 (Goulimari et al., 2005) which binds the fast polymerizing ends of actin filaments promoting actin polymerization (Chesarone et al., 2010).

Adhesion dynamics are regulated by complex feedback loops involving the Rho GTPases. The activation of these proteins is mediated by both GEFs and GAPs (Cote and Vuori, 2007; Lim et al., 2008; Premont et al., 2004; Sanz-Moreno et al., 2008). The scaffolding protein Tyr kinases such as Src and FAK can phosphorylate adhesion proteins such as paxillin which will then regulate the activation of Rho GTPases by modulating GEFs and GAPs localization and activation states (Mitra and Schlaepfer, 2006; Parsons, 2003; Tomar and Schlaepfer, 2009). Because the phosphorylation of Tyr residues in FAK and paxillin leads to the recruitment of regulators of Rho GTPases and of other signalling proteins, FAK and paxillin work as ‘switchable’ scaffolds (Parsons and Parsons, 2004).
1.6.3 \textbf{Ca}^{2+} \text{ in cell adhesion and migration}

Cell adhesion to the substratum and migration are complex cellular processes in which \text{Ca}^{2+} plays an important regulatory role at several levels. Many different steps in these processes have been shown to be directly or indirectly regulated by \text{Ca}^{2+} because many signalling and structural molecules involved in these processes are themselves regulated by \text{Ca}^{2+}. The findings that \text{Ca}^{2+} is differentially distributed in the cytosol of migration cells, being present at higher concentrations at the cell rear, suggest a role in cell detachment (Brundage et al., 1991; Marks and Maxfield, 1990). This differential distribution has been suggested to be partially due to \text{Ca}^{2+} influx through stretch-activated \text{Ca}^{2+} channels (SACC) at the cell rear and to be essential for rear detachment and retraction (Lee et al., 1999). At the leading edge transient microdomains of elevated \text{Ca}^{2+} concentration (flickers) can also be detected and these were at least partially linked to the activity of the F-actin and myosin II binding transient receptor potential M7 (TRPM7) SACC (Clark et al., 2006; Wei et al., 2009). The involvement in cell adhesion of store operated \text{Ca}^{2+} channels has also been more recently suggested by the fact that the flickering caused by TRPM7 can be amplified by IP$_3$R \text{Ca}^{2+} release from cellular stores (Wei et al., 2009). Moreover \text{Ca}^{2+} influx through TRPM7 was shown to promote \text{Ca}^{2+}-induced \text{Ca}^{2+} release through RYR during cell migration (Chen et al., 2010). STIM1 was identified as the sensor for ER \text{Ca}^{2+} content and was shown to activate Orai at the plasma membrane upon ER \text{Ca}^{2+} depletion and to allow the entry of extracellular \text{Ca}^{2+} to fill the cellular stores (Varnai et al., 2009). The important role of Orai and STIM1 in both store-operate \text{Ca}^{2+} entry and cell migration highlights the importance of store-operate \text{Ca}^{2+} entry in adhesion and migration regulation (Varnai et al., 2009; Yang et al., 2009).

1.6.3.1 Calpain in cell adhesion and migration

The fact that localized \text{Ca}^{2+} oscillations can trigger focal adhesion disassembly points to an important role of \text{Ca}^{2+} in focal adhesion turnover (Giannone et al., 2004; Giannone et al., 2002). The link between \text{Ca}^{2+} and adhesion disassembly was later shown to be at least partially due to the activity of some members of the intracellular \text{Ca}^{2+}-activated cysteine protease family, known as calpain (reviewed in Franco and Huttenlocher (2005)). The two most studied members of this family are the two ubiquitously expressed proteins calpain1 and calpain2, also known as \text{µ}-calpain and m-
calpain, respectively because they are activated by µM or mM concentrations of Ca\(^{2+}\) (Molinari and Carafoli, 1997; Sorimachi et al., 1994). Besides Ca\(^{2+}\), the proteolytic activity of these proteases is also regulated by its intracellular inhibitor calpastatin and by phosphorylation by the mitogen-activated protein kinase, among others (Croall and DeMartino, 1991). The first hint that suggested a connection between calpain activity and adhesion came from the findings that pharmacological inhibition of calpain led to an inhibition of cell migration; the same inhibitors were also found to reduce cell retraction at the cell rear during migration (Huttenlocher et al., 1997). The influence of calpain2 on cell adhesions was shown later to be due to its ability to cleave several components of the focal adhesion molecular complex, such as FAK (Chan et al., 2010), talin (Franco et al., 2004b) or paxillin, among others, and to trigger its disassembly mainly at the cell rear (Franco et al., 2004a). Calpain may also play a role in adhesion assembly because calpain-mediated proteolytic cleavage of talin was also found to promote integrin activation as the talin head domain can bind integrin β-tail (Calderwood, 2004). Calpain inhibition can inhibit cell spread in several different cell types (Parnaud et al., 2005; Rock et al., 2000).

1.6.3.2 Ca\(^{2+}\) regulation of small GTPases – relevance for cell adhesion and migration

In addition to the examples referred to above, there are many more situations where a link between Ca\(^{2+}\) and cell adhesion/ migration is suggested. One of these is the Ca\(^{2+}\) regulation of the Rho GTPases that play a central regulatory role in cell adhesion/ migration as well as cell cycle and proliferation. The Ras-like GTPases are a protein superfamily that includes the Ras, Rab, ARF, Rac, Rho and Rad/Gem/Kir subfamilies (Ehrhardt et al., 2002). These proteins become active when bound to GTP and can hydrolyse GTP to GDP causing a conformational change that can now trigger the activation/inactivation of target proteins (Shields et al., 2000). The GTP hydrolysis rate of these molecules is very low and this activity can be either increased or decreased by GEFs or GAPs respectively, as previously referred in 1.6.2 (Ehrhardt et al., 2002). These two groups of proteins are the two main signalling determinants for GTPases activity, but spatial and temporal distribution of both GTPases and their regulators also plays an important role in regulating GTPases activity. The fact that many GTPase regulatory proteins contain Ca\(^{2+}\) binding motifs, such as EF domains,
C1, C2 and IQ motifs points to a possible role of Ca\(^{2+}\) in regulating GEFs and GAPs activity. GEFs and GAPs can themselves regulate GTPases which are involved in many crucial steps during the adhesion and migration processes (Cullen and Lockyer, 2002; Ying et al., 2009).

Recently a new link between extracellular Ca\(^{2+}\) and cell adhesion and migration was suggested by studies made with the nutrient sensor Ca\(^{2+}\)-sensing receptor (CaSR). This receptor was found to promote cell migration and adhesion by being present in signalling complexes with integrins, in thyroid carcinoma cells, that can activate phospholipase C and the release of intracellular Ca\(^{2+}\) (Tharmalingam et al., 2011). The importance of these findings is highlighted by the important role that CaSR plays in both cell migration in the development of the nervous system and its recent implication in cancer metastasis (Saidak et al., 2009; Vizard et al., 2008).

### 1.6.4 Modulation of adhesion and migration by poxviruses

Poxviruses can promote host cell motility by inducing cell migration and the development of projections in the infected cells (Sanderson et al., 1998). During this motile phase the virus also induces Ca\(^{2+}\)-independent (not sensitive to EGTA) adhesion of the cell to the ECM proteins (Sanderson and Smith, 1998). Increased actin-dependent cell migration found in VACV-infected cells 8-10 h post infection coincides approximately with the period in the virus life cycle when the highest number of virus particles is released (Salzman, 1960; Sanderson et al., 1998). This process depends on the viral F11 protein that can inhibit RhoA signalling by interacting with its downstream effectors Rho-associated kinase and mDia (Valderrama et al., 2006). The development of cellular projections is a late event and is dependent on the expression of late genes because it is inhibited by AraC (Sanderson et al., 1998). The deletion of the C2L gene from VACV genome was found to affect the development of cellular projections and Ca\(^{2+}\)-independent adhesion of the infected cell to the ECM proteins during late infection (Pires de Miranda et al., 2003).

The induction of motility in poxvirus-infected cells could be an attempt by the virus to induce virus particle dissemination, as CEV particles found on the cell surface would in theory stand a much better chance of infecting a non-infected cell if the initially infected cell could move in to an area of non-infected cells. Alternatively the induction
of cell motility by the virus could be a consequence of virus-induced alterations in the cellular processes to create the appropriate conditions to allow the completion of the viral life cycle and the production of infectious particles (reviewed in Sanderson and Smith, (1999)). The first hypothesis seems less likely because MVA expressing F11 can induce cell motility, while MVA lacking F11 cannot, but does not show an increase in plaque size (Zwilling et al., 2010). Although the role that these processes might play in an in vivo situation is still not fully understood, as most of the data described above were mostly obtained in the context of a cell monolayer infection.

1.7 Project aims

GAAP is a novel membrane protein from the TMBIM family that possesses both anti-apoptotic and Ca\(^{2+}\) modulation properties. The discovery of hGAAP and vGAAP in our lab was followed by an initial analysis of their function (de Mattia et al., 2009; Gubser et al., 2007). This work seeks to further our understanding of GAAP and other members of the TMBIM family, including BI-1. The study of the TMBIM4 protein subfamily will allow us to improve our understanding of Ca\(^{2+}\) involvement in cellular processes such as apoptosis, response to viral infection and cell adhesion. Research on apoptosis, Ca\(^{2+}\) and cell adhesion is important to understand normal cellular processes as well as diseases such as cancer. The high degree of conservation between hGAAP and other members of the family found in other mammals, invertebrates and even plants, suggests that GAAP has a fundamental role in cells, making these proteins strong candidates for further investigation.

The aims of this project are:

- Purify TMBIM family proteins for functional and structural analysis
- Determine the molecular mechanism behind GAAPs ability to modulate Ca\(^{2+}\)
- Characterize GAAP oligomerization mechanism
- Investigate hGAAP involvement in cell adhesion
Chapter II

Material and Methods
2.1 Molecular Biology

2.1.1 Polymerase chain reaction (PCR)

To create C-terminally tagged GAAP mammalian expressing vectors, oligonucleotide primers were designed so that they would include the desired restriction enzyme cleavage sites to allow the cloning of the PCR product in the selected mammalian expression vector. A sequence encoding a flexible linker (amino acid sequence: KRGGSGGSGGSG) was also included between the end of GAAP and the tag.

All PCRs were carried out in a 50 µl volume containing 1x high fidelity PCR buffer (Invitrogen), 2 mM MgSO$_4$, 0.2 mM dNTP mix (Invitrogen), 0.2 µM of each primer (Table 2.1) and 1U Platinum HiFi Taq DNA polymerase (Invitrogen). Ten ng of plasmid DNA was used as a template.

Table 2.1 - Oligonucleotides used to amplify DNA fragments of interest by PCR. F and R refer to the forward and reverse primers, respectively.

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence 5’-3’</th>
<th>Allele</th>
</tr>
</thead>
<tbody>
<tr>
<td>hGAAP-GFP/Tom F</td>
<td>ctagctagctagatggctgaccccgacccccg</td>
<td>hGAAP</td>
</tr>
<tr>
<td>hGAAP-GFP/Tom R</td>
<td>cctcttttatattactgcttcca</td>
<td></td>
</tr>
<tr>
<td>vGAAP-GFP/Tom F</td>
<td>ctagctagctagatggccatgccgagcctctc</td>
<td>vGAAP</td>
</tr>
<tr>
<td>vGAAP-GFP/Tom R</td>
<td>cgaccggtgctgcgcgcgcgcggctgccccg</td>
<td></td>
</tr>
<tr>
<td>hGAAP TMD1-4.HA F</td>
<td>cgcggatccgcgccgatggctgaccccgacccccg</td>
<td>hGAAP TMD1-4</td>
</tr>
<tr>
<td>hGAAP TMD1-4.HA R</td>
<td>tggttaagcgtaatcagcttcagcttaatggag</td>
<td></td>
</tr>
<tr>
<td>hGAAP TMD5-7-HA F</td>
<td>cgggaatccgggtaaagctgtcgcggcctctc</td>
<td>hGAAP TMD5-7</td>
</tr>
<tr>
<td>hGAAP TMD5-7-HA R</td>
<td>ttagcttattggtag</td>
<td></td>
</tr>
<tr>
<td>vGAAP TMD1-4.HA F</td>
<td>cgcggatccgcgccgatggctgaccccgacccccg</td>
<td>vGAAP TMD1-4</td>
</tr>
<tr>
<td>vGAAP TMD1-4.HA R</td>
<td>tggttaagcgtaatcagcttcagcttaatggag</td>
<td></td>
</tr>
<tr>
<td>vGAAP TMD5-7-HA F</td>
<td>cgggaatccgggtaaagctgtcgcggcctctc</td>
<td>hGAAP TMD5-7</td>
</tr>
<tr>
<td>vGAAP TMD5-7-HA R</td>
<td>ttagcttattggtag</td>
<td></td>
</tr>
</tbody>
</table>
PCR were performed in a thermocycler using an initial denaturing temperature of 94 °C for 5 minutes (min) followed by 30 cycles of 94 °C for 30 s, annealing at 55 °C for 1 min and extension at 68 °C for 1 min. Finally the temperature was kept at 68 °C for 5 min for the final extension. The annealing temperature of the primers was calculated by the formula: 

$$T_m = \left[ n(C/G) \times 4 ^\circ C + n(A/T) \times 2 ^\circ C \right] - 5 ^\circ C$$

Where G, C, A and T correspond to the 4 different nucleotides in each primer that hybridize with the template DNA.

PCR products were resolved by agarose gel electrophoresis to confirm the fragment sizes.

2.1.2 **PCR-based site-directed mutagenesis**

Single amino acid mutations were generated using PCR-based site-directed mutagenesis. A forward primer (Table 2.2) was designed containing the desired nucleotide change, and a PCR as well as subsequent transformation was carried out using the QuikChange multi site-directed mutagenesis kit (Stratagene) following the manufacturer’s instructions. The selection of the correct clones was made by automated DNA sequence of plasmid DNA extracted from different clones.

2.1.3 **Agarose gel electrophoresis**

DNA samples were mixed with 1/5 volume of 6x loading buffer [0.25 % bromophenol blue, 0.25 % xylene cyanol, 30 % (v/v) glycerol, 50 mM EDTA pH 8] and loaded on a 1-2 % agarose gel. Agarose gels were prepared in tris-acetate ethylene diamine tetraacetic acid (EDTA) buffer (TAE) (40 mM Tris base, 40 mM sodium acetate, 1 M EDTA pH 8.0) and containing approximately 5 µl SYBR® Safe (Invitrogen) per 100 ml of gel. The electrophoresis was carried out in TAE at 60-100 V for the time necessary to achieve a good fragment resolution (depending on DNA fragment size). The resolved DNA was visualized using the Chemi Doc gel documentation systems (Bio-Rad) with Quantity One software (Bio-Rad).
Table 2.2 - Oligonucleotides used for site directed mutagenesis PCR. All gene mutagenesis was done in pcDNA3.1.

<table>
<thead>
<tr>
<th>Sequence 5’-3’</th>
<th>Allele</th>
</tr>
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<tr>
<td>tgtttgtcttttgtggatatgacctggtcaggattct</td>
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<td>vGAAP C9S-HA</td>
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<td>vGAAP C42S-HA</td>
</tr>
<tr>
<td>tgcagttttttatacttggacagtgtgacatggaaatattttataacaagg</td>
<td>vGAAP C60S-HA</td>
</tr>
<tr>
<td>cttgaactgtacgctatggtgttggatttacatctatg</td>
<td>vGAAP C101S-HA</td>
</tr>
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<td>ctttgggagcttcgagttcatcatctatgac</td>
<td>vGAAP C190S-HA</td>
</tr>
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<td>gttggttacatcgtgcatctcttaacgctggccctc</td>
<td>vGAAP E107Q-HA</td>
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<td>vGAAP D147N-HA</td>
</tr>
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<td>gagatttcgaaacctcagcagcaggttttgctgc</td>
<td>vGAAP G152N-HA</td>
</tr>
<tr>
<td>gcgaaaagagacagcagctggctgtcctgctgc</td>
<td>vGAAP G178Q-HA</td>
</tr>
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<td>cttgtccttgtgcttttggcggcccttttgatttc</td>
<td>vGAAP G185A-HA</td>
</tr>
<tr>
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<td>vGAAP D196N-HA</td>
</tr>
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<td>vGAAP EE207-208QQ-HA</td>
</tr>
<tr>
<td>gccctcatcattctacttgaatatcatctgttcttgc</td>
<td>vGAAP D219N-HA</td>
</tr>
</tbody>
</table>

2.1.4 Isolation of DNA from agarose gels

DNA resolved in an agarose gel was visualized under a long wave ultraviolet (UV) transillumination and the band of interest excised with a clean scalpel. The QIAquick gel extraction kit (Qiagen) was used according to the manufacturer’s protocol to extract the DNA from the agarose.

2.1.5 Restriction enzyme digest

DNA endonuclease restriction enzyme digestions were carried out in a volume of 20-50 µl and according to the manufacturer’s instructions. Briefly, a mix containing 1-2 U of enzyme per µg of DNA and the appropriate buffer (Roche or NEB) was left to incubate at 37 °C for 1-3 h. Digested DNA was purified from agarose gels using the gel extraction kit (Qiagen) after electrophoresis.
2.1.6 Quantification of DNA

A NanoDrop ND1000 spectrophotometer (Labtech International) was used to determine the concentration of DNA solutions using the OD_{260} reading. DNA purity was assessed using the OD_{260}/OD_{280} ratio, with a ratio between 1.8 and 2.0 being ideal.

2.1.7 DNA ligation

DNA fragments and linearised vector, both with sticky ends, in a molar ratio of 3:1 were ligated in a reaction volume of 20 µl in the presence of 1 U of T4 DNA ligase (Roche) in 1x T4 DNA ligase buffer (Roche). The ligation reaction was incubated overnight at 16 °C.

2.1.8 Transformation of competent bacteria with plasmid DNA

Chemically competent XL-10 cells (30 µl) were thawed on ice and 3 µl from a DNA ligation reaction was added to the competent cells. The mix was incubated on ice for 30 min and then heat shocked for 90 seconds (s) at 42 °C. After 5 min incubation on ice, 200 µl of Super optimal catabolite medium was added and the mix was placed in a shaking incubator at 37 °C for 1 h. The cells were spread on agar plates containing the appropriate antibiotic and left to incubate overnight at 37 °C.

2.1.9 Small and large scale preparation of plasmid DNA

Plasmid DNA was purified by the QIAGEN miniprep or maxiprep method according to the manufacturer’s instructions. Minipreps were prepared from 5 ml *Escherichia coli* cultures and were used for screening plasmids. Maxipreps were prepared from 250 ml cultures and were used in assays described in the results chapters.
2.1.10 Automated sequencing of DNA

Each sequencing reaction was prepared with 150 - 300 ng DNA per 3 kb of plasmid DNA and 0.2 µM of sequencing primer (Table 2.3), made up to 10 µl with water. Sequencing was carried out by the Advanced Biotechnology Centre (part of Imperial College London, Hammersmith Campus).

Table 2.3 - Oligonucleotides used in sequencing reactions. Universal primers used to sequence genes cloned in Invitrogen pcDNA3.1 and Clontech fluorescent protein vectors (pEGFP-C1 and pTom-C1).

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Oligonucleotide</th>
<th>Sequence 5'-3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>pcDNA3.1</td>
<td>CMV FWD</td>
<td>taatacgactcactataggg</td>
</tr>
<tr>
<td></td>
<td>BGH REV</td>
<td>ctagaaggcacagtcgaggc</td>
</tr>
<tr>
<td>pEGFP-C1</td>
<td>EGFP-N</td>
<td>gcagcggcaggctgagctggtc</td>
</tr>
<tr>
<td></td>
<td>EGFP-C</td>
<td>catggtctgctggagttcttg</td>
</tr>
</tbody>
</table>

2.1.11 Preparation of glycerol stocks

A small-scale culture containing the appropriated antibiotic was inoculated with a single colony from an agar plate and left to grow at 37 °C overnight. The next day equal volumes of the culture and 30 % (v/v) glycerol were combined and stored at -80 °C.

2.1.12 Plasmids

All plasmids used in this work are listed below in Tables 2.4 and 2.5. All the PCR generated fragments used to construct the listed plasmids were sequenced by automated DNA sequencing and were shown to be correct.
Table 2.4 - Mammalian expression plasmids used in this study.

<table>
<thead>
<tr>
<th>Plasmid Name</th>
<th>Description</th>
<th>Source/Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pcDNA3.1 hGAAP-HA</td>
<td>C terminal HA tagged hGAAP</td>
<td>Gubser et al., 2007</td>
</tr>
<tr>
<td>pcDNA3.1 hGAAP TMD1-4-HA</td>
<td>hGAAP aa 1-146</td>
<td>N. Saraiva</td>
</tr>
<tr>
<td>pcDNA3.1 hGAAP TMD5-7-HA</td>
<td>hGAAP aa 147-238</td>
<td>N. Saraiva</td>
</tr>
<tr>
<td>pcDNA3.1 hGAAP C164S-HA</td>
<td>hGAAP cysteine 164 mutated to serine</td>
<td>N. Saraiva</td>
</tr>
<tr>
<td>pcDNA3.1 hGAAP C191S-HA</td>
<td>hGAAP cysteine 191 mutated to serine</td>
<td>N. Saraiva</td>
</tr>
<tr>
<td>pcDNA3.1 hGAAP CXS-HA</td>
<td>hGAAP with all cysteines mutated to serines</td>
<td>N. Saraiva</td>
</tr>
<tr>
<td>pcDNA3.1 vGAAP-HA</td>
<td>C terminal HA tagged vGAAP</td>
<td>Gubser et al., 2007</td>
</tr>
<tr>
<td>pcDNA3.1 vGAAP TMD1-4-HA</td>
<td>vGAAP aa 1-145</td>
<td>N. Saraiva</td>
</tr>
<tr>
<td>pcDNA3.1 vGAAP C9S-HA</td>
<td>vGAAP cysteine 9 mutated to serine</td>
<td>N. Saraiva</td>
</tr>
<tr>
<td>pcDNA3.1 vGAAP C42S-HA</td>
<td>vGAAP cysteine 42 mutated to serine</td>
<td>N. Saraiva</td>
</tr>
<tr>
<td>pcDNA3.1 vGAAP C60S-HA</td>
<td>vGAAP cysteine 60 mutated to serine</td>
<td>N. Saraiva</td>
</tr>
<tr>
<td>pcDNA3.1 vGAAP C101S-HA</td>
<td>vGAAP cysteine 101 mutated to serine</td>
<td>N. Saraiva</td>
</tr>
<tr>
<td>pcDNA3.1 vGAAP C190S-HA</td>
<td>vGAAP cysteine 190 mutated to serine</td>
<td>N. Saraiva</td>
</tr>
<tr>
<td>pcDNA3.1 vGAAP C9 + 60S-HA</td>
<td>vGAAP cysteines 9 and 60 mutated to serines</td>
<td>N. Saraiva</td>
</tr>
<tr>
<td>pcDNA3.1 vGAAP C'all'S-HA</td>
<td>vGAAP with all cysteines mutated to serines</td>
<td>N. Saraiva</td>
</tr>
<tr>
<td>pcDNA3.1 vGAAP E107Q-HA</td>
<td></td>
<td>N. Saraiva</td>
</tr>
<tr>
<td>pcDNA3.1 vGAAP D147N-HA</td>
<td></td>
<td>N. Saraiva</td>
</tr>
<tr>
<td>pcDNA3.1 vGAAP G152N-HA</td>
<td></td>
<td>N. Saraiva</td>
</tr>
<tr>
<td>pcDNA3.1 vGAAP E178Q-HA</td>
<td></td>
<td>N. Saraiva</td>
</tr>
<tr>
<td>pcDNA3.1 vGAAP G185A-HA</td>
<td>vGAAP &quot;ion channel&quot; mutants</td>
<td>N. Saraiva</td>
</tr>
<tr>
<td>pcDNA3.1 vGAAP D196N-HA</td>
<td></td>
<td>N. Saraiva</td>
</tr>
<tr>
<td>pcDNA3.1 vGAAP EE207-208QQ-HA</td>
<td></td>
<td>N. Saraiva</td>
</tr>
<tr>
<td>pcDNA3.1 vGAAP E207Q-HA</td>
<td></td>
<td>N. Saraiva</td>
</tr>
<tr>
<td>pcDNA3.1 vGAAP E208Q-HA</td>
<td></td>
<td>N. Saraiva</td>
</tr>
<tr>
<td>pcDNA3.1 vGAAP D219N-HA</td>
<td></td>
<td>N. Saraiva</td>
</tr>
<tr>
<td>pC1 hGAAP-EGFP</td>
<td>C terminal GFP tagged hGAAP</td>
<td>N. Saraiva</td>
</tr>
<tr>
<td>pC1 vGAAP-EGFP</td>
<td>C terminal GFP tagged vGAAP</td>
<td>N. Saraiva</td>
</tr>
<tr>
<td>pC1 vGAAP-EGFP C9S</td>
<td>vGAAP-EGFP cysteine 9 mutated to serine</td>
<td>N. Saraiva</td>
</tr>
<tr>
<td>pC1 vGAAP-EGFP C60S</td>
<td>vGAAP-EGFP cysteine 60 mutated to serine</td>
<td>N. Saraiva</td>
</tr>
<tr>
<td>pC1 vGAAP-EGFP C960S</td>
<td>vGAAP-EGFP cysteines 9 and 60 mutated to serines</td>
<td>N. Saraiva</td>
</tr>
<tr>
<td>pC1 vGAAP-tdTomato</td>
<td>C terminal tdTomato tagged hGAAP</td>
<td>N. Saraiva</td>
</tr>
<tr>
<td>pC1 vGAAP-tdTomato</td>
<td>C terminal tdTomato tagged vGAAP</td>
<td>N. Saraiva</td>
</tr>
<tr>
<td>pC1 vGAAP-tdTomato C9S</td>
<td>vGAAP-tdTom cysteine 9 mutated to serine</td>
<td>N. Saraiva</td>
</tr>
<tr>
<td>pC1 vGAAP-tdTomato C60S</td>
<td>vGAAP-tdTom cysteine 60 mutated to serine</td>
<td>N. Saraiva</td>
</tr>
<tr>
<td>pC1 vGAAP-tdTomato C9+60S</td>
<td>vGAAP-tdTom cysteines 9 and 60 mutated to serines</td>
<td>N. Saraiva</td>
</tr>
<tr>
<td>pcDNA3.1(+)</td>
<td></td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pEGFP-C1</td>
<td></td>
<td>Clonetech</td>
</tr>
<tr>
<td>pt tdTomato-C1</td>
<td></td>
<td>Clonetech</td>
</tr>
<tr>
<td>pEYFP-C1</td>
<td></td>
<td>Clonetech</td>
</tr>
<tr>
<td>ER-YFP</td>
<td></td>
<td>Clonetech</td>
</tr>
<tr>
<td>Golgi-GFP</td>
<td></td>
<td>Clonetech</td>
</tr>
<tr>
<td>YFP-LamB1</td>
<td></td>
<td>Clonetech</td>
</tr>
<tr>
<td>VSVI-G-FGP</td>
<td></td>
<td>Clonetech</td>
</tr>
<tr>
<td>pcDNA CD20</td>
<td>CD20 mammalian expression plasmid</td>
<td>Daniele Bergamaschi</td>
</tr>
<tr>
<td>GFP-Vinculin</td>
<td></td>
<td>Susan Craig</td>
</tr>
<tr>
<td>GRASAP65-GFP</td>
<td></td>
<td>Barr et al. 1998</td>
</tr>
<tr>
<td>p IP_j-R-YFP</td>
<td>A series of YFP tagged truncated IP_j type 1</td>
<td>Parker et al., 2004</td>
</tr>
<tr>
<td>p IP_j-YFP truncations</td>
<td></td>
<td>Parker et al., 2005</td>
</tr>
</tbody>
</table>
Table 2.5 - Yeast expression vectors used in this study. TEVp refers to the TEV protease cleavage site.

<table>
<thead>
<tr>
<th>Plasmid Name</th>
<th>Description</th>
<th>Source/Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>p426 GAL1</td>
<td>Yeast expression vector with Galactose inducible</td>
<td>Mumberg et al., 1995</td>
</tr>
<tr>
<td></td>
<td>promoter</td>
<td></td>
</tr>
<tr>
<td>p426 GAL1 TEVp GFP-8His</td>
<td>p426 GAL1 with a TEVp cleavage site before the GFP and 8His tag</td>
<td>David Drew et al., 2006</td>
</tr>
<tr>
<td>p426 GAL1 vGAAP CMLV TEVp GFP-8His</td>
<td>p426 TEVp GFP-8His with vGAAP CMLV cloned before the TEVp site</td>
<td>N. Saraiva</td>
</tr>
<tr>
<td>p426 GAL1 hGAAP T88I TEVp GFP-8His</td>
<td>p426 TEVp GFP-8His with hGAAP T88I cloned before the TEVp site</td>
<td>N. Saraiva</td>
</tr>
<tr>
<td>p426 GAL1 AtGAAP3 TEVp GFP-8His</td>
<td>p426 TEVp GFP-8His with AtGAAP3 cloned before the TEVp site</td>
<td>N. Saraiva</td>
</tr>
<tr>
<td>p426 GAL1 BI-1 TEVp GFP-8His</td>
<td>p426 TEVp GFP-8His with BI-1 cloned before the TEVp site</td>
<td>N. Saraiva</td>
</tr>
</tbody>
</table>

2.1.13 RNA extraction

RNA was extracted from U2OS and HeLa cells using the QIAgen RNeasy RNA extraction kit according to the manufacturer’s instructions.

2.1.14 Reverse transcription-PCR (RT-PCR)

cDNA was synthesized by reverse transcription from total cellular mRNA. For each reaction 1 μg RNA, 2.5 μM oligo dT, 500 μM dNTP, and water nuclease-free to make up to 13 μl were mixed. The reactions were incubated at 65 °C for 5 min and transferred in to ice for 1 min. To each reaction 4 μl 5x First-strand buffer (Invitrogen), 5 mM DTT, 40 U RNase OUT (Invitrogen) was added, 50 U Superscript III reverse transcriptase (Invitrogen) and made up to a total of 20 μl with nuclease-free water. The reactions were incubated at 50 °C for 1 h, followed by 70 °C for 15 min.

The cDNA obtained (2-4 μl) was then used in a standard PCR using primers specific for hGAAP or for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Table 2.6). A reaction where the reverse transcriptase was not added was used as a control for DNA contamination of the RNA samples.
Table 2.6 - Oligonucleotides used to amplify endogenous hGAAP and GAPDH.

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence 5'-3'</th>
<th>Allele</th>
</tr>
</thead>
<tbody>
<tr>
<td>hGAAP 117F</td>
<td>aggacgacttcaactatggc</td>
<td>hGAAP</td>
</tr>
<tr>
<td>hGAAP 773R</td>
<td>ccagaaaccgtaacaggtgc</td>
<td>hGAAP</td>
</tr>
<tr>
<td>GAPDH F</td>
<td>cacagtccatgccatcactg</td>
<td>GAPDH</td>
</tr>
<tr>
<td>GAPDH R</td>
<td>tacctctggaggccatgtg</td>
<td>GAPDH</td>
</tr>
</tbody>
</table>

2.2 Cells and virus

2.2.1 Tissue culture

The mammalian cell lines used in this study were: human cervical carcinoma cells (HeLa) (including HeLa puro, HeLa vGAAP-V5 and HeLa hGAAP-V5 generated by Benjamin Johnson), human embryonic kidney cells with SV40 T antigen (HEK 293T), African green monkey kidney fibroblast cells (COS-7), human osteosarcoma U2OS cells (including U2OS neo, U2OS hGAAP, U2OS vGAAP and U2OS hGAAP Ctmut created by Caroline Gubser), a sarcoma osteogenic cell line (Saos-2), african green monkey epithelial cell line of kidney origin (BSC-1) and rabbit kidney cells (RK13). The HEK 293T, U2OS, COS-7, Saos-2 and BSC-1 cells were kept in Dulbecco’s modified Eagle’s medium (DMEM [Gibco]) with 10 % heat-treated (56 °C for 1 h) foetal bovine serum (FBS [Gibco]), 50 μg/ml penicillin and 50 μg/ml streptomycin. The HeLa cells were grown in minimum essential medium (MEM [Gibco]) with 10 % heat-treated FBS, 50 μ/ml penicillin and 50 μg/ml streptomycin and 2 mM L-glutamine. RK-13 cells were maintained in MEM supplemented with 10 % (v/v) FBS and penicillin/streptomycin (P/S) (50 μg/ml). All cell lines were kept at 37 °C in a humidified incubator (Heraeus) with 5 % CO₂. All the stable cell lines used in this study are listed in table 2.7.
Table 2.7 - List of U2OS and HeLa stable cell lines.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Description</th>
<th>Source/Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>U2OS neo</td>
<td>clonal U2OS cell line expressing the neomycin resistance gene (from empty pcDNA3.1)</td>
<td>Gubser et al., 2007</td>
</tr>
<tr>
<td>U2OS hGAAP-HA</td>
<td>clonal U2OS cell line expressing hGAAP-HA</td>
<td>Gubser et al., 2007</td>
</tr>
<tr>
<td>U2OS hGAAP-HA Ct mut</td>
<td>clonal U2OS cell line expressing hGAAP-HA Ct mut (E233AVNKK&gt;A233AVAAA)</td>
<td>C. Gubser, unpublished</td>
</tr>
<tr>
<td>U2OS vGAAP-HA</td>
<td>clonal U2OS cell line expressing vGAAP-HA</td>
<td>Gubser et al., 2007</td>
</tr>
<tr>
<td>HeLa puro</td>
<td>polyclonal HeLa cell line expressing the puromycin resistance gene from empty pDL vector</td>
<td>B. Johnson</td>
</tr>
<tr>
<td>HeLa hGAAP-V5</td>
<td>polyclonal HeLa cell line expressing hGAAP-V5</td>
<td>B. Johnson / N. Saraiva</td>
</tr>
<tr>
<td>HeLa vGAAP-V5</td>
<td>polyclonal HeLa cell line expressing vGAAP-V5</td>
<td>B. Johnson / N. Saraiva</td>
</tr>
<tr>
<td>HeLa A36-V5</td>
<td>polyclonal HeLa cell line expressing A36-V5</td>
<td>V. Doceul</td>
</tr>
</tbody>
</table>

2.2.2 DNA Transfection

For all transfection methods cells were seeded so that they would be 70-90% confluent the next day. The medium was replaced 1 hour (h) before transfection by medium containing 1% FBS with no antibiotics. All methods were used to induce transient expression of target genes.

Polyethylinimine (PEI) transfection reagent (Sigma-Aldrich). The DNA to transfection agent ratio used was 1:2 (2 µg of DNA per 1 µl of PEI). The DNA was diluted in Opti-MEM (Gibco), mixed, PEI was added to the mix and left to incubate at room temperature (RT) for 15 min. The DNA, PEI and Opti-MEM mix was added to the cells drop wise.

Lipofectamine 2000 (Invitrogen). The protocol used was as indicated by the manufacturer, ratios of transfection agent volume to µg of DNA were generally 1:2 (2 µg of DNA per 1 µl of lipofectamine 2000). The DNA and the Lipofectamine 2000 were mixed with Opti-MEM (Gibco) separately and incubated for 5 min at room temperature. The mix containing DNA was added to the mix containing Lipofectamine 2000 and left to incubate for 30 min at RT. The final mix was added to the cells drop wise.

Fugene 6 (Roche). The protocol used was as indicated by the manufacturer, ratios of transfection agent volume to µg of DNA were 1:3 (1 µg of DNA per 3 µl of Fugene 6). The DNA and the Fugene 6 were mixed with Opti-MEM separately and incubated for 5 min at RT. The mix containing the DNA was added to the mix.
containing Fugene6 and left to incubate for 30 min at RT. The final mix was added to the cells drop wise.

### 2.2.3 Small interfering RNA (siRNA) transfection

HeLa or U2OS cells were seeded so that they would be 20-30 % confluent the following day. The siRNA oligonucleotides duplexes (Table 2.8) (Invitrogen) and the Oligofectamine (Invitrogen) were diluted in OptiMEM separately and incubated for 8 min at RT. The content of the siRNA containing tube was added to the tube containing oligofectamine and left to incubate for 25 min at RT. The final mix was added to the cells drop wise and cells were analysed at 24 h and 36 h after transfection.

<table>
<thead>
<tr>
<th>siRNA</th>
<th>Sequence 5'-3' strand</th>
<th>Gene KD</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>hGAAP siRNA 1</td>
<td>cgaucgaggaacgacuucaacu</td>
<td>hGAAP</td>
<td>Gubser et al 2008</td>
</tr>
<tr>
<td>hGAAP siRNA 2</td>
<td>cuguacggacauuuguacauug</td>
<td>hGAAP</td>
<td>Gubser et al 2008</td>
</tr>
<tr>
<td>Control siRNA</td>
<td>proprietary sequence form Invitrogen</td>
<td>GFP</td>
<td>Invitrogen</td>
</tr>
</tbody>
</table>

### 2.2.4 Amplification of crude virus stocks

For preparation of working stock crude virus, five 175 cm² flasks of RK-13 cells per virus were infected at 0.1 plaque forming unit (p.f.u.) per cell for 48 h. The cells were collected as described above and resuspended in 5 ml infection medium. Recombinant viruses were stored at -80 °C.

### 2.2.5 Virus titration

Viruses were titrated by plaque assay, on monolayers of BSC-1 cells grown in six- well plates. The viruses were diluted in 2.5 % FBS MEM (10⁻⁵ to 10⁻⁹ dilutions, total volume 500 µl) and were incubated with the cells for 90 min at 37 °C, rocking the plate every 15 min. After this time the virus was removed and 2 ml 50 % 2x MEM
(supplemented with 5 % (v/v) FBS and 100 µg/ml P/S): 50 % 3 % (w/v) carboxy methyl cellulose was added to each well. The cells were incubated for 48-72 h to allow for plaque formation. The MEM/carboxy methyl cellulose mix was removed, cells were washed once with phosphate saline buffer (PBS) and stained for 30 min with crystal violet (5 % (v/v) crystal violet solution (Sigma), 25 % (v/v) ethanol). Wells were then washed with water and the number of plaques was counted to determine the virus titre.

2.2.6 VACV infection of mammalian cells

COS-7 cells were infected with VACV Evans Rev vGAAP-HA or ΔvGAAP at 3-5 plaque-forming units (p.f.u.) / cell for different periods of time (from 2-24 h). The single sucrose cushion purified viruses were stored until use at -80 °C. When needed they were thawed, sonicated, vortexed, diluted in 2.5 % FBS MEM or DMEM and added to 4 ml of 2.5 % FBS MEM per 10 cm petri dish or 500 µl per 12 well plate well. The dishes were rocked every 20 min for 2 h, after which the inoculum was aspirated and replaced with fresh medium.

2.3 Protein analysis

2.3.1 Preparation of cell lysates

Cell lysates were prepared by washing cells twice on ice with ice-cold PBS followed by scraping in an appropriate volume of cell lysis buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 2 mM EDTA, 1 % 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) (w/v), supplemented with protease inhibitors (Roche), and phosphatase inhibitors (Roche) for immunoblotting with phospho-specific antibodies). The cells were collected in eppendorf tubes and the cell debris were removed by centrifugation at 14,000 g for 15 min, 4 °C. The cleared lysate was retained and transferred to a new tube.
2.3.2 Protein quantification

To quantify the protein content of cell lysates a bicinchoninic acid (BCA) protein assay kit (Pierce) was used according to the manufacturer’s protocol.

2.3.3 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins were resolved by SDS-PAGE using vertical electrophoresis units from BioRad or Invitrogen. The gels consist of a ~1 ml staking gel layer (125 mM Tris-HCL pH 6.8, 5 % acrylamide, 0.1 % SDS, 0.1 % ammonium persulphate and 0.1 % TEMED) on top of ~4 ml of 10-12 % resolving layer (375 mM Tris pH 8.8 10–12 % acrylamide, 0.1 % SDS, 0.1 % ammonium persulphate and 0.04 % TEMED). Protein samples were mixed with 20 % (v/v) of 5x sample loading buffer (50 mM Tris-HCl pH 6.8, 2 % [w/v] SDS, 10 % [v/v] glycerol, 0.1 % [w/v] bromophenol blue, 10 % reducing agent ([β-mercaptoethanol (BME) or Dithiothreitol (DTT)]) before being loaded on the gel. Samples were electrophoresed at 120 V in running buffer (25 mM Tris, 250 mM glycine, 0.1 % SDS) alongside pre-stained molecular weight markers (Bio-Rad). When non-reducing conditions were required neither DTT nor BME were added to the protein samples prior to electrophoresis.

When the resolution of both GAAP and IP$_3$R or talin was required, precast Bis-Tris 4-12 % polyacrylamide gradient gels (Invitrogen) were used.

All protein resolution required during the yeast expression screen and purification process was done in 12 % polyacrylamide Tris-glycine precast gels (Invitrogen) without heating the samples, because this type of gel allows in-gel green fluorescent protein (GFP) fluorescence detection and good resolution of membrane proteins.

2.3.4 Imperial and Coomassie staining

Imperial staining (Pierce) was used to stain proteins resolved by SDS-PAGE according to the manufacturer’s instructions. The gel was washed with distilled water,
incubated for 1-2 h with the stain solution and washed with distilled water until a good contrast was achieved.

Acrylamide gels (or membranes) were stained for 30 min in Coomassie blue stain [0.25 % (w/v) Coomassie brilliant blue R-250 [BDH], 10 % (v/v) acetic acid, 45 % (v/v) methanol] and de-stained in de-stain buffer (10 % acetic acid (v/v), 20 % methanol (v/v)).

**2.3.5 Silver staining**

When low concentrations of protein were present the SilverQuest Silver Staining kit (invitrogen) was used, according to the manufacturer’s instructions, to stain proteins resolved by SDS-PAGE.

**2.3.6 Immunoblot (IB) analysis**

Proteins resolved by SDS-PAGE were electro-transferred at 20 V for 25 min onto a nitrocellulose membrane (Hybond ECL, Amersham) using a Trans Blot Semidry cell system (BioRad) in transfer buffer (25 mM Tris, 190 mM glycine, 0.037 % [w/v] SDS and 20 % [v/v] methanol). The membrane was then blocked at RT for 1 h in blocking buffer (5 % non-fat dried milk or 5 % bovine serum albumin [BSA] and 0.1 % [v/v] Tween-20 [Sigma] in PBS). The membrane was immersed in primary Ab diluted in blocking buffer to an appropriate concentration (Table 2.9) and gently rocked overnight at 4 °C. The membrane was washed with PBS-T (0.1 % (w/v) Tween-20 in PBS), incubated for 1 h with the appropriate secondary Ab (Table 2.9) followed by a new wash in PBS-T.
Table 2.9 - Antibodies used for immunoblot (IB), immunofluorescence (IF) and flow cytometry (FACS). Monoclonal Ab (mAb), polyclonal Ab (pAb).

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Source/Reference</th>
<th>Description</th>
<th>Species</th>
<th>IB dilution</th>
<th>IF dilution</th>
<th>FACS dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-HA</td>
<td>Sigma</td>
<td>mAb</td>
<td>Rabbit</td>
<td>1 : 10 000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-HA</td>
<td>Cambridge Bioscience</td>
<td>mAb</td>
<td>Mouse</td>
<td>1 : 200</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-GFP</td>
<td>Abcam</td>
<td>pAb</td>
<td>Mouse</td>
<td>1 : 25 000</td>
<td>1 : 3 000</td>
<td></td>
</tr>
<tr>
<td>α-V5</td>
<td>AbD Serotec Ltd.</td>
<td>mAb</td>
<td>Mouse</td>
<td>1 : 5000</td>
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<tr>
<td>α-VSV-G11</td>
<td>Doug Lyles</td>
<td>conformational mAb</td>
<td>Mouse</td>
<td>1 : 200</td>
<td>1 : 5 000</td>
<td></td>
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<tr>
<td>α-Tubulin</td>
<td>Millipore</td>
<td>mAb</td>
<td>Mouse</td>
<td>1 : 10 000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-GM 130</td>
<td>BD Biosciences</td>
<td>mAb</td>
<td>Mouse</td>
<td>1 : 1000</td>
<td>1 : 300</td>
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<tr>
<td>α-FAK</td>
<td>Santa Cruz</td>
<td>pAb</td>
<td>Rabbit</td>
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<td>Invitrogen</td>
<td>mAb</td>
<td>Mouse</td>
<td>1 : 1000</td>
<td></td>
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<tr>
<td>α-Talin (N)</td>
<td>Chemicon International</td>
<td>mAb</td>
<td>Mouse</td>
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</tr>
<tr>
<td>α-Talin (C)</td>
<td>Invitrogen</td>
<td>mAb</td>
<td>Mouse</td>
<td>1 : 5000</td>
<td>1 : 300</td>
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<tr>
<td>α-Vinculin</td>
<td>Invitrogen</td>
<td>mAb</td>
<td>Mouse</td>
<td>1 : 1000</td>
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</tr>
<tr>
<td>α-GAAP</td>
<td>Gubser et al, 2007</td>
<td>pAb serum generated against the N-terminal region of hGAAP and vGAAP (peptide: SSIEDDFNYGSSV)</td>
<td>Rabbit</td>
<td>1 : 500</td>
<td>1 : 50</td>
<td></td>
</tr>
<tr>
<td>α-Rabbit</td>
<td>Stratech Scientific</td>
<td>HRP conjugated anti-rabbit secondary</td>
<td>Goat</td>
<td>1 : 10 000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-Mouse</td>
<td>Stratech Scientific</td>
<td>HRP conjugated anti-mouse secondary</td>
<td>Goat</td>
<td>1 : 10 000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-Rabbit 800CW</td>
<td>Odyssey</td>
<td>IRD Dye 800CW fluorophore conjugated</td>
<td>Goat</td>
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<tr>
<td>α-Mouse 800CW</td>
<td>Odyssey</td>
<td>IRD Dye 800CW fluorophore conjugated</td>
<td>Goat</td>
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<tr>
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<td>Invitrogen</td>
<td>Alexa fluor 488 conjugated</td>
<td>Goat</td>
<td>1 : 500</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-Mouse 488</td>
<td>Invitrogen</td>
<td>Alexa fluor 488 conjugated</td>
<td>Goat</td>
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<td></td>
<td></td>
</tr>
<tr>
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<td>Alexa fluor 546 conjugated</td>
<td>Goat</td>
<td>1 : 500</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-Mouse 456</td>
<td>Invitrogen</td>
<td>Alexa fluor 546 conjugated</td>
<td>Goat</td>
<td>1 : 500</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-CD20 FITC</td>
<td>BD Biosciences</td>
<td>FITC conjugated mAb</td>
<td>Mouse</td>
<td>1/20</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Finally the membranes were incubated with horseradish peroxidase (HRP) conjugated secondary Abs and were developed by incubating with enhanced chemiluminescence (ECL) reagents (Amersham/Pierce) as directed by the manufacturers and exposure to film (X-OMAT/LS, Kodak) for 30 s to 1 h. Membranes incubated with fluorophore-conjugated secondary Abs (Li Cor) were developed using an Odyssey infrared imaging system (Li Cor). This last developing method allows for quantitative western blot.

2.3.7 Immunoprecipitation

HeLa or COS-7 cells were seeded 24 h prior to transfection so that they would be 70-80% confluent the next day. The cells were transfected with Lipofectamine 2000 or PEI and/or infected with different strains of VACV. The cells were collected and lysed the next day in 1% CHAPS buffer (50 mM Tris-HCl pH 7.5, 500 mM NaCl, 2 mM EDTA, 1% CHAPS w/v, protease inhibitors and phosphatase inhibitors). After centrifugation for 15,000 g for 15 min at 4 °C the supernatant was retained and the total protein content was determined by a Bradford Assay (BioRad). Some of the supernatant was kept for gel analysis. After 2 h incubation with protein G sepharose beads (Roche), the Abs were added to the cell lysates and incubated overnight with gentle rocking. Protein G sepharose beads (40 µl) was added the next day and left to incubate for 2 h. The beads were washed 4 times with 1% CHAPS buffer, re-suspended in 20 µl of 5x SDS sample loading buffer heated at 37 °C for 15 min and resolved on a SDS-PAGE.

2.3.8 In gel fluorescence

The presence of GFP tagged proteins in protein samples resolved on 12% polyacrylamide Tris-glycine precast gels (Invitrogen) was tested by in-gel GFP fluorescence. The gel was exposed to a blue light set at 460 nm with a cut-off filter of 515 nm for 10 s to 1 min and the image captured with a LAS-1000-3000 charge-coupled device (CCD) imaging system (Fujifilm).
2.3.9  **Protein N-terminal sequencing**

Protein run on Tris-glycine gels was transferred to an immobilon P membrane (Millipore). The membrane was stained with 0.1 % Coomassie Blue R in 50 % methanol for 5 min and de-stained with several changes, 2 to 5 min each, of 50 % methanol, 10 % acetic acid.

The dried and stained membranes were sent to the Protein Sequence Analysis (Edman degradation) service in the University of Cambridge.

2.3.10  **Protein cross-linking**

Cell lysates from HeLa cells that had been transfected with pcDNA3.1 vGAAP-HA or pcDNA3.1 hGAAP-HA were prepared as described in 2.3.1. To 40 µl of cell lysate the lysine-reactive aryl halide 1,5-difluoro-2,4-dinitrobenzene (DFDNB) (water insoluble, membrane permeable, spacer arm length 3 angstroms) was added at a range of concentrations of 2.5 to 160 nM. The DFDNB was prepared in Dimethyl sulfoxide (DMSO) immediately before use. The mix was incubated for 20 min at 37 °C and the reaction was stopped by the addition of 20 mM Tris pH 7.5.

2.4  **Protein production and purification**

The method here described was based on the work of Drew et al. (2006), published in Nature Methods and was carried out in the Membrane Protein Crystallography Group (Imperial College London, South Kensington campus). This method takes advantage of a GFP tag placed at the C terminus of the protein of interest to follow the recombinant protein expression in yeast, extraction and further purification steps.
2.4.1 Yeast transformation

The pep4 deletion *Saccharomyces cerevisiae* strain FGY217 (MATa, ura3-52, lys2Δ201, pep4Δ) (Kota et al., 2007) was used to obtain a yeast strain expressing a target gene fused with GFP-8His. The vGAAP, hGAAP, hGAAP T88I and BI-1 open reading frames were amplified by PCR using primers (Table 2.10) that include 5′ overhangs of 35 bp complementary to the upstream and downstream sequences of the *Sma*I site in the GFP-8Histidine (His) vector. This 2µ vector based plasmid (p424 GAL1) (Mumberg et al., 1995) includes the yeast EGFP15 gene (without the start methionine) with a 8His tag, a GAL1 promoter, a uracil (URA) selection marker and a tobacco etch virus (TEV) protease cleavage site between the target gene and GFP. The PCR products were cloned into the GFP fusion vector by homologous recombination after transformation of both plasmid and PCR product into the yeast (for schematics see Figure 3.1).

Using a sterile loop, 5 ml of YPG medium (yeast peptone medium (YP) + 2 % glucose) was inoculated with the yeast strain FGY217 and the culture was left to shake at 290 rpm overnight at 30 °C. The overnight culture was diluted into 50 ml of YPG to an OD₆₀₀ of 0.1 and was left to grow to an OD₆₀₀ of 0.5-0.6. To make the cells competent they were washed with water and then with 100 mM lithium acetate (LiAc), re-suspended in 100 mM LiAc and kept on ice until use.

A tube with 50 % (wt/vol) PEG 3350 and competent cells was vortexed for 5 s and kept on ice. Another tube with the *Sma*I-digested vector p424 GAL1 and the PCR product was vortexed for 30 s and kept on ice. The content of the first tube was mixed with the content of the second tube and salmon sperm DNA was added. The final mix was vortexed for 1 min. Cells were incubated at 30 °C for 30 min and transferred to 42 °C for 25 min. Cells were washed in water, re-suspended in water and spread onto URA minus selective plate and incubated for 2-3 days at 30 °C.

The yeast strain expressing *Arabidopsis thaliana* GAAP3 (AtGAAP3)-GFP-8His fusion protein, generated by the same method described above, was a kind gift from Dr. Bart Feys (Imperial College London).
Table 2.10 - Primers used in PCR to amplify the genes of interest to be cloned into the p426 GAL1 TEVp GFP-8His yeast expression vector.

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence 5'-3'</th>
<th>Allele</th>
</tr>
</thead>
<tbody>
<tr>
<td>hGAAP TEV F</td>
<td>tcgacggattctagaactagtggatcccccagcttgacccccggccc</td>
<td>hGAAP TEVpGFP-8His</td>
</tr>
<tr>
<td>hGAAP TEV R</td>
<td>aaccttgaaatataatcctctctctctctctttattagatacttcc</td>
<td>hGAAP-TEVpGFP-8His</td>
</tr>
<tr>
<td>vGAAP CMLV TEV F</td>
<td>tcgacggattctagaactagtggatcccccagcttgacccccggccc</td>
<td>vGAAP TEVpGFP-8His</td>
</tr>
<tr>
<td>vGAAP CMLV TEV R</td>
<td>aaccttgaaatataatcctctctctctctctttattagatacttcc</td>
<td>vGAAP TEVpGFP-8His</td>
</tr>
<tr>
<td>BI-1 TEV F</td>
<td>tcgacggattctagaactagtggatcccccagcttgacccccggccc</td>
<td>BI-1 TEVpGFP-8His</td>
</tr>
<tr>
<td>BI-1 TEV R</td>
<td>aaccttgaaatataatcctctctctctctctttattagatacttcc</td>
<td></td>
</tr>
</tbody>
</table>

2.4.2 Large-scale protein production

Five or ten litres of URA\textsuperscript{*} medium (1.92 g of yeast synthetic drop out medium w/o Uracil and 6.7 g of Dibco yeast nitrogen base w/o amino acids per litter) with 0.1 % glucose were inoculated with an overnight culture of the strain of interest at an OD\textsubscript{600} of 0.12. The culture was incubated at 30 °C and induced with 2 % galactose when the OD\textsubscript{600} reached 0.5-0.6. The culture was incubated for 24 h, the cells were collected by centrifugation at 4,000 g for 10 min and re-suspended in 25 ml of cell resuspension buffer (CRB) (50 mM Tris-HCL pH 7.6, 1 mM EDTA, 0.6 M sorbitol) per litre of culture. A sample of the cell suspension was used to measure the GFP counts on a SpectraMax M2e microplate reader (Molecular Devices). The cells were flash frozen in liquid nitrogen and kept at -80 °C.

2.4.3 Large-scale membrane extraction

Protease inhibitor (PI) (Roche) was added to the cell suspension and the cells were broken with four passes through a cell disruptor (Constant Systems TS series cell disruptor from Constant Systems) at 25, 30, 32 and 35 kPSI. A sample of cells was used to measure the GFP counts. The unbroken cells and debris were removed by centrifugation at 10,000 g for 10 min and the supernatant was centrifuged at 150,000 g for 1 h to collect the membranes. The pellet was re-suspended in 6 ml of membrane re-suspension buffer (MRB) (20 mM Tris-HCL pH 7.6, 0.3 M sucrose, 0.1 mM CaCl\textsubscript{2}) per
litre of culture using a 21-gauge needle. A sample of membrane suspension was used to measure the GFP counts. The membranes were flash frozen and kept at -80 °C.

2.4.4 Small-scale protein production

Two litres of URA* medium supplemented with 0.1 % glucose was inoculated with the appropriate yeast strains and grown to a final OD$_{600}$ of 0.12, grown at 30 °C until an OD$_{600}$ of 0.6 was reached and induced with 2 % galactose. After 20-24 h incubation at 30 °C (25 °C for hGAAP T88I) the cells were collected and re-suspended in CRB, flash frozen and kept at -80 °C.

2.4.5 Small scale membrane extraction

A tablet of protease inhibitor was added per 2 litres of culture. The cells were broken by passing four times at 25, 30, 32 and 35 PSI through a cell disruptor. The cell lysate was centrifuged at 15,000 g for 10 min and the membranes were collected by centrifuging the supernatant at 150,000 g for 1 h. The membrane pellet was re-suspended in MRB, flash frozen and kept at -80 °C.

2.4.6 Detergent Screen

2.4.6.1 Solubility efficiency

Membrane extract (250 µl) from small scale cultures were solubilized in the presence of 1 % of two different detergents: n-dodecyl-β-D-maltopyranoside (DDMα) and N,N-dimethyldodecylamine N-oxide (LDAO) from Anatrace. The tubes were left to agitate for 1 h at 4 °C. The final mix was centrifuged at 50,000 g for 1 h at 4 °C to exclude unsolubilized material. The GFP counts were measured before and after solubilization and the solubilization efficiency determined by the following formula:

\[
\text{Solubilization Efficiency} = \left( \frac{\text{GFP counts after solubilization}}{\text{GFP counts before solubilization}} \right) \times 100
\]
2.4.6.2 Fluorescence size-exclusion chromatography (FSEC)

Protein solubilized with DDMα or LDAO (as described in 2.4.6.1) were loaded into a Superose 6 10/300 GL Tricorn size exclusion chromatography column (GE Healthcare) equilibrated in gel filtration buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl and 3x the critical micelle concentration [CMC] of detergent of choice). Fractions of 200 µl were collected in a 96-well plate and the GFP counts were measured at 512 nm by excitation at 470 nm. The GFP counts were plotted against the fraction number.

2.4.7 Protein purification from yeast membrane extracts

2.4.7.1 Protein solubilization

Protein from membrane samples obtained from large scale cultures were solubilized in the presence of 1 % LDAO or DDMα for 2 h with gentle mixing at 4 °C in 1x PBS, 10 % glycerol, 100 mM NaCl and protease inhibitors. Samples were centrifuged for 1 h at 150,000 g to remove unsolubilized material. The supernatant was collected and tested for the presence of GFP and the solubilization efficiency calculated.

2.4.7.2 GFP-8His tagged proteins purification

Imidazole was added to the solubilized protein to a final concentration of 15 mM. The samples were either loaded on a 5 ml Nickel His-Trap column previously equilibrated with 1x PBS, 15 mM imidazole and 3x CMC of the detergent of choice or incubated with 20 ml of Ni-NTA resin (slurry) (for schematics see Figure 3.4). The slurry was poured into an Econo-Column (BioRad). The flowthrough from the His-Trap column and from the slurry was tested for GFP counts and the binding efficiency calculated by the following formula:

\[
\text{Binding Efficiency} = 100 - \left(\frac{\text{GFP counts flowthrough}}{\text{GFP counts before binding}}\right) \times 100
\]

Both columns were washed with 15 column volumes (CV) of wash buffer 1 (100 mM NaCl, 1x PBS, 30 mM Imidazole and 3x CMC of detergent of choice) and with 15
CV of wash buffer 2 (150 mM NaCl, 20 mM Tris pH 7.5, 30 mM imidazole and 3x CMC of detergent of choice). The protein was eluted from the columns with 150 mM NaCl, 20 mM Tris pH 7.5, 250 mM imidazole and 3x CMC of detergent of choice. The sample was diluted in dilution buffer (150 mM NaCl, 20 mM Tris pH 7.5, 0 mM imidazole and 3x CMC of detergent of choice) to a final imidazole concentration of 30 mM.

2.4.7.3 TEV protease cleavage

His-tagged TEV protease (produced in yeast, purified using His-Trap columns and kept at -80 °C until used) was added to pure GFP-8His tagged protein sample to a molar ratio of 1:1 of GFP and TEV and left to digest overnight at 4 °C.

2.4.7.4 Reverse His-Trap purification

The sample was diluted in dilution buffer to a final imidazole concentration of 15 mM, filtered through a 0.22 µm filter and loaded on a 5 ml His-Trap column equilibrated previously with PBS, 15 mM imidazole and 3x CMC of detergent of choice. The flowthrough was collected and concentrated with an Amicon 15 with a molecular weight cut off (MWCO) of 30 kDa up to 1 ml.

2.4.7.5 Gel filtration

The concentrated protein was loaded on a Superdex 200 SEC column equilibrated previously with gel filtration buffer (20 mM Tris pH7.5, 150 mM NaCl, 3x CMC of detergent of choice). The 1 ml fractions were collected and a small sample of those corresponding to the peak was loaded on a 12 % polyacrylamide Tris-glycine gel. The gel was analyzed by in gel fluorescence and with Coomassie staining.

The fractions containing pure protein were concentrated to 50-250 µl with an Amicon 15 centrifugal concentrator with a MWCO of 30 kDa. The final protein concentration was determined by the BCA protein assay (Pierce) using a BSA standard curve as reference.
2.5 Microscopy

2.5.1 Immunofluorescence

Cells were seeded over a sterile glass cover slip. After aspirating the cell medium and washing the cells twice with cold PBS, the cells were fixed with cold 4 % PFA (4 % paraformaldehyde, 250 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES] pH 7.4 in PBS) for 20 min at RT or with -20 °C methanol for 4 min at 4 °C and washed again with PBS. Ammonium chloride (50 mM) was added to the fixed cells for 5 min to quench free aldehydes, which can auto fluoresce. After being washed with PBS, the cells were permeabilized with 0.1 % Triton X-100. Before incubation with the primary Ab (Table 2.9) cells were blocked with blocking buffer (10 % FBS in PBS) for 30 min at RT. The cover slips were then washed in the blocking buffer before incubating with the secondary antibody (Table 2.9). After this incubation the cover slips were washed with PBS and distilled water. Finally the coverslips were mounted in mounting medium (Moviol4-88) containing 4',6-diamidino-2-phenylindole (DAPI).

Cell imaging was done by confocal microscopy using a LSM 5 PASCAL (Zeiss). Image acquisition was done using the LSM image browser software (Zeiss).

2.5.2 Focal adhesions turnover (live cell imaging)

Focal adhesion turnover measurements were done based on the work of Webb et al. (2004). U2OS cells were transfected with 1 µg of GFP-vinculin using Fugene 6. Cells were reseeded 24 h later in 35 mm glass bottom culture chambers (MatTek Corporation), coated with fibronectin, in 1 % FBS DMEM conditioned medium. Cells were kept in a humidified 37 °C incubator with 5 % CO₂ for 1 h before carrying out the imaging. GFP fluorescence was monitored at the edges of the cell for approximately 2 h per cell with pictures being taken every 2 min using a LSM 510 META (Zeiss) microscope. Image acquisition was done using the LSM image browser software (Zeiss).
The duration of adhesions was calculated by determining the amount of time elapsed between the first and last frames in which an individual adhesion was observed.

### 2.5.3 Calcium release (Fura-2-AM)

This work was undertaken by Dr. David Prole, Department of Pharmacology, University of Cambridge. HEK 293T cells were transfected with 5 µg of GAAP plasmids along with 1 µg of mCherry-C1 (Clontech) plasmid as a marker for transfected cells. Twenty-four h after transfection, cells were loaded with Fura-2-AM (Invitrogen) for 1 h and kept in a Ca²⁺-free solution. Ca²⁺ release as a response to ionomycin (10 µM) was measured in transfected cells i.e. mCherry positive cells. Traces representing the average Ca²⁺ responses of fields of transfected cells to ionomycin were obtained from 20-50 cells. The values obtained were corrected for background that was measured after quenching with Mn²⁺.

### 2.5.4 Förster resonance energy transfer (FRET)

HeLa cells were seeded on cover slips so that they would be 20 % confluent the next day. One day after seeding cells were transfected with a total of 2 µg of different sets of plasmids encoding GFP- or tdTomato (Tom)-tagged proteins. A ratio of 3:1 of acceptor encoding plasmid (Tom) to donor encoding plasmid (GFP) was used in the transfection.

Transfected cells on glass-bottomed culture dishes were fixed in 4 % PFA for 20 min, 16 h after transfection, and placed on the stage of an Olympus IX81 microscope equipped with 150 x/1.45 NA and 60 x/1.45 NA total internal reflection fluorescence (TIRF) objectives. Cells were illuminated with 488 nm or 561 nm diode-based lasers via appropriate filters and images were acquired with an Andor iXON 897 camera. Images were processed using Cell^R software (Olympus, Milton Keynes, UK). Cells were visualised using TIRFM with a 60 x/ 1.45 NA TIRF or 150 x/1.45 NA TIRF objective.
To determine FRET efficiency (as measured by the increase in donor fluorescence intensity after acceptor photobleaching) pictures of the same cell were taken on the Tom (561 nm) and on the GFP channels (488 nm) before and after Tom photobleaching. At least 10 cells were photographed for each condition and for each cell a region of interest (ROI) corresponding to the Golgi was selected and the fluorescence intensity was determined. Photobleaching of Tom expressing cells was achieved by exposure to an un-attenuated 561 nm laser for 60 s. Background subtraction and correction for crosstalk were applied and FRET efficiency (E) was determined using the following formula:

\[
FRET \text{ efficiency (E) } = 1 - \left( \frac{\text{GFP}_B - (\text{Tom}_B \times C \times \text{GFP}_\text{exp}) / \text{Tom}_\text{exp}}{\text{GFP}_A - (\text{Tom}_A \times C \times \text{GFP}_\text{exp}) / \text{Tom}_\text{exp}} \right)
\]

- \(\text{GFP}_B\) – GFP fluorescence intensity before photobleaching
- \(\text{GFP}_A\) – GFP fluorescence intensity after photobleaching
- \(\text{Tom}_B\) – tdTomato fluorescence intensity before photobleaching
- \(\text{Tom}_A\) – tdTomato fluorescence intensity after photobleaching
- \(\text{GFP}_\text{exp}\) – GFP units of exposure
- \(\text{Tom}_\text{exp}\) – tdTomato units of exposure
- \(C\) - Fraction of the tdTomato excitation that the 488 laser gives, relative to the 561 laser, per unit of exposure.

Grey value images were obtained by pixel-by-pixel subtraction of GFP pre-bleach from GFP post-bleach images using Image J software.
2.5.5 Interference reflectance microscopy (IRM)

U2OS cells were seeded in fibronectin-coated glass bottom dishes. Cells were fixed with 4 % PFA and stained with phalloidin. The dishes were filled with PBS and placed on the microscope. Interference reflection images were collected using a LSM 510 META (Zeiss) microscope (Holt et al., 2008). Images treatment and analysis was done using Image J using the phalloidin stain to help define the cell area.

2.6 Apoptosis assays

2.6.1 Transient assay - Sub G1 population

Saos-2 cells were seeded the day before on 6 cm dishes to achieve 80 % confluency at the time of transfection. Cells were transfected the next day with a plasmid expressing CD20 (1.5 µg) and 5 µg of pcDNA3.1, pcDNA3.1 vGAAP or a series of mutant pcDNA3.1 vGAAP.

Apoptosis was induced using 1.5 µM STS at 18 h post transfection and the cells (floating and adherent) were collected 6 h later. Cells were washed in cold PBS and stained with FITC-conjugated anti-CD20 antibody (Table 2.9) for 2 h on ice. After this step, cells were washed once in PBS and fixed in cold 96 % methanol for 18 h. Cells were washed again in PBS and resuspended in propidium iodide (PI) staining solution (0.05 mg/ml PI and 0.05 mg/ml RNase A in ml PBS) for 10 min at RT.

Flow cytometry was performed using the DAKO Cyan flow cytometer and data were analysed using the DAKO summitv4.3 software.

2.7 Cell attachment, spread and migration assays

2.7.1 Cell attachment assay

Non-confluent HeLa or U2OS cells were detached with trypsin and washed twice with 1 % FBS medium. The cells were counted and the same number of cells
was seeded in 12-well plates in 1 % FBS medium that had been kept at 37 °C with 5 % CO₂. After different time points after seeding, cells were gently washed twice with warm PBS and fixed with 4 % formaldehyde (4 % PFA + 250 mM HEPES pH7.4).

To quantify the percentage of cells that had already attached to the wells, these were stained with 0.1 % crystal violet in 200 mM HEPES for 1 h. The wells were washed 3 times with water and the dye was eluted using 10 % acetic acid. Samples were transferred to a 96-well plate and absorbance at 560 nm was measured a spectrophotometer. The absorbance was plotted against time to compare the rate of adhesion between the different cell lines.

2.7.2 EDTA/ethylene glycol tetraacetic acid (EGTA) cell detachment assay

HeLa or U2OS cells were seeded in 12-well plates so that they were 60 % confluent the next day. Cells are washed with 1 ml of warm PBS twice and incubated with 1 ml of warm PBS for 15 min. EDTA or EGTA was added to the cells and 20-40 min after incubation (at 37 °C) with the drug cells were washed gently with PBS to remove any non-adherent cells and the remaining adherent cells were fixed using 4 % PFA for 20 min.

To quantify the percentage of cells remaining attached to the wells after treatment, cells were stained with 0.1 % crystal violet in 200 mM HEPES for 1 h. The wells were washed 3 times with water and the dye eluted using 10 % acetic acid. Samples were transferred to a 96-well plate and the absorbance was read at 560 nm. Taking the untreated cells as 100 % adherent, the level of cell detachment was calculated for the different conditions.

2.7.3 Wound healing assay (WHA) – migration assay

U2OS cells were seeded in 10 % FBS media and cultured to confluence. Cells were rinsed twice with PBS and starved overnight in serum free medium. A horizontal
line was drawn using a marker on the bottom of the dish to be used as a guide. Using a sterile 200 µl pipette tip the cell monolayer was scratched perpendicularly to the line drawn in the step above, in three separate places per well, to create three wounds. The cells were rinsed and the medium was replaced with fresh FBS free medium or medium containing 10 % FBS. Regions above and below the point where horizontal line crosses the different wounds were acquired using a CCD camera with a 10x objective. Each region was imaged every 15 min for 12 h.

Distance between the two sides of the wound over time was measured using Image J and plotted against time to calculate cell migration speed.

2.7.4 Cell spread assay

U2OS cells were detached and rinsed twice in FBS-free medium. Cells were seeded on fibronectin-coated slides and left to attach for different times. Cells were then fixed with 4 % PFA at different time points post-seeding. Image acquisition was performed using a LSM 510 META (Zeiss) microscope with a 63x objective. The cell area was determined using Image J and the mean was plotted against time.

2.8 Electrophysiology

All patch clamp lipid bilayer recordings done with GAAP and BI-1 proteins purified by me, were performed by Guia Carrara in the laboratory of Prof. Colin Taylor at the Department of Pharmacology, University of Cambridge. The approach used was based on the work published by Kreir et al. (2008)

2.8.1 Making giant unilaminar vesicles (GUV)

The production of GUVs was done using electroformation from a 1,2-diphytanoyl-sn-glycero-3-phosphocholine (DPhPC) and cholesterol lipid mix. The lipid mix used (20 µl of a 1:10 mM cholesterol (Sigma) to DPhPC (Avanti) in chlorophorm)
was spread on the conductive side of an indium tin oxide-coated (ITO-coated) slide and the solvent left to evaporated. An O-ring was placed around the area of dried lipid film and 1 M sorbitol was placed over the lipids. A second ITO-coated slide was placed over the lipids, and the cassette was connected to the Vesicle Prep Pro (Nanion). The electroformation programme was initiated under the following parameters: a frequency of 5 Hz, 3 V voltages peak to peak, for 128 min at RT. GUVs were then re-suspended and kept at 4 °C.

### 2.8.2 Testing GUVs and forming GΩ planar bilayer seals

To test GUVs for their ability to form planar lipid bilayers these were tested on a Port-a-Patch (Nanion). Patch clamp buffer (140 mM KCl pH 7, 200 nM CaCl₂, 10 mM HEPES) were deposited on the inner side of a chip with an aperture of approximately 1 micron in diameter. The chip was mounted onto the Port-a-Patch system, and Patch clamp buffer was deposited on the outer side of the chip. A membrane potential of 20 mV was applied, and following the addition of GUVs in solution, suction was applied. A change in seal resistance from an order of magnitude of MΩ to GΩ, proved that GUVs have formed a planar bilayer seal over the chip opening.

### 2.8.3 Reconstituting purified protein into GUVs

To introduce purified protein into GUVs, purified protein was added to 50 µl of GUVs solution to a final concentration of 2 mg/ml, to which 100 µl of biobeads were added, and incubated for 15 min at RT. The beads were then discarded and the protein containing GUVs were used to form GΩ planar bilayer seals as described previously.

### 2.8.4 Single ion channel recordings

The PatchMaster software (Nanion) was used in combination with the EPC 10 Patch clamp amplifier (HEKA) to collect electrophysiological recordings in response to varying membrane potentials ranging between -100 mV and 100 mV. Recordings were
made using a standard electrophysiology buffer (140 mM KCl, 20 nM CaCl₂, and 10 mM HEPES, pH 7).

**2.9 Protein alignments**

All amino acid sequence alignments were done using the CLUSTAL W (1.83) multiple sequence alignment program.

**2.10 Statistical analysis**

Statistical analysis of data was performed using GraphPad Prism 5 software. For FRET, cell detachment, attachment, protein cleavage, focal adhesion duration and flow cytometry, the data were analysed using an unpaired Student’s T-test. For number, area and size of adhesions and cell size the data were analysed using a Mann-Whitney non-parametric test. * P<0.05, ** P<0.01, *** P<0.001
Chapter III

Purification of TMBIM family proteins
In an attempt to gain a better understanding of the biochemical and structural features of GAAP and other TMBIM family proteins a subset of these were expressed in yeast and purified. Ideally, purified protein would be used in crystallization trials with the objective of solving the protein structure. Because there is still no solved structure for any member of the TMBIM family this could provide a structure upon other TMBIM members could be modelled, and this could provide some clues about GAAP function. Purified protein would also be used for biochemical characterization and for functional assays such as testing ion channel activity in planar lipid bilayers. All the work described in this chapter was performed in Dr. Bernadette Byrne laboratory at Imperial College London.

A GFP-8His tag-based method (Drew et al., 2006) was selected for expression and purification of the eukaryotic TMBIM membrane proteins in *Saccharomyces cerevisiae*. This method was selected over others for a number of reasons. Firstly, yeast has proven to be one of the most successful heterologous overexpression systems for the production and purification of eukaryotic membrane proteins (Carpenter et al., 2008). Secondly, the use of a C-terminal GFP tag allowed the measurement of membrane integrated protein as the C-terminal GFP will only fold and fluoresce if the upstream protein folds properly and integrates the membrane. This allows an easy and quick detection of protein production in liquid cultures, protein gels and detergent solubilized protein. The use of a tobacco etch virus protease (TEVp) cleavage site between the protein of interest and the GFP-8His tag allows the removal of the GFP-8His tag to obtain native protein. Finally, the GFP-8His based system also allows the initial screening of many different proteins, which allowed an initial small screen to be made of 5 different TMBIM alleles, to find those that were most stable and highly expressed. The proteins selected for the initial screen were *Arabidopsis thaliana* GAAP3, vGAAP from CMLV, hGAAP and BI-1.

This method consists on the insertion of the desired PCR fragments, by homologous recombination, in to a yeast expression vector under the control of an inducible promoter (GAL1) (Figure 3.1). After induction with galactose the cells are collected, disrupted and the membrane fraction was extracted by ultracentrifugation. Membrane proteins were extracted from the membrane fraction using a detergent and then affinity purified using a His-Trap column. The purified protein was digested with
TEV protease to release the GFP-8His tag, and subjected to a reverse affinity purification, where the protein of interest (untagged) does not bind the column.

### 3.1 Creation of GAAP and BI-1 yeast expression strains

BI-1, vGAAP, hGAAP and hGAAP T88I, which includes a single point mutation found in an ovarian cancer cell line (Benjamin Johnson, unpublished data), were amplified by PCR with primers that included sequences to allow homologous recombination with the p426 GAL1 TEVp GFP-8His plasmid once co-transformed into yeast (Figure 3.1). GAAP3 from *Arabidopsis thaliana* (AtGAAP3) had been cloned by our collaborator, Dr. Bart Feys, Imperial College London. The reverse primers used also included a TEV protease cleavage site to allow the removal of the GFP-8His tag. The plasmid p424 GAL1 TEVp GFP-8His was derived from p426 GAL1 by Drew et al. (2006) by cloning a GFP-8His tag and the desired homologous recombination sites. Downstream from the homologous recombination site is GFP (codon optimized for expression in yeast) to allow easy protein detection and an 8His tag for protein purification using nickel. The plasmid obtained after homologous recombination is shown in Figure 3.1. The GAL1 promoter allows the controlled induction of protein expression by the addition of galactose to the culture medium.

Plasmid DNA was extracted from all yeast strains created, expanded in a bacterial system and sequenced to confirm the correct cloning and the absence of mutations that could have occurred during PCR. Clones where these conditions were verified were selected and used in subsequent experiments.

### 3.2 Protein expression and sub cellular localization in yeast

To determine whether the proteins produced had the correct size and hence were expressed correctly, a protein Tri-glycine gel was run with membrane extracts obtained by ultracentrifugation from cell extracts (as described in 2.4.5) form two clones of each transformation after a 24 h induction with galactose (Figure 3.2A). AtGAAP3 was also used as a positive control for both protein expression and
approximate protein size in SDS-PAGE as previous preliminary results from Dr. Bart Feys (unpublished data) showed that this protein could be expressed using this system. Using this approach it was clear that all the proteins run at ~38 kDa (Figure 3.2A), close to the predicted size of 40 kDa (20 kDa [vGAAP experimental size] + 23 kDa [approximately the GFP-8His experimental size, see Figure 3.5]).

Figure 3.1 - Strategy used to clone the genes of interest into the p424 GAL1 vector. The PCR products and the linearized vector (with Smal) were transformed into the parental yeast strain. The gene of interest in the PCR product was cloned into the vector by homologous recombination. As shown in the diagram, the final plasmid allowed the expression of the gene of interest fused to GFP and to an 8His tag with a TEV protease cleavage site just before the GFP to allow the removal of both tags during the purification process.
Figure 3.2 - Protein expression in yeast. Yeast strains engineered to express the TMBIM proteins were induced with galactose for 22 h to activate protein expression from the GAL1 promoter. Protein expression levels was determined by (A) in gel fluorescence of membrane extracts from two yeast clones of each construct resolved by SDS-PAGE and (B) relative GFP units (arbitrary units) from total cell cultures. The highest expressing clone values are shown for each strain. * Correspond to an endogenous fluorescent 'background' protein, L - Fluorescent ladder. The position of size markers in kDa is shown on the left of the gel. (C) Membrane localization of TMBIM family proteins fused to GFP was tested under confocal microscopy. Two representative cells for each strain are shown. Phase contrast was used to determine the cell outline. Scale bar corresponds to 2 µm.
Protein expression levels were determined by measurement of whole cell GFP fluorescence emission from liquid cultures (Figure 3.2B). vGAAP and BI-1 had the highest expression levels, comparable with AtGAAP3. Both human GAAP alleles tested failed to express at high enough levels to proceed with large scale expression and purification although their expression could be confirmed with the in gel fluorescence and by confocal microscopy. Band intensity detected by in gel fluorescence correlated well with the GFP fluorescence emission.

Using confocal microscopy on immobilized live yeast cells from different strains, which had been induced with galactose for 24 h to induce protein expression it was possible to observe a discrete, intracellular and cytoplasmic pattern compatible with a cytoplasmic (BI-1), ER (GAAPs and BI-1) or Golgi (GAAPs and BI-1) subcellular localization of the fusion proteins tested (Figure 3.2C). The subcellular distribution pattern is more consistent with either an ER or Golgi localization (Newstead et al., 2007) in all cases. Although further experiments using organelle specific markers would be required to dissect the specific subcellular localization, this simple and quick approach suggests that the proteins are being integrated into the membrane as expected.

Given that only AtGAAP3, vGAAP and BI-1 expressed at high enough levels to enable protein purification, these three alleles were selected for further characterization before protein purification was attempted.

3.3 Protein monodispersity and solubility in DDMα and LDAO

After yeast membrane purification using 1 L cultures (as described in 2.4.5) the GFP-fusion proteins were solubilized using different detergents to extract the membrane-associated proteins. The solubilisation efficiency for the different proteins in different detergents was calculated as described in methods section 2.4.6.1. As a first approach the two detergents tested were (DDMα) and (LDAO).

Both detergents used were able to solubilize BI-1-GFP and GAAP-GFP fusion proteins from yeast membrane extracts with an efficiency of around 70 to 80 % (Figure
3.3A). LDAO solubility efficiency appeared to be slightly higher that DDMα for all three proteins.

![Graph A: Solubility efficiency comparison between DDMα and LDAO for TMBIM proteins](image1)

![Graph B: Fluorescent size exclusion chromatography (FSEC) analysis for AtGAAP3, vGAAP, and BI-1](image2)

**Figure 3.3 - Detergent screen.** (A) Solubilisation efficiency of TMBIM proteins in two detergents, DDMα and LDAO. Membrane extracts from yeast cultures 22 h post galactose induction were solubilized in either DDMα or LDAO and protein solubilisation efficiency was determined (see methods for more details). (B) Fluorescent size exclusion chromatography (FSEC) analysis of AtGAAP3, vGAAP, and BI-1. Membrane extracts from small-scale cultures were solubilized using LDAO or DDMα and analysed by size exclusion chromatography (SEC) as described in materials and methods. The GFP emission for each fraction was measured and the GFP relative fluorescent units (RFU) were plotted against the fraction number.
Given that all TMBIM proteins were fused to GFP it was possible to test protein monodispersability after detergent solubilisation using fluorescence size exclusion chromatography (FSEC). Detergent solubilized proteins that are monodispersed are typically stable after purification (Kawate and Gouaux, 2006). LDAO or DDMα solubilised protein was loaded onto a SEC column, and 200 µl fractions from the column were collected in a 96-well plate and tested for GFP expression by measuring total GFP fluorescence (Figure 3.3B). The criterion used to access monodispersity was that the fusion protein main peak was symmetric and larger than either the peak corresponding to the aggregated protein (small peak on the left hand side of the main peak) or free GFP peaks (expected between the main and the aggregate peak). All three proteins tested appeared to be monodispersed in both detergents used (Figure 3.3B) because all curves are symmetrical and no other significantly large peaks are detected. Only a small peak corresponding to aggregated protein was detected for vGAAP and BI-1. In all cases, the GFP RFU values indicate that there were good levels (>1000 RFU) of monodispersed protein. LDAO gave higher and more symmetrical peaks when compared to DDMα. Free GFP was not detected in this experiment.

One of the main purposes of this experiment was to screen for the best detergent to be used during large-scale protein purification. Taking all these data into consideration it was decided to proceed with the production in a large scale of the three proteins using LDAO as the extraction detergent.

### 3.4 Protein purification strategy

To obtain pure protein from large-scale cultures, cells were induced with galactose, lysed 24 h later and the membranes were purified. Protein was solubilized using LDAO, because this was the detergent that showed the best results both for protein solubilisation efficiency and monodispersability. Following this step, the preferred method used to purify membrane proteins would be binding the protein to a Ni-NTA resin (“slurry”) (Figure 3.4) where proteins are allowed to bind to the nickel resin in solution with agitation. This mix is then poured into an empty glass column for washing and eluting steps. However, this method gave a very poor binding efficiency
(calculated as the difference in GFP fluorescence between the initial sample and the column flow through as described in 2.4.7.2), which was less than 15 % for vGAAP.

Figure 3.4 - Schematic of the purification protocol used to purify AtGAAP3, BI-1 and vGAAP from yeast membrane extracts. After solubilisation, the membrane proteins were either bound to slurry Ni-NTA resin (“slurry”) or to a 5 ml His-Trap column, washed and eluted with imidazole. The GFP-fusion proteins were then cleaved with TEV protease to remove the GFP-8His tag. TEV protease (that contains an 8His tag) and GFP-8His were removed from the mix in a reverse purification step using a 5 ml His-trap column. The purity and monodispersity of the final cleaved protein was assessed by SEC and SDS-PAGE.
An extended overnight incubation with the resin increased the protein binding to the resin but only up to ~30%. Given the poor binding to the “slurry” we decided to try a His-Trap column (Figure 3.4) to purify the fusion proteins, although we were aware that this approach could increase the protein lipid removal and lead to an increase in protein instability.

After this step the bound protein was washed and the visible green band, mainly corresponding to the GFP fusion protein, was eluted with 250 mM midazole. The sample was diluted until the imidazole concentration reached 30 mM, Pure TEV protease-8His was added to the sample and incubated for 24 h to allow cleavage of the GFP fusion protein. To remove the uncleaved GAAP-GFP-8His, TEVprotease-8His, free GFP and other possible contaminants the sample was further diluted and passed again through a new His-Trap column in what is called a “reverse purification” (Figure 3.4). In this case the bound fraction was discarded and the flow through retained. The final sample was concentrated and run through a SEC column.

3.5 Camelpox virus vGAAP purification

The CMLV vGAAP protein was purified from 5 L cultures using the protocol described above in Figure 3.4 with LDAO as solubilizing detergent and using a His-Trap column to bind the GFP-8His tagged protein. Using this protocol a binding efficiency of 60-70 % was consistently obtained for vGAAP.

When the final cleaved vGAAP purified sample was analysed by SEC four peaks were observed (Figure 3.5A). The first peak (at ~8 ml of elution volume) corresponded to aggregated protein while the other three to different oligomers of vGAAP (at 12.5 ml, 13.5 ml and 15.5 ml). Most protein was found to elute in second and third peaks (Figure 3.5C) and when the protein from all three peaks was concentrated and analysed by non-reducing SDS-PAGE at least four intense bands (labelled c, d, e and g) were observed in addition to a band corresponding to free GFP (labelled as f) and a band corresponding to uncleaved GAAP-GFP. All four bands that might correspond to different vGAAP oligomeric forms were detected by the anti-GAAP Ab by immunoblot (Figure 3.5D), as well as two additional bands that were probably
monomeric and dimeric uncleaved GAAP-GFP because these are also detected by in-gel fluorescence (Figure 3.5B).

Although there was some clear contamination with both uncleaved GFP fusion and with some free GFP (labelled a and b, respectively) (Figure 3.5 B) these seemed to represent a small fraction of the total final concentrated protein as showed in the Coomassie stain (Figure 3.5C). GFP was used as an additional control for both size and GFP fluorescence detection. Some additional bands that do not correspond to either putative vGAAP or vGAAP-GFP oligomeric forms can be seen but these represent a small fraction of the total protein sample and can possibly correspond to some cleaved form of vGAAP (see 3.8 for more details). The final yields obtained were usually around 0.35 mg of pure protein per litre of starting yeast culture. This amount was enough to allow the setting up of crystallization trials at 10 mg/ml of protein in both the MemGold™ ICL6 and ICL9 conditions (Molecular Dimensions under license from Imperial College of Science Technology & Medicine, London, (Newstead et al., 2008)) at 4 °C and 20 °C. Unfortunately no crystal formation was observed for over 3 months for all conditions tested.
Figure 3.5 - vGAAP (CMLV) purification in LDAO. vGAAP was purified from a 5 L yeast culture using LDAO as the extraction detergent and using a 5 ml His-Trap strategy to bind the protein to the nickel resin. A filter with a molecular weight cut off (MWCO) of 30 kDa was used to concentrate the protein after the reverse purification step. The final protein sample was submitted to gel filtration in a Superdex 200 column. (A) The fractions obtained corresponding to the UV peaks were analysed by (B) in gel fluorescence and (C) Coomassie stain. The final concentrated protein was visualized using Coomassie staining and in parallel an (D) anti-GAAP immunoblot was also performed. L - ladder, FL - fluorescent ladder. * Corresponds to the aggregation peak and the arrows with the letters a-g correspond to: a- vGAAP-GFP, b- free GFP, c- putative 4x vGAAP, d- putative 3x vGAAP, e- putative 2x vGAAP, f- free GFP and g- 1x vGAAP. The position of size markers in kDa is shown on the left of each gel.
3.6 **Arabidopsis thaliana GAAP3 purification**

Using a very similar purification protocol as the one used to purify vGAAP it was possible to obtain purified AtGAAP3 protein. The His-Trap column allowed a binding efficiency of 50-60%, which although lower than that obtained for vGAAP was still high enough to allow a final yield of 0.25 mg of protein per litre of starting yeast culture.

The final pure protein obtained after reverse purification was concentrated through a 50-kDa molecular weight cut off (MWCO) filter (and here referred as the 50-kDa sample) and the flow through further concentrated using a 30-kDa MWCO filter (and here referred as the 30-kDa sample). Both samples were analysed by SEC (Figure 3.6A). For the 30-kDa sample there was no detectable aggregation peak and there were only two visible peaks (at 14.5 ml and 15.5 ml) that might correspond to the dimeric and monomeric forms of the protein by analogy with the results found for vGAAP. In the 50 kDa sample SEC profile there was one visible predominant peak (at 14.5 ml) that overlapped with the putative dimer peak observed in the 30-kDa sample. The Tris-glycine PAGE followed by Coomassie stain analysis of the samples revealed that the concentrated 50-kDa sample (that contained all the fractions from 10 to 17.5 ml) showed a higher number and intensity of higher molecular size bands when compared with the 30-kDa concentrated sample (that also contained all the fractions from 10 to 17.5 ml) where only one band was clearly visible (Figure 3.6C). A small contamination with the AtGAAP3-GFP and free GFP was detectable by in gel fluorescence for the 50 kDa concentrated sample (Figure 3.5B) and could also be detected by Coomassie stain (Figure 3.5C, bands g and e). Considerably less additional bands that could not be explained by either oligomerization of uncleaved protein were detected for both the 30-kDa and 50-kDa AtGAAP3 concentrated samples when stained with Coomassie (Figure 3.6C) when compared with vGAAP, although the final protein concentration showed here was considerably lower (~1 mg/ml) than vGAAP.
Figure 3.6 - AtGAAP3 purification in LDAO. AtGAAP3 was purified from a 5 L culture using LDAO as the extraction detergent and using a 5 ml His-Trap strategy to affinity purify the protein. After the reverse purification step the protein was concentrated through a 50-kDa MWCO filter and the flow through was further concentrated through a 30-kDa MWCO filter. Both samples were run through a Superdex 200 column and (A) the fractions corresponding to the UV peaks of the 30-kDa MWCO sample and the concentrated 50-kDa sample were analysed by (B) in gel fluorescence and (C) Coomassie stain. L - ladder, FL - fluorescent ladder. * Corresponds to the aggregation peak and the arrows with the letters a-g correspond to: a- AtGAAP3-GFP, b- free GFP, c- putative 4x AtGAAP3, d- putative 3x AtGAAP3, e- AtGAAP3-GFP, f – putative 2x AtGAAP3, g- free GFP and h- 1x AtGAAP3. The position of size markers in kDa is shown on the left of each gel.
3.7 BI-1 purification in LDAO

BI-1 is the most extensively studied member of the TMBIM family and has also been showed to modulate \( \text{Ca}^{2+} \) (Xu et al., 2008), to have \( \text{Ca}^{2+}/\text{H}^{+} \) antiporter activity when reconstituted in liposomes (Ahn et al., 2010) and a peptide containing its C-terminal portion was recently showed to be able to form a \( \text{Ca}^{2+} \) permeable channel pore (Bultynck et al., 2011). Using the same approach described above for vGAAP we were able to achieve a final yield of 0.1 mg of BI-1 protein per litre of culture. This considerably lower yield was partially due to a lower binding efficiency of the protein to the His-Trap column (50-60 %).

The final purified BI-1 sample obtained after the reverse purification was concentrated using a 50-kDa MWCO filter (here referred as the 50-kDa sample) and the flow through was concentrated further using a 30-kDa MWCO filter (here referred as the 30-kDa sample). The SEC profile obtained for both samples showed several peaks or shoulders that were consistent with BI-1 being an oligomer, as seen for GAAPs. Three peaks/shoulders, other than the aggregation peak, were visible at \( \sim 12.5 \) ml, 14 ml and 15 ml (Figure 3.7A). The peak corresponding to the lower molecular size species (15 ml) and the next peak to the left (14 ml) probably corresponded to a monomeric and diametric BI-1, respectively. The shoulder on the 14 ml peak corresponded to either aggregating protein or most likely to either a trimeric or tetrameric species. The very high aggregation peak and the low yield obtained suggested that the protein was highly unstable in the conditions used.

Using in-gel fluorescence analysis, only residual BI-1-GFP was detected in the final protein sample (Figure 3.7B labelled as band a). Since BI-1 could barely be detected with Coomassie stain, silver stain was used instead (Figure 3.7C). The overall purity of the final protein sample was very difficult to assess due to the very low yield obtained but it was clear that the final sample was far from pure. Most of the protein detected by silver stain was found to run at a size that could correspond to a dimeric form of the protein, (Figure 3.7B, band labelled as b), while almost no monomeric form was observed.
Figure 3.7 - BI-1 purification in LDAO. BI-1 was purified from a 5 L yeast culture using LDAO as the extraction detergent and using a 5 ml His-Trap strategy to bind the protein to the resin. After the reverse purification step the protein was concentrated through a 50 kDa MWCO filter and the flow through was concentrated further through a 30-kDa MWCO filter. Both samples were run through a Superdex 200 column and (A) the fractions corresponding to the UV peaks were analysed by (B) in gel fluorescence and (C) silver stain. L - ladder, FL - fluorescent ladder. * Corresponds to the aggregation peak and the arrows with the letters a-f correspond to: a- BI-1-GFP, b- free GFP, c- putative 4x BI-1, d- putative 2x BI-1, e- free GFP, f –1x BI-1. The position of size markers in kDa is shown on the left of each gel.
3.8 BI-1, vGAAP and AtGAAP3 protein stability

Protein stability of the TMBIM proteins purified from yeast was tested at the two temperatures most commonly used for protein crystal growth, 4 °C and 20 °C, over a period of several days.

vGAAP appeared to suffer some form of cleavage or degradation because both putative dimer and monomer bands showed a clear reduction in size over time as shown by Coomassie stain (Figure 3.8A). This reduction in size happened at a faster rate for samples kept at 20 °C compared to 4 °C. When the same samples were immunoblotted with an Ab raised against the N-terminal region of vGAAP it became apparent that the N-terminal region of the protein was being cleaved because the signal obtained with the anti-GAAP Ab was reducing with time (Figure 3.8B), although a slightly smaller form of the protein could still be detected with the Coomassie stain. A similar cleavage pattern was observed for AtGAAP3, but the degradation observed occurred at a much slower rate, only being truly clear after 6 days at 20 °C (Figure 3.8C). The band corresponding to putative monomeric BI-1 showed no cleavage or degradation. However, given the very low amounts of protein the bands could not be clearly detected, so it was possible that degradation was still occurring but in these conditions it was not visible (Figure 3.8B).

Protein N-terminal sequencing was used to confirm the vGAAP protein identity and to determine to what degree the protein was being degraded (Figure 3.8C). This approach identified the 20-kDa band as vGAAP and also showed the expected N-terminal protein degradation/cleavage when the sample was kept for 7 days at 20 °C prior to N-terminal sequencing. In 66 % of the sequences obtained the first 25 amino acids had been removed, some other starting sequences were also found but in lower abundance.
Figure 3.8 - Temperature stability assay. vGAAP (A and B) BI-1 and AtGAAP3 (C) were incubated for several days at 4 °C or 20 °C. After different times of incubation samples of each protein were collected and stored at -80 °C. All the samples were run in a Tris-glycine gel and this was stained by Coomassie stain (A and C) or (B) immunoblotted using a polyclonal Ab raised against a N-terminal peptide of vGAAP. (D) N-terminal amino acid sequence of CMLV vGAAP, before and after 7 days incubation at 20 °C, obtained from protein N-terminal sequencing. The percentage of protein that has each one of the represented amino acids as the first amino acid of the peptide chain is shown. The position of size markers in kDa is shown on the left of each gel.
3.9 Summary

The SEC profile of the final concentrated purified vGAAP protein is consistent with either the presence of two contaminants, because three peaks other than the aggregation peak are observed, or with vGAAP behaving as an oligomer (Figure 3.5A). The second option seems to be the more likely because all four bands obtained on a Coomassie stained gel of purified protein are reactive with the anti-GAAP Ab (Figure 3.5D). It seems very unlikely that the residual GFP fusion protein by itself could account for any of the SEC peaks, because this was almost undetectable by in gel fluorescence (Figure 3.5B) and hardly visible by Coomassie stain (Figure 3.5C). Nevertheless this was not formally ruled out by any of the experiments performed. Further experiments were done in a mammalian cell expression system with both hGAAP and vGAAP to further explore the GAAP oligomerization hypothesis. These results are presented in chapter V of this thesis.

The yeast-based method chosen to express TMBIM proteins enabled the production and purification of three different members of the TMBIM family, vGAAP, AtGAAP3 and BI-1. Using this approach and taking into account data shown above for each protein purification process, it became clear that AtGAAP3 was the most pure and stable of all the three TMBIM proteins tested.

The final protein obtained allowed some very elementary biochemical characterization as well as lipid bilayer patch clamp experiments (described in chapter IV) to be performed. However all crystallization trials attempted did not generate any crystals. Further optimizations to improve protein purity, stability and solubility are probably needed if the crystallization of any of these proteins is ever to be attempted again.
Chapter IV

GAAP is an ion channel
Previous work reported that overexpressed hGAAP interacted with endogenous IP$_3$R type three (de Mattia et al., 2009). IP$_3$R but not SERCA was able to co-immunoprecipitate with hGAAP. However, it was not determined if this interaction was direct or indirect and one difficulty with this model was that the majority of IP$_3$R is located in the ER, whereas GAAP is present in the Golgi. Endogenous hGAAP was not used, as there was not then and still there is not a good anti-GAAP Ab that is able to immunoprecipitate GAAP.

The finding that hGAAP can modulate intracellular stores Ca$^{2+}$ content led to the two alternate hypotheses to explain the effects of GAAP overexpression and knock down in Ca$^{2+}$ homeostasis. The ability of hGAAP to co-immunoprecipitate with IP$_3$R combined with the fact that hGAAP can modulate IP$_3$-induced Ca$^{2+}$ signaling (de Mattia et al., 2009) led to the hypothesis that hGAAP could modulate Ca$^{2+}$ by regulating IP$_3$R channel activity, perhaps as part of a larger protein complex. The alternative hypothesis was that GAAP could itself function as an ion channel and regulate Ca$^{2+}$ content by allowing the flow of Ca$^{2+}$ ions (Figure 4.1).

![Figure 4.1](image_url)

**Figure 4.1** – Two possible mechanisms by which GAAP is able to reduce the Ca$^{2+}$ filling state of intracellular stores. GAAP could either exert its effect on the Ca$^{2+}$ filling state of intracellular stores by modulating another Ca$^{2+}$ channel activity (left) or by acting as a Ca$^{2+}$ channel itself (right).
4.1 Interaction between GAAP and IP$_3$R was observed by co-immunoprecipitate when both proteins were overexpressed

As a first approach to understand GAAP’s anti-apoptotic and Ca$^{2+}$ modulating mechanisms, a further characterization of the nature of the GAAP-IP$_3$R interaction was attempted. The existence of a similar interaction between vGAAP and IP$_3$R was also tested.

To further explore and characterize the interaction of GAAP with the IP$_3$R a yellow fluorescent protein (YFP)-tagged IP$_3$R type 1 was used in co-immunoprecipitation experiments (kindly provided by Prof. Colin Taylor, University of Cambridge). This protein has a single residue change in the pore region (VG$^{2550}$DVLR to VGAVLR) (Parker et al., 2004) that inactivates the channel, allowing stable overexpression without deleterious effects for the cells. COS-7 cells were transfected with a plasmid that encodes YFP-IP$_3$R and 24 h later cells were either infected with a recombinant VACV strain Evans lacking vGAAP ($\Delta$vGAAP), or a VACV strain Evans with a C-terminally HA-tagged copy of vGAAP (rev-vGAAP-HA), or the cells were transfected with an expression plasmid encoding an HA-tagged hGAAP (pcDNA3.1-hGAAP-HA). The YFP-IP$_3$R was immunoprecipitated using an anti-GFP Ab and to determine if vGAAP or hGAAP were co-immunoprecipitated, the immunoprecipitates were immunoblotted with an anti-HA Ab. The reciprocal experiment was also performed for hGAAP: hGAAP was immunoprecipitated using an anti-HA Ab and the co-immunoprecipitation of IP$_3$R was assessed by immunoblotting using an anti-GFP Ab. To test for non-specific precipitation of ER or Golgi membrane proteins the immunoprecipitates were also tested for the presence of SERCA.

Data obtained showed that it was possible to co-immunoprecipitate vGAAP and hGAAP using an anti-GFP Ab when co-expressed with YFP-IP$_3$R but not when expressed on their own (Figure 4.2A and B lane 1). YFP-IP$_3$R was also found to co-immunoprecipitate with hGAAP when an anti-HA immunoprecipitation was performed (Figure 4.2B lane 1), although the YFP-IP$_3$R band observed was very weak. SERCA was not co-immunoprecipitated in any of the samples tested, reducing the possibility of the above results being due to an unspecific pull down of Golgi and ER membranes. The tubulin immunoblots for the whole cell extracts (WCE) show that the initial total
protein content of the samples used in the immunoprecipitations was very similar in all the samples. GAAP and YFP-IP₃R expression was confirmed by immunoblot of the WCE using anti-HA and anti-GFP Abs, respectively.

Figure 4.2 - Overexpressed vGAAP and hGAAP can interact with overexpressed IP₃R. Co-immunoprecipitation between vGAAP or hGAAP and YFP-tagged type 1 IP₃R. (A) COS-7 cells were transfected with pYFP-IP₃R and 24 h later cells were infected with either ΔvGAAP or rev vGAAP-HA virus and collected after 16 h. (B) Cells were transfected with pYFP-IP₃R and 24 h later cells were retransfected with either pcDNA3.1 or pcDNA3.1hGAAP-HA and collected after 16 h. (A and B) The cell lysates were immunoprecipitated with either an anti-HA Ab or an anti-GFP Ab and the immunoprecipitates were analysed by SDS-PAGE and immunoblotted (IB) using anti-HA, anti-YFP Ab, anti-SERCA and anti-tubulin Abs as indicated. Tubulin was used as loading control. The position of size markers in kDa is shown on the left of each gel.
4.2 Attempt to map GAAP-IP$_3$R interaction

In an attempt to map the IP$_3$R domain involved in the interaction with vGAAP, a series of plasmids encoding for different portions of the transmembrane regions of the IP$_3$R with an YFP tag were used (Figure 4.3A and B) (Parker et al., 2004). These expressed proteins were used in co-immunoprecipitation assays with vGAAP. Additional proteins tagged with YFP or GFP that localize to different cellular organelles were included as additional controls for non-specific co-immunoprecipitation due to the presence of the YFP tag.

Full length (fl) and all seven truncated forms of YFP-IP$_3$R were expressed in COS-7 cells for 24 h. After this time cells were infected with either a ΔvGAAP or a rev vGAAP-HA VACV for 16 h and cell lysates were prepared for immunoprecipitation. All YFP-IP$_3$R proteins were expressed but not all at the same levels. In an overexposed film it was visible that vGAAP could be co-immunoprecipitated by YFP-IP$_3$R as shown before. But vGAAP was also co-immunoprecipitated with all seven truncated YFP-IP$_3$R proteins tested. It was noted that even in cases like TMD1-2 and TMD5-6 that do not share any overlapping regions, both truncated forms of YFP-IP$_3$R immunoprecipitated vGAAP efficiently.

The fact that a positive vGAAP co-immunoprecipitation could also be obtained with all of the negative control GFP- or YFP-tagged molecules (Figure 4.3C - overexposed HA blot) raised the possibility that the co-immunoprecipitation between vGAAP and fl YFP-IP$_3$R detected previously was probably non-specific.

A similar experiment was conducted in which the cells were infected with VACV for just 6 h, in an attempt to reduce the vGAAP expression levels, hoping that this could increase the IP specificity (Figure 4.4). This time no vGAAP co-immunoprecipitation could be detected with any of the negative control proteins but still a vGAAP co-immunoprecipitation could be detected with most of the YFP-IP$_3$R protein fragments tested confirming the non-specific nature of the co-immunoprecipitation.
Figure 4.3 - vGAAP is co-immunoprecipitated with all of IP₃R TMDs. Co-immunoprecipitation (co-IP) between vGAAP and full length and truncated versions of YFP-tagged type 1 IP₃R (plasmids were kindly provided by Colin Taylor, University of Cambridge). Schematic representation of (A) the full length and (B) the truncated forms and YFP-IP₃R used to map the interaction. COS-7 cells were transfected with the different plasmids encoding all the IP₃R-YFP alleles and 18 h later cells were infected with either ΔvGAAP or rev vGAAP-HA VACV and collected after 16 h. (C) Following co-IP with the anti-GFP Ab the immunoprecipitates and the whole cell extracts (WCE) were resolved by SDS-PAGE and were immunoblotted with anti-YFP, anti-HA, anti-SERCA (used as control for ER and Golgi membrane proteins contamination) and anti-tubulin (loading control) Abs. Four additional Clontech vectors that express YFP- or GFP-tagged proteins that localize to different cell organelles were used as negative controls: ER-YFP (YFP tagged peptide that localizes to the ER), YFP-LamB1 (YFP tagged peptide that localizes to the nucleus), GFP-Tub (GFP tagged peptide that localizes to the cytosol) and pEYFP-C1 (expresses free YFP). The position of size markers in kDa is shown on the left of the gel.
**Figure 4.4 - vGAAP is co-immunoprecipitated with almost all of IP$_3$R TMDs at 6 h post infection.** Co-immunoprecipitation (co-IP) between vGAAP and full length and truncated versions of YFP-tagged type 1 IP$_3$R. COS-7 cells were transfected with the different IP$_3$R-YFP alleles and 18 h later the cells were infected with either ΔvGAAP or rev vGAAP-HA virus and cell lysates were prepared after 6 h. Immunoblots were performed following co-IP with the anti-GFP Ab and whole cell extracts (WCE). Membranes were immunoblotted with anti-GFP, -HA, -tubulin, and –SERCA Abs. ER-YFP and pEYFP-C1 were used as negative controls. The position of size markers in kDa is shown on the left of the gel.
4.3 GAAP can allow the passage of ions through a lipid bilayer

Given the lack of specificity showed for the vGAAP-IP$_3$R interaction it was unlikely that GAAP was modulating IP$_3$R and therefore we sought to test the second hypothesis for GAAP mediated Ca$^{2+}$ modulation in which GAAP would act as an ion channel itself. This would require vGAAP to have the ability to allow ions to flow through the membrane rather than controlling another ion channel such as IP$_3$R.

Taking advantage of the purified proteins generated for biochemical and structural purposes, it was tested, in collaboration with Prof. Colin Taylor, if CMLV vGAAP and AtGAAP3 could allow the passage of ions when loaded into an artificial lipid bilayer. CMLV vGAAP was tested instead of VACV Evans vGAAP because the latter could not be purified using the same approach as used for CMLV vGAAP, AtGAAP3 and BI-1. BI-1 was also tested at the time for completion because it had been proposed that BI-1 could itself allow the flow of Ca$^{2+}$ (Ahn et al., 2009; Bultynck et al., 2011). The adenosine A2 receptor that had been purified using the same protocol as GAAPs and BI-1 was used here as a negative control because no ion channel activity has been described for this protein (Lebon et al., 2011) (kindly provided by Bernadette Byrne, Imperial College London). All electrophysiological recording were done using the following buffer, 140 mM KCl, 20 nM CaCl$_2$ in 10 mM HEPES, pH 7. This means that the only three ions that can possibly flow through the channels to generate a measurable current are K$^+$, Cl$^-$ or Ca$^{2+}$.

When reconstituted in planar lipid bilayers both GAAP proteins and BI-1 allowed the passage of ions when different artificial membrane potentials were applied (Figure 4.5). The passage of ions is shown as “openings”, these openings are detected in amperage traces containing a baseline (corresponding to the closed channel) and are characterized by a sharp shift away from the baseline (channel opening) and back to the baseline (channel closing). These form very characteristic “square” openings.

Different amplitudes of opening were seen for vGAAP, AtGAAP3 and BI-1 and the absence of openings detected with both the detergent only and AA2R control suggest that the openings observed for the GAAP and BI-1 proteins are specific and are not due to any contaminants that remained during the purification procedure.
Figure 4.5 - CMLV vGAAP, AtGAAP3 and BI-1 allow the passage of ions when reconstituted into planar lipid bilayers. This experiment was carried out by Guia Carrara, Imperial College London, using proteins purified by me and is here reproduced with her permission. Artificial lipid bilayers patch clamping of human BI-1, AtGAAP3 and vGAAP from CMLV. The different proteins were reconstituted in GUV’s and added to a lipid bilayer. The artificial lipid bilayer was subjected to different membrane potentials using a single channel patch clamp system. Representative traces are shown and represent typical channel openings when the different membrane potentials were applied (-30 mV to -100 mM). To establish a base line, recordings were made in the absence of any protein (n=35). The dotted line defines the baseline amperage recorded in the absence of any protein. Any shifts below this line are considered to be due to channel openings, and the returning to the baseline represents closure of the channel. Adenosine A2 receptor (AAR) (n=6) and 1% LDAO (n=10) (detergent used for protein purification) were used as negative controls. Scale of amplitude in picoamperes (pA) over time in milliseconds (ms) is shown in the figure.
This was the first electrophysiological ion channel-like activity data recorded for any of these proteins. This includes BI-1 where Ca\(^{2+}/H^+\) anti-porter activity had been described indirectly through measurements of Ca\(^{2+}\) changes in liposomes where BI-1 was reconstituted (Ahn et al., 2009) or Ca\(^{2+}\) channel activity using a peptide corresponding to its C-terminal domain (Bultynck et al., 2011). These results therefore show that CMLV vGAAP and AtGAAP3 are probably ion channels or ion exchangers and that this function is conserved within the TMBIM4 subfamily of proteins and in BI-1, another TMBIM family member.

### 4.4 GAAP shown sequence similarity with other ion channels and pumps

In an attempt to identify key residues or domains in GAAP that could be involved in ion selectivity, gating and/or transport across the membrane, the BLAST algorithm was applied using individual GAAP TMDs and the full length amino acid sequence (Altschul et al., 1990). This approach allowed a further characterization of the ion channel. It would also suggest which amino acid residues might be mutated in an attempt to ablate or modify GAAP ion channel activity.

Most of the alignments obtained using this method were with Na\(^{2+}/H^+\) exchangers and with several ion channels (Figure 4.6). If GAAPs were Na\(^+\)/H\(^+\) anti-porters, then E108 of vGAAP would be a good candidate for the putative Na\(^+\) binding site, as it aligned with the Na\(^+\) binding residue of *E. coli* Na\(^+\)/H\(^+\) antiporter A (NhaA) D164 (Padan et al., 2009) (Figure 4.6A).

Another hypothesis is that GAAPs were cation channels. If this was the case then examination of the GAAP sequence might identify some regions that could correspond to the “selectivity filter” region similar to that found in Ca\(^{2+}\) and Na\(^+\) channels. Between vGAAP TMD4 and 5, TMD5 and 7 and TMD6 and 7 three clusters of highly conserved acidic residues were identified, D\(^{147}\)FS, ETVE\(^{178}\)L, and SPE\(^{207}\)E respectively. Each one of these three motifs aligned with either the sodium leak channel (NALCN) pore region (DFS and ETVEL vGAAP motifs) (Figure 4.6D) (Lu et al., 2007) or the potassium channel from *Streptomyces lividan* (KcsA) (SPEE vGAAP...
motif) (Figure 4.6B) and most of these acidic residues are very well conserved within the GAAP family (Figure 4.8).

vGAAP acidic residues D196 and D219 align with cation channels and are both absolutely conserved among GAAPs. Moreover D219 from vGAAP aligns perfectly with BI-1 D213 that has been showed to be essential for its C-terminal domain to form a Ca\(^{2+}\) channel (Bultynck et al., 2011) (Figure 4.7). A G\(^{153}\)AG\(^{155}\) motif similar to selectivity filter of K\(^{+}\) channels (GYG) (Jiang et al., 2002), IP\(_3\)R and other cation channels was found in GAAP's TMD5 (Figure 4.6C). The first G153 is absolutely conserved amongst GAAPs and second G155 is conserved in most GAAPs (Figure 4.8). The four COO\(^-\) groups provided by the highly conserved glutamic acids and aspartic acids (at positions 196, 207, 208 and 219 of vGAAP) at the N-terminal region of all GAAPs could correspond to the typical selectivity filter found in many Ca\(^{2+}\) channels (Kew and Davies, 2010).

A glycine hinge is often found in the pore-lining TMD segments, and these are predicted, by analogy with other ion channels, to be located just after the selectivity filter (Magidovich and Yifrach, 2004). Given this and its high degree of conservation within the GAAP family, G185 was also considered as a target for mutagenesis (Figure 4.6 and 4.8).

Using the information collected from these alignments eight residues were chose to be mutated in vGAAP. Glutamic acid residues were mutated to glutamine, aspartic acid to asparagine and glycines were mutated to alanines. This alteration in charge would affect ion channel conductivity if the mutated amino acid residue were located within the pore region and were involved in gating or ion selectivity. The localization of each mutated residue within vGAAP topology can be seen in Figure 4.9. In an attempt to narrow down the number of mutants to be purified to ultimately be tested for ion channel activity in artificial lipid bilayer experiments the mutant vGAAPs were tested for expression, subcellular localization, anti-apoptotic activity and ability to modulate Ca\(^{2+}\).
Figure 4.6 - BLAST searches of full length or individual GAAP TMDs. Representative alignments of vGAAP and hGAAP with known ion channels and anti-porters. (A) Alignment with E. coli Na⁺/H⁺ anti-porter NhaA reveals the E108 of hGAAP aligns with D164 of NhaA, this residue is part of Na⁺ binding site in NhaA making this a candidate residue for mutation if the GAAPs are Na⁺/H⁺ anti-porters. (B and C) GAAP TMD5 contains a 153GAG155 motif similar to selectivity filter of K⁺ channels, IP₃Rs, HCN channels and others. (D) GAAP D148 aligns with selectivity filters of NALCN and AtKCO1. Red labelled amino acid residues represent some of the positions mutated in vGAAP Evans whereas the blue residues represent residues previously described as having some role to play in ion transport/selectivity.

Figure 4.7 - Crustal X alignment of BI-1 and GAAP. Red labeled residues represent all the positions mutated in vGAAP Evans, the blue residues represent BI-1 amino acid residues involved in Ca²⁺ transport and the blue line delineates the region that was shown by Bultynck et al., (2011) to be sufficient to allow the flow of Ca²⁺ across the membrane.
Figure 4.8 - Alignment of some of the GAAP family proteins. The alignments show the degree of conservation of the residues chosen for mutagenesis. Alignment was performed using the clustal W multiple alignment tool. Green lines define the degree of conservation of the residues chosen.

**Vaccinia Evans**

**Camelpox**

**Homo sapiens**

**Pan troglodytes**

**Canis lupus**

**Bos taurus**

**Rattus norvegicus**

**Galus gallus**

**Danio rerio**

**Arabidopsis thaliana 3**

**Caenorhabditis elegans**

**Vaccinia Evans**

**Camelpox**

**Homo sapiens**

**Pan troglodytes**

**Canis lupus**

**Bos taurus**

**Rattus norvegicus**

**Galus gallus**

**Danio rerio**

**Arabidopsis thaliana 3**

**Caenorhabditis elegans**
Figure 4.9 - Diagram showing the localization of the single “ion channel mutations” introduced in vGAAP. Topology shown was defined by Guia Carrara et al, (submitted).

4.5 vGAAP “ion channel mutants” characterization – expression, dimerization and subcellular localization

Each residue identified in 4.4 (here referred to as “ion channel mutations”) was individually mutated in pcDNA3.1 vGAAP-HA using site directed mutagenesis PCR. All the mutant vGAAP-HA expression plasmids obtained were sequenced to confirm the presence of the desired mutation and the absence of additional mutations during the PCR. The vGAAP “ion channel mutant” proteins were checked for expression, dimerization and subcellular localization. Mutant and wt vGAAPs were expressed transiently in Saos-2 cells for 18 h before cell lysates were prepared and analysed by immunoblotting or fixed for immunofluorescence. All mutant proteins, apart from E107Q that was expressed to a much lower level, showed an expression level similar to wt vGAAP (Figure 4.10A). The dimerization ability of the mutant vGAAP alleles was then assessed using non-reducing (-DTT) SDS-PAGE analysis (Figure 4.10A). The EE207-208QQ dimer was more sensitive to DTT as shown by a lower dimer to monomer ratio in non-reducing conditions when compared to wt vGAAP.
Figure 4.10 - vGAAP mutant proteins subcellular localization and basic biochemical characterization. (A) GAAP protein expression levels and dimerization ability were determined by immunoblot analysis of cell lysates obtained 18 h after transfection of pcDNA3.1 with wt vGAAP or mutant gene into Saos-2 cells. Reducing (+DTT) and non-reducing (-DTT) SDS-PAGE were used to resolve the protein lysates. The position of size markers in kDa is shown on the left of the gel. (B) Saos-2 cells were transfected with vGAAP wt and mutant vGAAP expressing plasmids for 18h and subcellular localization was assessed by immunofluorescence stain using GM130 as a Golgi marker. The proportion of cells with GAAP in the Golgi, Golgi and ER or ER only was assessed by counting over 100 individual transfected cells. Data presented as mean percentage of total cells. IF was carried out by Dr. Benjamin Johnson, Imperial College London.
When tested for subcellular localization using an anti-GM130 Ab as a Golgi marker it was found that vGAAP mutants D147N and EE207-208QQ had an altered localization and were now present in the ER (Figure 4.10B). This could point to a possible incorrect folding of the proteins, which led them to be retained in the ER, or due to an altered Golgi localization signal. In both cases it could alter anti-apoptotic function and/or Ca\textsuperscript{2+} modulation. In an attempt to dissect these possibilities two single mutants were created E207Q and E208Q, of which only E207Q localized to the Golgi while E208Q had a predominant ER localization. Two other mutants, E107Q and E178Q, showed a less drastic altered subcellular localization and these two mutant proteins were found in both the Golgi and the ER in ~50 % of the cells.

All these data were taken into account when interpreting the mutant vGAAPs Ca\textsuperscript{2+} modulation and anti-apoptotic functional assays results.

4.6 vGAAP “ion channel mutants” characterization – anti-apoptotic activity and Ca\textsuperscript{2+} modulation

To test wt and mutant vGAAP anti-apoptotic activity a transient expression apoptosis assay with STS as the apoptotic stimulus was used. Wild type or mutant vGAAPs expression plasmids were co-transfected with cell surface marker CD20 expression plasmid in a 5:1 ratio in Saos-2 cells and 18 h post transfection cells were subjected to either STS or mock treatment. The use of CD20 allowed for the selection of transfected cells using a FITC-conjugated anti-CD20 Ab. After staining the cells with propidium iodide (PI), these were subjected to FACS analysis. Using this approach it was possible to quantify the sub-G1 proportion of the cell population, indicative of apoptotic and dead cells, within the CD20-positive cell population (Figure 4.11A and B).

The protection from STS-induced apoptosis obtained with wt vGAAP expression in Saos-2 cells was very modest but reproducible and statistically significant (Figure 4.12). When all vGAAP mutants were tested using this method it was clear that most of the mutations had no alteration of anti-apoptotic activity. Only mutations E178Q and D219N impaired vGAAP’s anti-apoptotic activity in this assay significantly. Mutant EE207-208QQ appeared to have some effect although this wasn’t statistically
significant. Mutants G152A and D196N showed a slightly increased protection against STS induced apoptosis.

Figure 4.11 - vGAAP transient STS apoptosis assay validation. (A) Saos-2 cells were co-transfected overnight with a plasmid expressing CD20 and either a plasmid expressing vGAAP-HA or empty vector pcDNA3.1. After treatment with STS or DMSO (0 µM STS) for 6 h, both attached and detached cells were collected. These cells were stained with a FITC-conjugated anti-CD20 mAb and then fixed and stained with propidium iodide (PI) prior to FACS analysis. The total population was gated for the CD20-positive cells. The X axis corresponds to the cell number and the Y axis corresponds to the PI stain levels. The histograms show the distribution of the PI stain intensity in the cell population allowing the identification of the sub-G1 population as shown by a vertical bar in each histogram. (B) Quantification of the sub-G1 portion from the CD20-gated population.
It has not been shown so far that the GAAP anti-apoptotic function is linked to its Ca\(^{2+}\) handling activity. Taking this into consideration Ca\(^{2+}\) release experiments were performed (by Dr. David Prole at University of Cambridge) with wt and mutant vGAAPs. HEK293T cells transiently expressing the wt or mutant vGAAP were loaded with Fura-2-AM and kept in a Ca\(^{2+}\) free solution. Ca\(^{2+}\) release as a response to ionomycin (that causes emptying of intracellular Ca\(^{2+}\) stores) was measured in cells expressing GAAP. The mean peak Ca\(^{2+}\) responses from 3-4 independent fields of cells was calculated. vGAAP was able, as was hGAAP (de Mattia et al., 2009), to reduce the amount of Ca\(^{2+}\) released in to the cytosol upon stimulation with ionomycin (Figure 4.13). This suggests that vGAAP, like hGAAP, can reduce the filling state of Ca\(^{2+}\) stores in the cell. Mutation in vGAAP positions 107, 147, 152, 207 and 219 impaired its ability to reduce Ca\(^{2+}\) release from intracellular stores upon ionomycin treatment or in other words impaired its ability to reduce the Ca\(^{2+}\) content on the intracellular Ca\(^{2+}\) stores.

Taking into account the protein expression, subcellular localization, anti-apoptotic activity and Ca\(^{2+}\) modulation ability of these mutant proteins, mutations D219N, D196N and G152A were proposed for further study because all these mutants had an expression level and subcellular localization comparable to wt vGAAP, all showed impaired Ca\(^{2+}\) modulation, but D219N was no longer anti-apoptotic and D196N and G152A showed an increased protection against STS-induced apoptosis. D207N was also chosen due to its high conservation within TMBIM4 members, good alignment with other ion channels and due to its inability to modulate Ca\(^{2+}\). All data obtained for each of the mutant vGAAP proteins are summarized on Table 4.1.
Figure 4.12 - vGAAP wt and mutants transient STS apoptosis assay. Saos-2 cells were co-transfected overnight with a CD20-expressing plasmid and either a plasmid expressing wt or mutant vGAAP-HA or with empty pcDNA3.1. After treatment with STS or DMSO (-STS) for 6 h, both attached and detached cells were collected. These cells were stained with a FITC-conjugated anti-CD20 mAb and then were fixed and stained with PI prior to FACS analysis. Total population was gated on the CD20-positive cells. Sub-G1 percentage from the CD20-gated population was determined (n=1 for -STS and n=3 for +STS). Data are representative of three experiments and are represented as mean ± SEM. Difference between the wt vGAAP was analysed using the unpaired Student’s T-test and is represented as * p<0.05.
Figure 4.13 - vGAAP reduces stores Ca²⁺ content and some “ion channel mutations” prevent this effect. This experiment was carried out by Dr. David Prole, University of Cambridge, using mutant vGAAP plasmids I generated and is here reproduced with his permission. HEK293T cells were transfected with pcDNA3.1, wt or mutant vGAAP together with mCherry-C1 as a transfection marker. Twenty-four hours post transfection, cells were loaded with Fura-2-AM, bathed in Ca²⁺-free medium, and Ca²⁺ responses to ionomycin (10 µM) were measured in transfected cells. The graph shows the mean peak Ca²⁺ responses from 3-4 independent fields of cells (n=5-48 cells in each field). Data are represented as mean ± SEM. Differences between the wt vGAAP and mutant GAAPs was analysed using the unpaired Student’s T-test and are represented as * p<0.05, ** p<0.01, *** p<0.001.
Table 4.1 - Summary table of data acquired for the wt and vGAAP “ion channel mutant” proteins. Crosses represent a different phenotype from the wt vGAAP, ticks represent a similar phenotype while +/- stand for an intermediate phenotype. (-) Was used when data was absent.

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4.7 Summary

After a first attempt to map the interaction of GAAP with the IP₃R it was shown that this was not a specific interaction. Although it is important to note that the experiments presented here were done using IP₃R type 1 over-expression but the experiments done by de Mattia et al. (2009) were done using IP₃R type 3 specific Abs. Also it is worth noting that these are all membrane proteins and that the concentration of salt (NaCl in this case) of the IP buffer, hence the stringency of the washes, is crucial to obtain a specific co-immunoprecipitation and to get rid of false positive pull downs. A high NaCl concentration should allow distinction between a specific or a non-specific co-immunoprecipitation, and this could have been attempted in the initial optimization stages of these co-immunoprecipitations. Later when looking at a GAAP-GAAP interaction by co-immunoprecipitation (chapter V) only at a NaCl concentration as high as 500 mM in the wash buffer could we detect a specific interaction. Taken together all the co-immunoprecipitation data and the limitations of membrane proteins co-
immunoprecipitations suggest that the previously found vGAAP (Caroline Gubser, unpublished) and hGAAP interactions with IP$_3$R could have been due to an artefact of the technique and probably are not a real interaction. Given this result a second hypothesis to explain GAAP function was considered, namely that GAAP is an ion channel that allows the passage of Ca$^{2+}$ ions. To address this hypothesis, purified vGAAP and AtGAAP3 were tested in artificial lipid bilayer experiments and shown to allow the passage of Ca$^{2+}$ ions through the bilayer when different membrane potentials were applied.

The GAAP amino acid sequence was also shown to align with some previously described ion channels and ion exchangers (Figure 4.6 and 4.7). In an attempt to formally prove that GAAP was indeed an ion channel and to characterize its pore and selectivity filter domains, a set of single point mutants was generated based on alignments with other channels and conservation within the TMBIM4 sub-family. These mutants were tested for expression, subcellular localization and function. The vGAAP mutants G152A, D196N, E207Q and D219N were chosen to be tested in artificial lipid bilayer experiments to try and find a mutant with an altered conductivity (Table 4.1). Three mutants (D196N, E207Q and D219N) are part of the proposed GAAP putative Ca$^{2+}$ selectivity filter made up of the four amino acid residues (D or E) that could provide the four COO$^-$ groups usually found in Ca$^{2+}$ selectivity filters (Sather and McCleskey, 2003).

All data collected so far point to a possible ion channel conserved function in the TMBIM4 family and maybe for the entire TMBIM family, because pure BI-1 also showed ion channel activity. However, these data does not rule out that GAAPs might be ion exchangers. These can behave as ion channels in lipid bilayers if both its gates are left open: in this case the molecule will appear as an ion channel with these experimental settings.
Chapter V

GAAP is able to form tetramers
vGAAP and AtGAAP3 produced in and purified from yeast showed additional bands when analyzed by SDS-PAGE, and these bands were of an appropriate size for oligomeric forms of the protein (see chapter III). The fact that BI-1, another member of the same protein family, has been shown to oligomerize when overexpressed (Ahn et al., 2010; Xu et al., 2008) was also consistent with our findings for GAAPs.

Moreover, given our findings on vGAAP ion channel function in vitro (detailed in chapter IV) it would be expected, by comparison with most ion channels described so far, that GAAP would require some form of oligomerization to achieve a comparable number of TMDs and thereby have ion channel activity.

Ultimately, structural determination of GAAP is required to understand its oligomeric structure, however it was very difficult to obtain sufficient quantities of this highly hydrophobic protein and initial attempts to crystallize vGAAP were unsuccessful. Therefore several different approaches were taken to confirm if GAAP can indeed form oligomers in mammalian cells.

5.1 GAAP-GAAP interaction detected by co-IP

The first approach taken was to investigate a possible GAAP-GAAP interaction by co-immunoprecipitation (Figure 5.1). A plasmid expressing GAAP-HA was transfected into a cell line that had been engineered previously to express GAAP with a C-terminal V5 tag (GAAP-V5) (see Table 2.7 for more details). A cell line engineered to express A36 (see Table 2.7 for more details), another VACV membrane protein, with a V5 C-terminal tag was used as a control for nonspecific pull down of membrane proteins during the immunoprecipitation. An immunoprecipitation assay was then performed on cleared lysates from these cells using an anti-V5 Ab coupled to protein G sepharose beads. The immunoprecipitates were immunoblotted with anti-HA and anti-V5 Abs. The WCE were immunoblotted with an anti-HA Ab to confirm plasmid transfection and protein expression from the transfected plasmid.

HA-tagged vGAAP expressed from the plasmid was found to co-immunoprecipitate with V5-tagged vGAAP, expressed from the cell line, but not with V5-tagged A36 although this protein was immunoprecipitated to similar levels as
vGAAP-V5 (Figure 5.1). HA-tagged hGAAP also co-immunoprecipitated with V5-tagged hGAAP but not with V5-tagged A36. Whole cell extracts show similar expression levels from the plasmids that encode for hGAAP-HA and vGAAP-HA in both GAAP-V5 and A36-V5 expressing stable cell lines. This experiment indicates that both vGAAP and hGAAP are able to interact with each other, although a co-immunoprecipitation cannot prove that this interaction is direct.

**Figure 5.1 - hGAAP and vGAAP can interact with themselves.** HeLa cells stably expressing hGAAP-V5 or vGAAP-V5 were transfected with pcDNA3.1 hGAAP-HA or vGAAP-HA for 16 h. Cells were collected, lysed and subjected to immunoprecipitation (IP) using an anti-V5 Ab. A HeLa cell line expressing A36-V5 (~37 kDa), a VACV membrane protein, was used as a control for non-specific immunoprecipitation of membrane proteins. Immunoblots (IB) using anti-HA or anti-V5 Abs were performed to detect both HA and V5 tagged-GAAP proteins. Whole cell extract (WCE) was also immunoblotted with anti-HA. The position of size markers as kDa is shown on the left of the gel.
5.2 Crosslinking with DFDNB revealed the presence of a GAAP tetramer

In an attempt to characterize the stoichiometry of GAAP oligomerization, several crosslinking chemicals were tested. The best results came from the use of the lysine-reactive aryl halide DFDNB (Figure 5.2A). This water insoluble, membrane permeable crosslinker has a very short spacer arm and the nature of this chemical allows it to get inside membrane or lipid micelles making the crosslinking process much more efficient for membrane proteins (Kornblatt and Lake, 1980).

When incubated with DFDNB, both hGAAP and vGAAP showed an increased intensity of higher molecular weight bands detected with the anti-HA Ab. Although these bands were already visible with DMSO treatment only, or when untreated, they became more pronounced with the addition of the crosslinker (Figure 5.2A). With higher concentrations of DFDNB the proteins tended to aggregate and get trapped in the wells, indicated by an open arrowhead. In the case of hGAAP the two higher molecular size bands were very week and were reproducibly found only when hGAAP was treated with higher concentrations of DFDNB.

Membrane proteins tend to run on SDS-PAGE gels at a size different from that predicted from their molecular mass. To confirm if the bands observed with DFDNB treatment represent GAAP oligomeric forms, the predicted monomer copy number of each band was plotted in a semi-logarithmic scale with its relative gel mobility (Figure 5.2B). The linear relationship found for both vGAAP and hGAAP indicates that the relative mobility of each band corresponds to its assigned subunit stoichiometry. The main bands detected were of ∼20-23 kDa, ∼37-45 kDa, ∼60 kDa and ∼80 kDa, and these are consistent with monomeric, dimeric, trimeric and tetrameric forms of GAAP.

5.3 GAAP oligomerization in vitro is pH-dependent

It was reported previously that BI-1 oligomerization is regulated by pH (Kim et al., 2008) and that this could be linked to its function in the ER as a Ca\(^{2+}\) modulator.
Figure 5.2 - hGAAP and vGAAP crosslink with DFDNB. HeLa cells were transfected with (A) pCDNA3.1-vGAAP-HA or (B) pCDNA3.1-hGAAP-HA. Cell lysates were prepared 18 h post transfection and crosslinked with increasing concentrations of DFDNB, untreated (lane 1 in each gel) or treated with DMSO only (lane 2 of each gel) and subjected to SDS-PAGE under non-reducing conditions prior to immunoblotting with an anti-HA Ab (A and B). The predicted oligomeric states of GAAP monomer (1x), dimer (2x), trimer (3x) and tetramer (4x) are shown by full arrows. (C and D) The relative mobility of the different oligomeric forms was determined and plotted against the predicted oligomeric state. Data shown comes from three independent experiments and is represented as mean ± SEM.
To investigate if GAAP oligomerization was also dependent on pH, hGAAP-HA and vGAAP-HA were expressed in mammalian cells, extracted from membranes using CHAPS-based buffers with varying pH and analysed by non-reducing SDS-PAGE. It was clear that both vGAAP and hGAAP showed best oligomerization levels at pH 7 or higher (Figure 5.3). vGAAP oligomerization levels peaked at pH 7 while hGAAP oligomerization levels increased until pH 8, the highest pH tested. Bands corresponding to the monomer (~20 kDa) and putative dimer (~37 kDa), trimer (~60 kDa) and tetramer (~80 kDa) could be identified for both vGAAP and hGAAP although in the case of hGAAP a series of other bands were also visible. In these non-reducing conditions the vGAAP and hGAAP band that migrates at ~37 kDa is in fact composed of two bands.

5.4 vGAAP, but not hGAAP, dimerization is sensitive to DTT

When vGAAP and hGAAP were analysed by non-reducing SDS-PAGE two very close and intense bands of ~37 kDa (putative dimer) were visible, even in the absence of any crosslinker (Figure 5.4, untreated lanes). When treated with increasing amounts of the reducing agent DTT these bands gradually disappear in the case of vGAAP but the hGAAP bands showed no reduction in intensity even when high DTT concentrations were used (Figure 5.4). The very weak bands corresponding to the higher oligomeric states of vGAAP also mostly disappear after DTT treatment.

These observations led us to hypothesise that the dimerization mechanism in vGAAP might be dependent on cysteine disulphide bounds, whereas hGAAP dimerization might not rely on disulphide bonds. Alternatively, the hGAAP disulphide bonds could be inaccessible to DTT.
Figure 5.3 - GAAP oligomerization is pH-dependent. Lysates from HeLa cells transfected with pCDNA3.1-vGAAP-HA or pCDNA3.1-hGAAP-HA for 18 h were incubated at varying pH, prior to SDS-PAGE in non-reducing conditions and the membranes immunoblotted with an anti-HA Ab. Arrows show the different oligomerization states of GAAP. Monomer (1x), dimer (2x), trimer (3x) and tetramer (4x). The position of size markers in kDa is shown on the left of each gel.

Figure 5.4 - DTT is able to disrupt the vGAAP but not hGAAP dimer. HeLa cells were transfected with pCDNA3.1-vGAAP-HA or pCDNA3.1-hGAAP-HA. Cells lysates were prepared 18 h post transfection and treated with increasing concentrations of DTT prior to immunoblotting. Samples were resolved by SDS-PAGE and immunoblotted with an anti-HA Ab. Arrows show the different oligomerization states of GAAP. Monomer (1x), dimer (2x), trimer (3x) and tetramer (4x) U, untreated samples. The position of size markers in kDa is shown on the left of each gel.
5.5 vGAAP TMDs 5-6 and the C terminus are not required for oligomerization

BI-1 was shown to lose some of its oligomerization ability when the C terminus was deleted (Kim et al., 2008). Truncating vGAAP and hGAAP between TMD 4 and 5 (Figure 5.5A) allowed us to test if any of these truncated forms of GAAP retained the ability to oligomerize and hence map the domains required for this process.

Truncated vGAAP and hGAAP molecules, illustrated in Figure 5.5A, were resolved by reducing and non-reducing SDS-PAGE. Truncated vGAAP containing TMDs 1-4 still retains the ability to dimerize because a very intense band that runs at ~25-30 kDa, corresponding to a putative dimer of the 12-14 kDa monomeric form of the protein, can be detected at non-reducing conditions. These bands are also DTT sensitive because they are not detected when reducing SDS-PAGE conditions are used. Both hGAAP truncated proteins tested lost the ability to dimerize in both reducing and non-reducing conditions, although a faint band corresponding to the hGAAP TMD1-4 dimer could still be detected.

This result suggests that the region of vGAAP containing TMD1-4, the first 147 amino acid residues, is sufficient for protein dimerization and that the last two TMDs and the C-terminal portion of vGAAP are not required for dimerization.

5.6 vGAAP cysteines 9 and 60 are essential for oligomerization – SDS-PAGE analysis

In an attempt to map the essential residues involved in vGAAP oligomerization and taking in consideration data from Figures 5.4 and 5.5 an amino acid sequence alignment with representative members of the GAAP family was undertaken (Figure 5.6). This allowed analysis of the conservation level of cysteine residues within the GAAP family, which could be involved in creating disulphide bounds between GAAP molecules leading to dimerization and possibly to oligomerization.
Figure 5.5 - vGAAP TMDs 5-6 and the C terminus are not required for GAAP dimerization. (A) Schematics of the wild type and truncated forms of vGAAP and hGAAP. Transmembrane domains (TM) (B) Lysates from HeLa cells transfected for 18 h with plasmids that encode wild type or truncated forms of vGAAP-HA and hGAAP-HA were subjected to SDS-PAGE under reducing (+DTT) or non-reducing conditions (-DTT). Membranes were blotted with an anti-HA Ab to detected GAAP. The position of size markers in kDa is shown on the left of the gel.
Figure 5.6 – Alignment of GAAP family protein amino acid sequences showing the degree of conservation of the cysteine residues. The alignment was created using the Clustal W multialignment tool. Cysteine residues are highlighted in red and transmembrane regions are defined by green lines.
The sequence alignments showed that there was only one highly conserved cysteine in the GAAP family. Strikingly, it mapped to TMD 6, which was not included in the region that was defined as necessary and sufficient for vGAAP oligomerization (TMD1-4). Different vGAAPs differ considerably from all other GAAP family members in possessing many more cysteines. Close to the end of TMD 5 most GAAPs analysed, except vGAAP and AtGAAP3, show some degree of cysteine conservation.

Most of vGAAP unique cysteines were located closer to the N terminus of the protein. Considering GAAP topology, there are three cysteines located in the intermembrane loops, and not located deep inside the TMDs, and these are indicated in grey boxes and are present in both viral GAAPs and Danio rerio GAAP (Figure 5.7). Notably both cysteine residues in hGAAP are predicted to be deep within TMD5 and 6 (Figure 5.7). There are only two cysteines in vGAAP that are not embedded in the TMDs, cysteines 9 and 60.

These data might suggest that although the cysteine present in TMD 6 is essential for GAAP function or structure it might not be directly involved in GAAP-GAAP interaction.

To further investigate the importance of disulphide bonds in both vGAAP and hGAAP oligomerization all cysteine residues from both proteins were individually mutated to serine. A vGAAP and an hGAAP mutant with all cysteines mutated was also generated and was called either vGAAP CXS or hGAAP CXS.

All mutant proteins were tested for subcellular localization by immunofluorescence, as an indicator for correct protein folding (Figure 5.8). When compared with the wild type proteins and using an anti-GM130 Ab as a Golgi marker, it was clear that all mutant vGAAP and hGAAP proteins still localized to the Golgi complex because their localization overlapped well with that of GM130. Even the CXS mutant in which all cysteines have been replaced by serines showed a Golgi localization that was indistinguishable from wild type.
Figure 5.7 - Diagram of cysteine distribution in vGAAP and hGAAP. GAAP topology as described by Carrara et al, (submitted) with the localization of the cysteine residues that were subjected to site-directed mutagenesis highlighted by yellow crosses. Both vGAAP and hGAAP N and C terminus are located at the cytosolic side of the Golgi membrane. The portion after TMD6 was described to be either a re-entrant loop or to be free in the cytosol.
Figure 5.8 – A Golgi localization persists in hGAAP and vGAAP cysteine to serine mutants. HeLa cells were transfected with pcDNA3.1 wt or cysteine to serine mutant vGAAP-HA or hGAAP-HA and fixed in 4 % PFA after 16 h. An anti-HA Ab was used to detect GAAP and an anti-GM130 Ab was used as a Golgi marker. Cells were imaged using confocal microscopy. CXS mutants have all their cysteines mutated to serines. The right panels represent phase images. Scale bars correspond to 10 µm.
To investigate the contribution of each individual cysteine residue for hGAAP and vGAAP dimerization, each mutant was expressed in mammalian cells and analysed by both reducing and non-reducing SDS-PAGE (Figure 5.9A and B). None of the hGAAP cysteine mutants lost the ability to form dimers, as shown by the presence of a ~37 kDa band for all mutants in both reducing and non-reducing conditions. Even the CXS hGAAP mutant, with all the cysteines mutated, showed the presence of a band that migrated at a size corresponding to the dimeric form of the protein, in both reducing and non-reducing conditions (Figure 5.9A). For the vGAAP mutants, only cysteines 9 and 60 showed a reduced dimer to monomer band ratio in non-reducing conditions compared to the wild type vGAAP (Figure 5.9B). When these were mutated in combination the double mutant (C9+60S) failed to dimerize to the same degree as the protein lacking all cysteines (CXS), indicating that these are the only cysteines involved in vGAAP dimerization. Mutants C9S, C60S, C9+60S and CXS also showed a reduction in the intensity of putative trimer and tetramer bands. Upon addition of DTT, all mutant and wt vGAAP proteins were reduced to a monomeric state only, as shown by the absence of any higher molecular size bands, showing equal levels of protein and ruling out possible experimental artefacts due to differential protein expression.

Taken together these data suggested that cysteine residues 9 and 60 are likely to be involved in both dimerization and tetramerization of vGAAP.

As shown in Figure 5.2, wild type vGAAP can form dimers, trimers, and tetramers and the proportion of the high molecular size forms increases with the addition of a crosslinker. Using the same approach for vGAAP C9+60S mutant it was shown that these mutations abrogate the appearance of high molecular size bands when the protein is resolved by non-reducing SDS-PAGE, even when previously crosslinked with DFDNB (Figure 5.10). Putative dimer, trimer and tetramer bands are clearly seen for wt vGAAP when crosslinked in the same conditions as the mutant.
Figure 5.9 - vGAAP oligomerization is dependent on cysteine residues 9 and 60. Individual or multiple cysteine to serine mutations were made in (A) hGAAP-HA or (B) vGAAP-HA. Lysates from HeLa cells transfected with pcDNA3.1 wt or cysteine to serine mutant vGAAP-HA and hGAAP-HA were run on SDS-PAGE in the presence or absence of reducing agent (DTT) and immunoblotted (IB) with an anti-HA Ab. (B upper panel) The dimer and monomer bands intensity of the vGAAP-HA mutants was measured using Li-Cor and plotted as the ratio between dimer and monomer. The predicted oligomerization forms of GAAP are shown, by arrows on the side of the gel. Monomer (1x), dimer (2x), trimer (3x) and tetramer (4x). CXS mutants have all the cysteines mutated to serines. The position of size markers in kDa is shown on the left of each gel.
Figure 5.10 - vGAAP C9+60S is not able to oligomerize even when crosslinked. Lysates from HeLa cells transfected with either pcDNA3.1 wt or C9+60S vGAAP-HA were incubated in the presence of increasing concentrations of the crosslinker DFDNB. Samples were resolved by non-reducing SDS-PAGE and membranes were immunoblotted with an anti-HA Ab. The predicted oligomerization states of vGAAP are shown by arrows. Monomer (1x), dimer (2x), trimer (3x) and tetramer (4x). The position of size markers in kDa is shown on the left of the gel.

5.7 vGAAP cysteine’s 9 and 60 are essential for oligomerization – FRET analysis

To corroborate the SDS-PAGE analysis, hGAAP, vGAAP, vGAAP C9S, vGAAP C60S and vGAAP C9+60S were C-terminally fused with GFP or with tdTomato (Tom) for FRET analysis (Figure 5.11A). The use of FRET to analyse the GAAP-GAAP interaction offered two major advantages when compared to the non-reducing SDS-PAGE approach: it allowed whole cell analysis, this overcomes the possible membrane protein aggregation during extraction of membrane proteins using detergents; and, because the proteins remain in the Golgi membrane during FRET analysis, these are
more likely to retain their native conformation. As a control for proper expression and folding, the subcellular localization of each fusion protein was assessed by immunofluorescence using an anti-GM130 Ab as a Golgi marker (Figure 5.11B). All fusion proteins tested showed the expected Golgi localization as indicated by overlapping with GM130 localization. Therefore FRET could be used to investigate GAAP-GAAP interactions in a cell based assay where the protein would still be inserted in the Golgi membrane.

Wild type vGAAP fused with GFP (vGAAP-GFP) was co-transfected in mammalian cells with tdTomato fused vGAAP (vGAAP-Tom). This combination allowed the detection of a FRET signal as an increase in vGAAP-GFP fluorescence after vGAAP-Tom photobleach. This could be seen either by an increase in total cell vGAAP-GFP fluorescence after vGAAP-Tom photobleach, as showed by grey scale images (Figure 5.12A) or by the calculation of FRET efficiency (E) in regions of interest where both signals were detected (Figure 5.12A) (see materials and methods for FRET efficiency calculation). None of the vGAAP cysteine mutants tested were able to produce a FRET signal, giving similar results to the negative controls, GRASP65-GFP and free GFP both in combination with vGAAP-Tom. GRASP65-GFP was used as a negative control because it is known to localize to the Golgi and its GFP tag was engineered so it was localized in the cytoplasmic side of the Golgi membrane (Barr et al., 1998), as was the case for GAAP. These data confirm the results found using the non-reducing SDS-PAGE approach that cysteine 9 in vGAAP is required for vGAAP-vGAAP interactions. vGAAP cysteine 60 that had a weaker impact on vGAAP dimerization disruption when analysed by non-reducing SDS-PAGE, seems to be absolutely required when tested by FRET.

Using immunofluorescence it was possible to confirm Golgi localization for both wt hGAAP-GFP and hGAAP-Tom (Figure 5.11B). The FRET signal obtained with the wt hGAAP-GFP and the wt hGAAP-Tom co-transfection was significantly higher than the signal obtained with all the controls used (Figure 5.13A and B), indicating a positive interaction between the two proteins. Using this same approach, it was also possible to confirm the previously observed hGAAP-hGAAP interaction in cell lysates using non-reducing SDS-PAGE.
Figure 5.11 - Subcellular localization of GAAP-GFP and GAAP-Tom fusion proteins. (A) Diagram showing the localization of GFP and tdTomato (Tom) tagged-GAAP. (B) HeLa cells were transfected with plasmids encoding GFP or tdTomato tagged vGAAP and hGAAP or with GFP or tdTomato alone. Cells were fixed 16 h post transfection and imaged using confocal microscopy. Golgi localization of the GFP and the tdTomato fusion GAAP proteins was assessed by comparison with immunostaining with an anti-GM130 Ab. Scale bars correspond to 10 µm.
Figure 5.12 - vGAAP-vGAAP interaction assessed by FRET. FRET was detected by the increase in GFP fluorescence after tdTomato (Tom) photobleach in HeLa cells transfected with plasmids that express vGAAP-GFP and vGAAP-Tom or control proteins. Cells were fixed 16 h post transfection. GRASP65-GFP and free GFP were used as negative controls. (A) Representative images of GFP fluorescence before and after Tom photobleach. Grey values images were obtained by pixel-by-pixel subtraction of the GFP prebleach images from postbleach images. Scale bar, 10 µm (B) Regions where GFP and Tom colocalize from > 10 cells for each condition were selected and FRET efficiency was calculated (see materials methods for more details). Data are shown as mean ± SEM and were analyzed using an unpaired Student’s T-test and is represented as ** p<0.01 for comparison with GRASP65-GFP + vGAAP-Tom.
Figure 5.13 - hGAAP-hGAAP interaction assessed by FRET. FRET was detected by the increase in GFP fluorescence after tdTomato (Tom) photobleach in HeLa cells transfected with plasmids encoding hGAAP-GFP and hGAAP-Tom. Cells were fixed 16 h post transfection. GRAP65-GFP and free GFP were used as negative controls. (A) Representative images of GFP fluorescence before and after Tom photobleach. Grey values images were obtained by pixel-by-pixel subtraction of the GFP prebleach images from postbleach images. Scale bar, 10 µm (B) Regions where GFP and Tom colocalize from at least 10 cells for each condition were selected and FRET efficiency was calculated (see materials methods for more details). Data are shown as mean ± SEM and were analyzed using an unpaired Student’s T-test and is represented as *** p<0.001 for comparison with GRASP65-GFP + hGAAP-Tom.
5.8 Summary

All GAAP proteins analysed so far by SDS-PAGE exhibit anomalous gel mobility, because they tend to show increased gel mobility when analysed by SDS-PAGE compared to their known molecular masses. For example vGAAP, with a predicted size of 26.5 kDa (Gubser and Smith, 2002) runs with the 20-kDa marker on the protein ladder in SDS-PAGE. This could be attributed to the protein’s high hydrophobicity, to the possibility that lipids and/or detergent molecules could still be bound to the protein when running through the gel and to the absence of a strong protein denaturing step (95 °C for 10 min) in the cell lysate preparation protocol that will reduce protein denaturation. The binding of lipids or detergent to the protein and the incomplete denaturation will make the molecular complexes more globular, this will increase the protein gel mobility. It was also noted that both hGAAP and vGAAP (from both CMLV and VACV) expressed in mammalian cells showed several bands other than the predicted 20-kDa form when analysed by gel electrophoresis. One possible explanation is that these additional bands could correspond to dimers, trimers and tetramers of GAAP.

Using DFDNB it was shown that both vGAAP and hGAAP can form dimers, trimers and tetramers. Although not resolved in the conditions used, the existence of any species with higher molecular weight than the tetramer could not be completely ruled out and require further investigation.

It was possible to define the N-terminal region of vGAAP as essential and sufficient for vGAAP oligomerization. Yet it was not possible to rule out that the truncation made in hGAAP impaired the proper folding of the two truncated proteins and that this could account for the loss of oligomerization observed for both the hGAAP truncations in Figure 5.5.

Using both biochemical and cell-based assays, it was shown that both hGAAP and vGAAP can form oligomers, these are most likely tetrameric and that these are dependent on pH. Oligomerization of hGAAP, but not vGAAP was found to be independent of disulphide bounds. Using FRET and non-reducing SDS-PAGE it was possible to identify cysteines 9 and 60 in vGAAP as essential for oligomerization. Results obtained using the non-reducing SDS-PAGE approach were very clear and
identified cysteines 9 and 60 from vGAAP as important for vGAAP dimerization. Nevertheless this approach did not address the fact that GAAP oligomerization occurs within the Golgi membrane and that the protein had been extracted and solubilized with the help of a detergent (CHAPS). This issue was addressed by using FRET to analyse the GAAP-GAAP interaction because this technique allows the detection of the GAAP-GAAP interaction while the proteins were still embedded in the Golgi membrane and in their native state. The use of FRET confirmed the vGAAP-vGAAP and hGAAP-hGAAP interactions and the importance of vGAAP cysteine 9 for this interaction. vGAAP cysteine 60, that showed only a mild effect on dimerization disruption using the non-reducing SDS-PAGE technique was shown to be required to the vGAAP-vGAAP interaction by using FRET. The use of FRET enabled the clarification and confirmation of the results obtained with the non-reducing SDS-PAGE approach. FRET offers many advantages when analysing membrane protein-protein interactions because it does not require the extraction of the proteins from the membranes with detergent that usually lead to both unspecific interactions due to the high protein hydrophobicity and to the possible loss of interactions that are dependent on the correct protein insertion in the cellular lipid bilayers.

It is not known how vGAAP dimers assemble to form the higher molecular weight oligomer detected by SDS-PAGE. It may be that vGAAP forms a dimer through cysteine-based disulphide bonds and that a dimer of dimers is formed through other interactions, such as electrostatic forces.
hGAAP increases cell adhesion
So far hGAAP has been described as an anti-apoptotic protein that can modulate the amount of Ca\textsuperscript{2+} kept in intracellular stores. During apoptosis assays using ceramide, it was observed that cells often detached prior to apoptosis, with detachment and apoptosis being prevented in the cells in which hGAAP was overexpressed (Gubser et al., 2007). Given the essential role of Ca\textsuperscript{2+} in cell adhesion we therefore sought to test whether hGAAP expression affected cell adhesion.

### 6.1 hGAAP overexpression increases cell adhesion and reduces cell detachment

As a first approach to test a possible involvement of hGAAP in cell adhesion, a simple attachment assay was used to test if hGAAP overexpression affected cell adhesion. U2OS and HeLa cells either overexpressing hGAAP or control cell lines carrying the empty plasmids (U2OS neo or HeLa puro, see table 2.7 in material and methods for more details) were seeded in tissue culture plates and washed at different time points to remove non-adherent cells. The cells that had attached to the wells were stained with crystal violet and the amount of crystal violet taken up was measured by spectrophotometry, after this the crystal violet retained in the cells was eluted with acetic acid, to determine the number of cells present. The correlation between levels of crystal violet dye and cell number is only possible if the volume is similar between cell lines. To control for this parameter viable cell diameter after detachment was calculated using the Invitrogen automated cell counter and trypan blue as a marker for viable cells (U2OS neo: 11.65 ± 0.25 µm\textsuperscript{2}; U2OS hGAAP 11.68 ± 0.33 µm\textsuperscript{2} and U2OS hGAAP C-terminal mutant [Ctmut] 11.49 ± 0.29 µm\textsuperscript{2}), no significant differences in cell size were found between U2OS cell lines that could account for the differences observed in the attachment assay.

Using two independently generated hGAAP overexpressing cell lines in two different cell types (HeLa and U2OS hGAAP expressing cell lines were previously generated in our lab by Ben Johnson and Caroline Gubser, respectively) it was clear that hGAAP overexpression increased the speed of attachment to the substrate (Figure 6.1A and B). In HeLa cells between 60 and 120 min post seeding, there were statistically greater numbers of adherent cells if hGAAP was overexpressed. Similarly,
in U2OS cells there were more adherent hGAAP expressing cells at 45 and 60 min post seeding. In U2OS cells, a cell line expressing a mutated version of the hGAAP was analysed in parallel. The C-terminal region of the TMBIM family members is characteristically charged. A C-terminal mutant of hGAAP where these charged amino acids were replaced by alanines, (Figure 6.1C) was used as an additional control because it was shown previously to be unable to inhibit apoptosis using a variety of apoptotic stimuli (Caroline Gubser, unpublished). This mutant was shown to express to similar levels as the wt hGAAP protein (Figure 6.1D). hGAAP-V5 expression in HeLa was confirmed by immunoblot with an anti-V5 Ab (Figure 6.1E). Analysis of the adherence of cells expressing hGAAP Ctmut showed they were indistinguishable from control cells not overexpressing hGAAP, indicating that the C-terminal charged residues are important for induction of increased cell adhesion.

The adhesion of cells to the plastic matrix was also assessed using a detachment assay. Cell detachment was induced by addition of EDTA to the medium and cells remaining adhered to the plastic at different times thereafter were measured using the same crystal violet staining method used before. U2OS and HeLa cells seeded for 24 h were treated with different concentrations of EDTA and the cells remaining attached after treatment were quantified by crystal violet stain. In both EDTA concentrations used for both cell types hGAAP overexpression was able to reduce cell detachment to a considerable degree (Figure 6.2). Both methods used to measure the effect of hGAAP effect on cell adhesion let to the same conclusion: namely, that hGAAP overexpression increased the cell’s ability to attach to the substratum.

The C-terminal mutations in hGAAP rendered the protein unable to modulate Ca$^{2+}$ in a single cell Ca$^{2+}$ release assay (Figure 6.3). When Ca$^{2+}$ from intracellular stores was released by the action of ionomycin the amount released by the control cell line (neo) and the hGAAP Ctmut cell line were not different, while the cell line overexpressing hGAAP releases a significant lower amount of Ca$^{2+}$ as previously shown by de Mattia et al. (2009). The observed effects in cell adhesion, anti-apoptotic effect and Ca$^{2+}$ modulation caused by the C-terminal mutations cannot be accounted by a reduction in protein expression because both wild type and Ctmut hGAAP were expressed to similar levels when tested by immunoblot using anti-HA Ab (Figure 6.1D).
Figure 6.1 - hGAAP overexpression increases cell attachment and its charged C terminus is essential for this phenotype. (A) U2OS or (B) HeLa cells expressing hGAAP-HA, hGAAP Ctmut-HA or just the plasmid resistance gene (neo or puro) were detached using trypsin, counted and seeded in a 12-well plate (n=4). Cells were washed in PBS at different time points post seeding to remove non-adherent cells. The remaining cells were fixed with PFA and stained with crystal violet. The amount of crystal violet remaining after washing was determined by eluting with acetic acid and its absorbance measured. Data are representative of three individual experiments and are represented as mean ± SEM. Data were analyzed using the Student’s T-test and is represented as * p<0.05, **p<0.01 for comparison to the neo or puro cells. (C) Diagram showing the hGAAP mutated residues in the hGAAP Ctmut protein. (D and E) Cells were lysed and immunoblotted using (D) anti-HA or (E) anti-V5 Abs to confirm hGAAP expression. The position of size markers in kDa is shown on the right of each gel.
Figure 6.2 - hGAAP overexpression reduces cell detachment upon EDTA treatment and mutating the charged C terminus abolishes this phenotype. (A) U2OS or (B) HeLa cells were seeded in a 12-well plate for 24 h (n=4) and treated with different concentrations of EDTA for 20 min. After treatment, the wells were washed to remove detached cells and the remaining attached cells were fixed with PFA, stained with crystal violet, and the crystal violet eluted with acetic acid and its absorbance measured. Data are representative of three individual experiments and are represented as mean ± SEM of the percentage of cells still attached after EDTA treatment compared to untreated cells. Data were analyzed using an unpaired Student’s T-test and are represented as ** p<0.01, *** p<0.001 for comparison to the neo or puro cells.
Figure 6.3 - hGAAP reduces the Ca²⁺ content of intracellular stores and its charged C-terminal region is essential for this function. (A) HEK293T cells were transfected with plasmids expressing the indicated proteins along with mCherry-C1. Twenty-four hours after transfection, cells were loaded with Fura-2-AM and kept in Ca²⁺-free solution. Ca²⁺ release as a response to ionomycin was measured in transfected cells. Traces show representative average Ca²⁺ responses of fields of transfected cells to ionomycin (n=20-52 cells in each case). (B) Graph shows the means ± SEM of peak Ca²⁺ responses from three independent fields of cells (n=18-64 cells in each field). Data were analyzed using the Student’s T-test: * p<0.05, for comparison to pcDNA3.1 transfected cells. Ca²⁺ release assays were performed by David Prole, University of Cambridge.
6.2 hGAAP knockdown increases cell detachment

The above data indicated that hGAAP overexpression increased cell adherence to the substratum. Reduction in hGAAP expression might therefore be expected to do the converse. To test this hGAAP knockdown was achieved by transfection of siRNAs and the consequences of this on cell adhesion was measured using the EDTA detachment assay described above. Cells were transfected for 36 h with well-characterized hGAAP siRNA 1 or 2 (Gubser et al., 2007) or with a control siRNA. These cells were then incubated with either Ca^{2+} containing medium or EDTA containing medium. hGAAP knockdown efficiency was assessed by RT-PCR with hGAAP specific primers. When siRNA1 and 2 were used a reduction in hGAAP mRNA levels was obtained in the two different cell types tested (Figure 6.4A) although siRNA1 was reproducibly better at knocking down hGAAP levels than siRNA2. GAPDH mRNA levels were determined and used as a loading control. To address if hGAAP mRNA reduction caused a reduction in protein levels the levels of hGAAP-HA was measured 36 h post siRNA transfection in hGAAP-HA expressing U2OS cells by immunoblotting. This approach was selected because of the lack of a good Ab to easily measure the levels of endogenous hGAAP. It was possible to detect a reduction in hGAAP-HA protein levels in these conditions with both siRNA1 and siRNA2 but not with a control siRNA (Figure 6.4B). Given that the levels of hGAAP-HA are higher when compared to endogenous protein levels we can assume that both siRNA 1 and 2 are able to knockdown endogenous hGAAP in the conditions used.

Cells transfected with either hGAAP specific siRNAs showed an increased cell detachment compared to control siRNA, with EDTA concentrations of 0.5 mM, and 5 mM, and these differences were statistically significant (Figure 6.4A). No alterations in cell detachment were seen when 1 mM CaCl2 was used instead.
Figure 6.4 - hGAAP siRNA knockdown increases cell detachment upon EDTA treatment. (A) U2OS and HeLa cells or (B) U2OS expressing hGAAP-HA were seeded in a 12-well plate and transfected with the appropriate siRNA for 36 h. (B) U2OS and HeLa siRNA transfected cells were tested for downregulation of endogenous hGAAP by RT-PCR with hGAAP specific primers. GAPDH was used as a loading control and samples with no RT were used to control for DNA contamination. (B) U2OS hGAAP-HA cells were also tested for hGAAP-HA downregulation by immunoblot using an anti-HA Ab. The position of size markers in kDa or base pairs (bp) is shown on the right of each gel. (C) U2OS transfected with siRNA for 36 h were treated with different concentrations of EDTA or with Krebs Ringer buffer containing 1 mM CaCl$_2$ for 20 min. Wells were then washed to remove detached cells and the remaining cells were fixed with PFA, stained with crystal violet and the dye remaining eluted with acetic acid and absorbance measured. Data are representative of three individual experiments and are represented as mean ± SEM. Data were analyzed using the Student’s T-test and is represented as * p<0.05, ** p<0.01, for comparison to the control siRNA.
6.3 hGAAP affects cell spread on fibronectin

In an attempt to better understand and characterize the altered adhesion and detachment of cells mediated by hGAAP, the speed of cell spread on the ECM protein fibronectin was measured. U2OS cells were seeded on fibronectin-coated slides, fixed at different time points and stained with phalloidin. The cell area was measured using confocal microscopy and was plotted against time, showing how fast the cells could settle down and spread out. hGAAP overexpression made cells spread out faster when compared to both hGAAP Ctmur and neo cell lines (Figure 6.5A). In contrast, hGAAP knockdown using each hGAAP-specific siRNA slowed cell spread when compared to the control siRNA (Figure 6.5B).

The fact that hGAAP knockdown can impair cell spread and hGAAP overexpression can accelerate it suggests a possible involvement of hGAAP in focal adhesion dynamics or actin cytoskeleton reorganization. This is because cell spread in response to integrin activation, which in these experimental conditions is mainly triggered by integrin binding to fibronectin, is highly dependent on these two events.

6.4 hGAAP overexpression reduces cell migration in a wound-healing assay (WHA)

A WHA was used to test hGAAP influence in uni-directional cell migration in a confluent monolayer of cells. Wounds were generated in confluent monolayers of hGAAP, hGAAP Ctmur or neo U2OS cells seeded in fibronectin-free dishes, and cell migration was stimulated by addition of 10 % FBS. Wound healing caused by cell migration into the wound was monitored for 12 h by measuring the size of the wound every 15 min and plotted against time to calculate the speed of cell migration (Figure 6.6A). The speed at which cells overexpressing hGAAP migrate to fill the wound was higher than the speed of hGAAP Ctmur or neo cells (Figure 6.6B). The results obtained show that hGAAP overexpression decreased the ability of cells to move uni-directionally in a monolayer in a fibronectin-free condition.
Figure 6.5 - hGAAP overexpression increases cell spread speed whereas hGAAP knockdown has the reverse effect. (A) hGAAP, hGAAP Ctmut or neo U2OS cells were seeded in fibronectin-coated slides and left to adhere and spread for different times as shown. Cells were then fixed with PFA and stained with phalloidin for easier determination of cell area. (B) U2OS cells were transfected with the panel of siRNA showed for 36 h, after this time cells were re-seeded in fibronectin-coated slides and fixed at different times post seeding. Over 35 cells were imaged for each cell line and time points and their area (µm²) was measured using Image J software. Data are shown as mean ± SEM and significant differences were determined using an unpaired Student’s T-test and are represented as ** p<0.01, *** p<0.001 for comparison to the neo or control siRNA transfected cells.
Figure 6.6 - hGAAP overexpression reduces cell migration in a wound-healing assay. U2OS cells were seeded in 6-well plates and grown to confluence. After serum starving the cells overnight, three lesions were made per well by scratching a pipette tip across the cell monolayer and each wound was imaged every 15 min for 12 h. Cell migration was stimulated by replacing the FBS-free medium with 10 % FBS medium after creating the wounds. Two examples of typical wounds are shown in A. (B) The distance between both sides of the wound was measured for each time points and plotted against time to determine cell migration speed. Data shown corresponds to six individual wounds. Data are representative of three individual experiments and are represented as mean ± SEM. Data were analyzed using the Student’s T-test and is represented as ** p<0.01, for comparison to the neo cell line. Scale bar corresponds to 100 µm.
6.5 Overexpression or knockdown of hGAAP does not abolish protein trafficking to the plasma membrane

Given that the subcellular localization of hGAAP is in the Golgi, and the importance of this organelle for the correct traffic to the plasma membrane of many proteins involved in cell adhesion processes, it was important to test a possible effect of hGAAP overexpression or knockdown in these pathways. In an attempt to show that hGAAP overexpression or knockdown was not abolishing or significantly impairing the secretory pathway a control experiment was performed to complement data obtained by Gubser et al. (2007). Cells overexpressing hGAAP or hGAAP Ctmut, or in which hGAAP expression was knocked down by siRNA, were transfected with the vesicular stomatitis virus (VSV) glycoprotein G (gpG) fused with GFP (VSV-gpG-GFP). VSV-gpG is a well characterised type I membrane protein that is expressed on the cell surface (Katz et al., 1977). Cells transfected with VSV-gpG-GFP expressing plasmid were fixed, but not permeabilised, and then stained with an anti-VSV-G mAb that, in these circumstances, only detected plasma membrane VSV-gpG-GFP, and not internal VSV-gpG-GFP. The levels of VSV-gpG-GFP at the plasma membrane were very similar within the three different cell lines and also within all the three siRNA treated cells used in this experiment (Figure 6.7). The total expression levels were also not substantially affected as seen in the GFP channel that allows the detection of all VSV-gpG-GFP protein within the cell (Figure 6.7). Using this approach it was possible to show that neither hGAAP nor hGAAP Ctmut overexpression nor hGAAP knockdown drastically altered the ability of the secretory pathway to produce or export the VSV-gpG to the plasma membrane. Although further quantitative and functional assays would be necessary to completely rule out small and less obvious effects of this pathway.
Figure 6.7 - hGAAP knockdown or overexpression do not abolish protein trafficking to the plasma membrane. U2OS cells overexpressing hGAAP-HA or hGAAP Cutm-HA or hGAAP siRNA- or control siRNA-treated for 18 h were transfected with a plasmid expressing VSV-gpG-GFP for 18 h. After this time, cells were fixed, but not permeabilized, and immunostained with an anti-VSV-gpG Ab. Cells were imaged for both total VSV-gpG (GFP signal) and cell membrane bound VSV-gpG (Ab). Scale bars represented are 10 µm.
6.6 hGAAP overexpressing cells have more focal adhesions, but these are smaller

Given that hGAAP can increase both cell adhesion and cell spread on fibronectin, and reduce cell migration, we decided to explore a possible effect on focal adhesion dynamics. Focal adhesions are essential for cell to extracellular matrix adhesion and play an important role in many cellular processes such as migration. By determining the size, distribution and number of these structures it was possible to define if hGAAP was having an effect on focal adhesion dynamics.

As a first approach, the number of focal adhesions was estimated using vinculin as a focal adhesion marker because this is one of the best characterized components of the focal adhesion complexes. A plasmid expressing vinculin tagged with GFP was transfected into cells overexpressing hGAAP, and these were seeded on fibronectin-coated plates and the number of focal adhesions was measured by confocal microscopy. Phalloidin was used as an additional marker for focal adhesions because actin stress fibres are also found in such structures. The number of focal adhesions found using this approach for hGAAP overexpressing cell lines was slightly higher than the numbers found for the control neo and hGAAP Ctmut overexpressing cell lines (Figure 6.8). Although this difference was small, it was statistically significant.

The use of interference reflectance microscopy (IRM) allowed the quantification of the total number of focal adhesions per cell independently of a particular marker, by measuring the number of sites of contact between the cell and the substratum. This showed that the number of focal adhesions per cell was higher in the hGAAP overexpressing cells than the control cell lines (Figure 6.9A) backing up the data found using vinculin as a marker for focal adhesions. Additionally, the size of the focal adhesions was found to be significantly smaller in cells overexpressing hGAAP when compared to the control neo and the Ctmut cell lines (Figure 6.9B). The average percentage of cell-substrate contact area was found to be higher in hGAAP overexpressing cells (Figure 6.9C). This result, together with the increased cell spread speed observed for hGAAP U2OS cells, could explain the increased adhesion phenotype observed in Figure 6.1 with hGAAP overexpression, because the overall contact area is increased in these cells when compared with the control cell line.
Figure 6.8 - hGAAP overexpression increases the number of vinculin positive focal adhesion sites. U2OS cells overexpressing hGAAP-HA or hGAAP Ctmut-HA were seeded in fibronectin-coated coverslips for 24 h and hGAAP overexpressing cells were transfected with GFP-vinculin. Twelve hours later cells were fixed and stained with phalloidin. Confocal microscopy images were acquired and the number of focal adhesions was calculated for > 20 cells. Data are represented as mean ± SEM. Data were analyzed using the Student’s T-test and is represented as * p<0.05 for comparison to the neo cells.
Figure 6.9 - Analysis of the cell-substrate interface of hGAAP-overexpressing cells using interference reflectance microscopy (IRM). U2OS cells were allowed to attach for 24 h to glass-bottom dishes pre-coated with fibronectin. Cells were fixed, stained with phalloidin and kept in PBS. IRM images were acquired and (A) the number of adhesions per cell, (B) the average adhesion size and, (C) the average cell-substrate contact area values were calculated for > 30 cells. Data are represented as mean ± SEM. Data were analyzed using the Student’s T-test and is represented as * p<0.05, **p<0.01, ***p<0.001 in comparison to the neo cells. (D) Example of the image treatment done to determine focal adhesion number and size. Individual cells are imaged for (a) IRM and for phalloidin. IRM images are then put through a (b) band pass filter and (c) threshold to obtain the final image that can be used to determine the parameters shown in A, B and C.
6.7 hGAAP knockdown causes an elongated morphology, fewer and bigger focal adhesions

It was noticed that after hGAAP knockdown cells showed an elongated morphology (Figure 6.7 and 6.10A) and this phenotype was not observed when the control siRNA was used. In cells in which hGAAP was knocked down it was evident that the actin stress fibres were mostly positioned along the cell length and very few were actually facing the inside of the cell (Figure 6.10A). A possible explanation for this phenotype could be linked to the inability of the cells to make enough new focal adhesions to allow for cell spread and movement, or with the inability to disassemble focal adhesions at the cell rear. To test these possibilities the same strategy described in Figure 6.5 was used. Vinculin-GFP was used as a focal adhesion marker and the number of focal adhesions per cell was counted. An almost three-fold reduction in focal adhesion number per cell was observed in hGAAP siRNA (siRNA1 and siRNA2)-treated cells (Figure 6.10B). To confirm this result, IRM was used, and this technique also showed that the number of focal adhesions per cell was highly reduced after hGAAP knockdown when compared to cells transfected with the control siRNA (Figure 6.11A).

Interestingly, knockdown of hGAAP increased the size of individual focal adhesions (Figure 6.11B). Furthermore, when the levels of FAK phosphorylated at tyrosine 397 were measured there was a reduction of FAK phosphorylation upon hGAAP knockdown when compared to control siRNA (Figure 6.12A and B). This result supported the relevance of the reduction in the number of focal adhesions. (Figure 6.11A and B). The levels of FAK phosphorylated at tyrosine 397 were measured using both IF and immunoblotting (Figure 6.12A and B). Activation of FAK by integrin clustering leads to its autophosphorylation at Tyr397, which is a binding site for the Src family kinases (Calalb et al., 1995; Schaller et al., 1994) making this a good indicator for active focal adhesions. Talin was used in the IF experiment as a control, as it is also present in most focal adhesions because this is one of the first components of the focal adhesion structures to be recruited even before maturation into focal adhesions occurs (Campbell and Ginsberg, 2004).
Figure 6.10 - hGAAP knockdown reduces the number of vinculin-positive focal adhesions. hGAAP knockdown alters cell morphology. U2OS cells were seeded in fibronectin-coated coverslips and transfected with the indicated siRNA for 24 h. After this period cells were transfected with GFP-vinculin. Twelve hours later cells were fixed and stained with phalloidin. (A) Confocal microscopy images were acquired and (B) the number of focal adhesions (FAs) was calculated for > 20 cells. Data are represented as mean ± SEM. Data were analyzed using the Student’s T-test and is represented as ***p<0.001 for comparison to control siRNA transfected cells. Scale bars correspond to 10 µm.
Figure 6.11 - Analysis of the cell-substrate interface of hGAAP knockdown cells using interference reflectance microscopy. U2OS cells were transfected with the indicated siRNAs for 24 h. After this time the cells were detached and allowed to reattach for 12 h to glass-bottom dishes pre-coated with fibronectin. Cells were fixed, stained with phalloidin and kept in PBS. IRM images were acquired and (A) the number of adhesions per cell and (B) the average adhesion size values were calculated for >20 cells for each condition. Data are represented as mean ± SEM. Data were analyzed using the Student’s T-test and is represented as * p<0.05 and **p<0.01 compared to the control siRNA transfected cells.
Figure 6.12 - FAK activation (phosphorylation on Tyr 397) correlates with focal adhesion number per cell. U2OS cells in which hGAAP was knocked down by siRNA, were seeded on fibronectin-coated coverslips. (A) Cells were immunostained for pTyr^{397} FAK and for talin using specific Abs. Scale bars correspond to 5 µm. (B) Cells were also seeded for immunoblotting with anti-pTyr^{397} FAK Ab and anti-FAK Ab. Actin was used here a loading control. * Corresponds to the full length FAK band. The position of size markers in kDa is shown on the left of the gel.
6.8 hGAAP overexpression increases focal adhesion turnover and hGAAP knockdown reduces it

All the data obtained up to this stage pointed to a possible altered focal adhesion dynamics due to hGAAP overexpression or knockdown. To investigate this hypothesis, focal adhesion duration measurements were performed to determine if hGAAP was having any effect on focal adhesion turnover rate. To achieve this, GFP-vinculin was used as a marker for focal adhesion in live cell imaging recordings. Cells were allowed to spread in fibronectin-coated glass-bottom dishes for 30 min and after this time cells were imaged by confocal microscopy for 2 h and the duration of newly formed focal adhesions was determined. Focal adhesions in hGAAP overexpressing cells had a shorter duration compared to focal adhesions found in neo or hGAAP Ctmutil cell lines (Figure 6.13A and B). This is a reflection of an increased turnover rate of focal adhesions. In cells where hGAAP was knocked down the opposite effect was observed, and focal adhesions lasted for longer (Figure 6.14A and B). This means that the focal adhesion turnover was decreased in these cells. The differences observed in focal adhesion turnover rate could be due to either changes in disassembly or assembly rates or both.

Levels of endogenous vinculin in all cells used were determined by immunoblot to control for possible differences in vinculin levels that could influence the focal adhesion dynamics results. No differences in endogenous vinculin protein levels were observed for all the cells tested (Figure 6.13C and 6.14C).
Figure 6.13 - hGAAP overexpression reduces focal adhesion duration. (A) The indicated U2OS cell lines were transfected with GFP-vinculin for 18 h and reseeded in fibronectin-coated glass-bottom dishes for 30 min. After this period, an individual cell was imaged for 2 h for newly formed focal adhesions. Representative frames from time-lapse sequences used to quantify focal adhesion duration are shown. Arrows point to representative adhesions for each cell line. Scale bars correspond to 10 µm. (B) Data shown represent the mean duration of focal adhesions per cell ± SEM (n=6 cells, with 4-10 focal adhesion per cell). Data were analyzed using the Student’s T-test and is represented as *** p<0.001 for comparison to the neo cells. (C) Immunoblot showing endogenous levels of vinculin in the different cell lines used. The position of size markers in kDa is shown on the right of the gel.
**Figure 6.14 - hGAAP knockdown increases focal adhesions duration.** U2OS cells were transfected with the indicated siRNA for 20 h and with GFP-vinculin for another 16 h. After being seeded on fibronectin-coated glass-bottom dishes for 30 min an individual cell was imaged for 2 h for newly formed focal adhesions. (A) Representative frames from time-lapse sequences used to quantify focal adhesion duration are shown. Arrows point to representative adhesions for each cell line. Scale bars correspond to 10 µm. (B) Data shown represents the mean duration of focal adhesions per cell ± SEM (n=6 cells, with 4-10 focal adhesion per cell). Data were analyzed using the Student’s T-test and is represented as ** p<0.01 or *** p<0.001 for comparison to the control siRNA transfected cells. (C) Immunoblot showing endogenous levels of vinculin after treatment with different siRNAs. The position of size markers in kDa is shown on the right of the gel.
6.9 Calpain2-dependent talin cleavage following overexpression or knockdown of hGAAP

One of the key regulators of focal adhesion turnover is calpain2 (Guroff, 1964). This Ca^{2+}-activated protease controls focal adhesion disassembly and assembly by cleaving components of the focal adhesion such as talin, FAK, or paxillin (Franco et al., 2004a; Franco et al., 2004b). Calpain2 is highly dependent on Ca^{2+} for its activation and is regulated by intracellular Ca^{2+} levels among other factors. Given its important function in focal adhesion dynamics and its dependence on Ca^{2+}, it seemed to provide a possible link between hGAAP and cell adhesion.

The variety of sequences and factors affecting calpain2 proteolytic cleavage makes the establishment a consensus sequence for calpain2-specific cleavage difficult (Tompa et al., 2004). This fact has hampered the development of a reliable and specific assay to analyse calpain2 activity in vivo. Due to this fact, calpain2 dependent talin cleavage was analysed by immunoblot as an indirect measurement of calpain2 proteolytic activity in focal adhesions.

Using two different antibodies that recognize each of the two cleaved portions of talin, the N-terminal / head domain and C-terminal / rod domain (Figure 6.15A), the levels of cleaved talin were assessed in hGAAP overexpression or knockdown conditions. Cells overexpressing hGAAP, or in which hGAAP was knocked down by siRNA, were seeded for either 2 days or 30 min. Control cells were treated with ALLM, a specific calpain2 inhibitor (Franco et al., 2004b). Unfortunately, the calpain inhibitor ALLM was not very effective at inhibiting cleavage in these cells under the conditions used in this assay. However, in cells expressing hGAAP, but not in cells expressing hGAAP Ctmut, differences were seen in the levels of cleaved talin. There was a very modest increase in the levels of the talin cleaved rod portion 30 min after seeding (Figure 6.15B). There was also a more clear increase in the levels of the talin cleaved head after 30 min. No differences were seen in any of the 2 days post seeding samples as the levels of cleaved talin were very low.
Figure 6.15 - hGAAP overexpression has a modest effect on calpain2-dependent proteolytic cleavage of talin. (A) Schematic representation of talin and calpain2 cleavage site between talin amino acid 433 and 434 (Franco et al., 2004b). (B) U2OS cells expressing hGAAP or hGAAP Ctmut or (C) transfected for 36 h with hGAAP specific siRNAs were seeded in fibronectin-coated wells for 2 days or for 30 min. ALLM-treated cells were incubated with the peptide for 1 h at 50 µm, before being collected for immunoblot. Cell lysates were prepared and resolved by SDS-PAGE. Membranes were immunoblotted using an anti-talin-head (N-terminal) or anti-talin-rod domain (C-terminal) specific Abs. An immunoblot for actin was used as a loading control. * Indicates cleaved talin (head and rod) specific bands. The top bands in both talin immunoblots correspond to full-length talin. The position of size markers in kDa is shown on the left of each gel.
A more striking difference was observed when cells were transfected with hGAAP siRNA1. In this case the levels of cleaved talin were clearly reduced when compared to the control siRNA (Figure 6.15C). This was observed for both the head and the rod portions of the protein. An anti-actin Ab was used as a control for protein content of the samples and although a difference could be observed between the post 30 min and the post 2 days seeding samples, no difference can be detected within the post 30 min or within the post 2 days samples.

6.10 Summary

hGAAP overexpression led to an increased cell adhesion phenotype in U2OS and HeLa cells. This was analysed further in U2OS cells and showed that this was accompanied by an increased number of focal adhesion sites. These data were supported by the use of an hGAAP mutant in which the charged residues at the C terminus were mutated to alanines and which was shown to be unable to modulate the Ca\textsuperscript{2+} content of the intracellular stores or inhibit apoptosis. The focal adhesions from hGAAP overexpressing cells were smaller and had a higher turnover rate than the ones found in control cells. In contrast, cells in which hGAAP expression was knocked down by siRNA showed the opposite effects and in addition these cells showed a very elongated morphology. A plausible explanation for some of these observations is that calpain2 is less active in cells where hGAAP was knocked down and that this leads to reduction in focal adhesion turnover and adhesion. The reduced levels of calpain2-dependent talin cleavage observed upon hGAAP knockdown supported this hypothesis, although this requires further confirmation by testing other targets of calpain2 proteolysis and possibly by using one of the calpain2 specific activity assays that are currently being developed with some success.

These observations allowed us to propose a model whereby hGAAP overexpression would decrease the amount of Ca\textsuperscript{2+} stored in intracellular stores due to the release of Ca\textsuperscript{2+} from the stores and could also stimulate the entry of extracellular Ca\textsuperscript{2+} regulated by store-operate Ca\textsuperscript{2+} entry mechanisms, these were shown to play an important role in adhesion regulation (Varnai et al., 2009; Yang et al., 2009). Increased release of Ca\textsuperscript{2+} could increase the activity of calpain2, which requires Ca\textsuperscript{2+} for its
activity. With more active calpain2, the elements of the focal adhesions that depend on calpain2 for disassembly could be more easily cleaved. This would promote focal adhesion disassembly, which could account for the increased numbers and turnover rate of the focal adhesions found in hGAAP overexpressing cells. In turn hGAAP knockdown would result in reduced focal adhesion turnover and therefore resulting in reduced cell adhesion and spreading.

The cell migration results obtained using the WHA do not necessarily fit the increased focal adhesion turnover and the increased calpain2 activity caused by hGAAP overexpression. This is because the knockdown of calpain2 by siRNA is known to cause cells to lose motility and to increase adhesion. This result could be due to several factors such as the absence of fibronectin to drive focal adhesion formation and the fact that this assay only allows the measurement of unidirectional, and not multidirectional, migration. Further experiments looking at single cell migration in fibronectin-coated dishes or chemotaxis driven migration would be valuable and help determine if the results found using the WHA are or are not consistent with the proposed model.

The mechanism by which hGAAP is increasing cell adhesion remains incompletely defined and it might be more complex than expected due to Ca²⁺ central and sometimes apparently contradictory roles in many cell adhesion, migration and spread processes. Investigating not only the role of calpain2 in this phenotype but also exploring the possible effects in the actin cytoskeleton dynamics or the distribution and activity of small GTPases could lead to a better understanding of the mechanism behind hGAAP effects on cell adhesion and focal adhesion dynamics.
Chapter VII

Discussion and future work
The study of virus-host interactions and the mechanisms that the host and virus develop to counteract each other’s actions has led to important findings regarding cellular processes. Viruses can exploit the cellular machinery to their own purpose and at the same time they are forced to counteract the host cell responses. Different viruses use different strategies to avoid host defence mechanisms, which ultimately try to abort the initial infection, and thus prevent the completion of the virus life cycle and the spread of the virus to other cells.

The study of poxviruses has uncovered a large array of strategies used by these viruses to control the host immune response. This has not only revealed the importance of different cellular molecules and pathways in antiviral response mechanisms and contributed to the characterization and understanding of those pathways, but also allowed the identification and characterization of novel cellular proteins and processes. A good example came from the study of the CMLV genome that led to the identification of gene 6L (later known as vGAAP), that was also found to be present in some VACV strains, and was characterized as a Golgi anti-apoptotic protein (Gubser et al., 2007). A gene encoding a very similar protein was also identified in almost all eukaryotes analysed so far, including mammals, fish, amphibians, plants and yeast, showing that it is highly conserved and therefore likely to have some important cellular functions. The human equivalent of the viral GAAP protein called human GAAP was also shown to be a Golgi anti-apoptotic protein and to share the same broad range anti-apoptotic activity as both VACV strain Evans and CMLV vGAAP (Gubser et al., 2007). These proteins were included in the TMBIM protein family due to their six transmembrane domains, a charged C terminus and the high amino acid similarity with the members of this family, in particular with BI-1, the most studied member of the family (Reimers et al., 2008).

Cell death is one of the most basic and efficient methods that organisms have to control an infection in a multicellular host. This innate immune response leads to both containment of the pathogen in the apoptotic cell, by preventing the completion of the pathogen life cycle and so stopping the production of infectious pathogens and their release, and by generating stress signals on the cell surface that can be recognised by the immune system cells that will remove the infected cell. Although the role of vGAAP in preventing cell death during poxvirus infection is not formally proven due to the presence of several other viral genes involved in preventing apoptosis during poxvirus
infection, work is being currently done in our lab to address this question using the VACV Copenhagen strain 811 that contains large deletions in its genome resulting in loss of the anti-apoptotic activity of the virus (Benjamin Johnson, unpublished data) (Perkus et al., 1991). Using this approach it will be possible to test if introduction of vGAAP will have an anti-apoptotic effect during infection.

The fact that GAAPs are highly hydrophobic membrane Golgi proteins and have a broad spectrum of anti-apoptotic activity suggested a possible link with Ca^{2+} because this cellular secondary messenger has been show to play an important role in apoptosis. This hypothesis was shown to be correct by overexpression and knockdown of hGAAP (de Mattia et al., 2009) and vGAAP overexpression (Figure 4.13). Using this approach it was shown that hGAAP overexpression could reduce the Ca^{2+} content of both the Golgi and the ER Ca^{2+} stores, reducing the amount of Ca^{2+} released upon an apoptotic stimuli. These data suggested that the mechanism behind GAAP anti-apoptotic activity was related to its ability to modulate Ca^{2+}. It is important to note that this link has not been formally proven and it is possible that these are two independent functions of the protein.

7.1 Purification of TMBIM family proteins

Some of the challenges associated with membrane protein expression, purification and crystallization have been overcome in these last decades. The development of new and improved methods for the production and purification of recombinant mammalian membrane proteins allowed great progress in the determination of membrane protein 3D structures (Carpenter et al., 2008; Wagner et al., 2006). In an attempt to understand GAAP function and given that no structure is still available for other protein from the TMBIM family that could be used as the base to model GAAP structure, we attempted to express and purify GAAP with protein crystallization and structure determination as the long-term goal. The production of purified protein would also allow biochemical characterization, such as oligomeric state determination, and the use of functional in vitro assays to further our knowledge on GAAP function.
Using a panel of different TMBIM alleles, AtGAAP3, CMLV vGAAP, hGAAP, hGAAP T88I and BI-1, it was possible to produce and purify CMLV vGAAP, AtGAAP3 and BI-1 using a yeast-based method to produce recombinant proteins with C-terminal GFP-8His tags (Drew et al., 2006). After the production, subcellular localization (membrane targeting), protein size, detergent solubility and monodispersity after detergent solubilisation were assessed it was decided that vGAAP, AtGAAP3 and BI-1 were good targets to attempt protein purification. The use of GFP fluorescence to monitor protein levels, subcellular localization, detergent solubility efficiency, monodispersity and binding to nickel resin efficiency allowed the step-by-step optimization of the production and purification protocol. These optimizations included testing of different additives to the yeast cultures in an attempt to improve protein expression, different detergents used for protein solubilisation from membrane extracts, and different protein binding to nickel resins methods to improve binding efficiency and protein recovery.

Using the final optimized protocol it was possible to produce and purify CMLV vGAAP using LDAO in sufficient amounts and purity to attempt crystallization trials using a protein concentration of 10 mg/ml. However none of the tested MemGold™ ICL6 and ICL9 crystallization buffer conditions (Molecular Dimensions under license from Imperial College of Science Technology & Medicine, London, (Newstead et al., 2008)) at 4 °C or 20 °C allowed the development of any visible protein crystals. Although these preliminary results were discouraging, many more conditions could be tested to achieve the production of protein crystals, including the testing of different protein solubilising detergents to reduce protein aggregation and increase protein stability, different protein concentrations, and other crystallization buffer conditions. Although our initial favourite target for protein crystallization and 3D structure determination was vGAAP, the purification of AtGAAP3 revealed that this protein was more stable, as shown by stability assays performed at 4 °C and 20 °C (Figure 3.8), and purer when compared with vGAAP, making this a much more attractive target for protein crystallization attempts.

As stated above, further optimizations on the protein purification protocol and the crystallization conditions, including the screening of more detergents and buffer conditions, are probably required to obtain higher amounts of more stable, less aggregated and more highly purified protein samples. Other approaches to allow
protein crystallization of TMBIM members could include the testing of other TMBIM alleles including the related yeast protein Ybh3p (Buttner et al., 2011). It was clear from our small screen that the only plant GAAP tested, AtGAAP3, was a lot more stable and easy to purify when compared to viral or mammalian proteins, suggesting that other plant GAAPs could be tested. Given the high degree of conservation of these proteins, it is likely that an elementary function (such as ion channels or transporters) and structure are conserved within the family (Hu et al., 2009), making this a possible good structure upon which vGAAP, hGAAP and other TMBIM members could be modelled.

In addition to structural studies the purified proteins proved to be useful reagents for biochemical and functional characterization. Size exclusion chromatography and non-reducing SDS-PAGE analysis of purified vGAAP and AtGAAP3 provided some hints to its oligomeric state. Several protein bands could be detected in a SDS-PAGE analysis, that in the case of vGAAP were all positive in an immunoblot analysis using an anti-GAAP Ab (Figure 3.5D); and several peaks were observed when the purified proteins were subjected to SEC analysis. Based on all the in vitro data collected for purified vGAAP and AtGAAP3 it was hypothesised that these proteins were oligomers (possibly including tetramers, see bellow). Using transient or stable expression of vGAAP and hGAAP in mammalian cells oligomers could also be detected (discussed in more detail in 7.3). By immunoblotting, the anti-GAAP Ab detected the monomer signal much better than any of the other oligomeric forms, although the levels detected with a Coomassie stain were not so strikingly different. This may be because the epitope recognised by the Ab (N-terminal region) might be less accessible due to oligomerization, given that the oligomerization domain was mapped to this region. Further biochemical characterization of GAAPs might arise from the use of techniques such as circular dichroism (Kelly et al., 2005) or SEC coupled to multi angle light scattering (MALS) that could provide important information regarding alpha-helix content or oligomeric state of these proteins, respectively. Both techniques were attempted using the purified protein samples, but with inconsistent results. Further optimizations of the purification protocol would generate more stable and pure protein, which could then be subjected to such tests and provide important biochemical information.

The most important finding regarding GAAP function was obtained by the use of purified vGAAP and AtGAAP3 in artificial lipid bilayer patch-clamp experiments. It was
shown that both proteins allowed passage of ions when reconstituted in an artificial lipid bilayer. This property was also observed for BI-1 confirming recently published data that suggests that BI-1 has ion exchanger (Ahn et al., 2009; Ahn et al., 2010; Yun et al., 2011) or ion channel (Bultynck et al., 2011) activity and is further discussed in 7.2.

### 7.2 GAAP is an ion channel

Previous data from our lab suggested a possible link between the Ca\(^{2+}\) modulation function of hGAAP and the IP\(_3\)R. The hypothesis that hGAAP Ca\(^{2+}\) modulation was occurring through IP\(_3\)R regulation was supported by both co-IP found between hGAAP and IP\(_3\)R and also by the fact that hGAAP could modulate IP\(_3\)-dependent Ca\(^{2+}\) release (de Mattia et al., 2009). Studies with other proteins involved in apoptosis regulation such as Bcl-2 (Rong et al., 2008) and Cyt c (Boehning et al., 2003) suggested that IP\(_3\)R Ca\(^{2+}\) release modulation by apoptosis regulatory proteins was indeed a recurrent phenomenon, making it an even more attractive hypothesis.

However, a thorough investigation of the IP\(_3\)R and vGAAP interaction showed that this observation was non-specific and therefore most likely due to an artefact of the immunoprecipitation technique used. Notably, no specific interaction was detected when the region of IP\(_3\)R involved in binding vGAAP was attempted to be mapped. This strengthened the alternative theory that GAAP could allow Ca\(^{2+}\) to flow out of the Golgi by functioning itself as an ion channel or as an exchanger. Using purified CMLV vGAAP, AtGAAP3 and BI-1 from yeast cultures this second hypothesis was tested and data obtained (in collaboration with Prof. Colin Taylor laboratory, University of Cambridge) showed that all these proteins, but not a control protein (Adenosine A2 receptor) purified from yeast by a very similar process, showed ion channel-like activity in artificial lipid bilayers when subjected to different voltages that created an artificial membrane potential (Figure 4.5). It is important to highlight that although GAAP induced openings detected in these experiments that correspond to an ion channel-like activity, these observations are also compatible with activity from a constitutively open ion exchanger, because this would behave as an ion channel in these conditions. The biggest caveat of this experiment is that if GAAPs co-purify with another yeast protein
that has ion channel activity we could be detecting that activity and not GAAP specific ion channel activity. This was improbable given that Adenosine A2 receptor that was expressed in yeast and purified in a very similar manner did not give ion channel activity. In addition, preliminary data obtained by Guia Carrara showed that the single channel opening amplitudes of CMLV vGAAP, AtGAAP3 and BI-1 at a given membrane potential applied to the bilayer are not the same. This suggests that the conductivity of the protein responsible for the openings in the three purified proteins samples is different, making the presence of a common contaminant that is responsible for the openings even less likely.

To further support data obtained with the artificial bilayer recordings, site directed mutagenesis of amino acid residues found to align with other known ion channels or exchangers and to be conserved within the TMBIM4 family was carried out so that a mutant with altered conductivity (altered current-voltage regression slope) could be found and so the ion channel-like activity could be directly linked to GAAP. So far all the mutants have been generated and tested for biochemical and subcellular properties. The function of these mutant vGAAPs was tested using the two functional assays available for GAAP: Ca\textsuperscript{2+} storage content release and anti-apoptotic activity. This allowed us to narrow down the number of mutants to purify and eventually test for ion channel activity in artificial lipid bilayers. Using this approach and taking in consideration the protein amino acid alignments with other ion channels and exchangers performed, a small number of mutants were selected and are being currently purified and tested for ion channel activity by Guia Carrara. This approach can also bring important information on both ion gating and ion selectivity because it can contribute to the identification of the key residues involved in the pore and filter formation.

One question still unanswered about GAAP ion channel activity is its gating mechanism. It was proposed for BI-1 that it could be a leaky channel and function as a “safety valve” for Ca\textsuperscript{2+} content of the ER, therefore it is possible to speculate that GAAP might have the same function in the Golgi. This hypothesis could explain the effects observed in intracellular store Ca\textsuperscript{2+} content upon hGAAP and vGAAP overexpression and upon hGAAP knockdown (de Mattia et al., 2009).
Given that vGAAP and hGAAP can modulate Ca$^{2+}$, the presence of a highly conserved charged acidic sequence motif (4x COO\(^{-}\)) closer to the C terminus of all GAAPs tested (Figure 4.8), that could fulfil the needs of a Ca$^{2+}$ filter, the presence of Ca$^{2+}$ in the artificial bilayer experiment buffer, and the recent finding that a peptide corresponding to the BI-1 C terminus is capable of allowing Ca$^{2+}$ to flow across a lipid bilayer (Bultynck et al., 2011), we can speculate that GAAP might allow the flow of Ca$^{2+}$ from the Golgi. This hypothesis is further supported by the finding that a highly conserved amino acid residue present in both BI-1 and GAAP (vGAAP D219) was shown to be absolutely essential for BI-1 Ca$^{2+}$ channel activity (Bultynck et al., 2011). The ion channel selectivity could be investigated by different techniques using pure protein reconstituted in either lipid bilayers and using different buffers with different ions present or by using GUVs loaded with a Ca$^{2+}$ indicative dye. This would be very important to allow a more complete understanding of GAAP function.

We can also speculate on the conservation throughout evolution of GAAP and BI-1 ion channel activity, because while a plant GAAP did show this same activity, a C-terminal peptide of a plant BI-1 was not able to do so (Bultynck et al., 2011). Recently the TMBIM4 subfamily was proposed as the ancestral gene for some of the TMBIM family genes (Hu et al., 2009). It would be very interesting to investigate the level of conservation of GAAP ion channel-like activity not only within the TMBIM4 genes but also within the whole TMBIM family.

### 7.3 GAAP is able to form tetramers

Most proteins capable of selective ion transport across the membrane (mostly ion channels and ion transporters) show either oligomerization or homologous repeat domains (Kew and Davies, 2010). In a very crude and simplistic analysis we can say that most ion channels tend to form tetrameric structures while ion exchangers can assemble into different oligomeric structures that can improve but are not absolutely required for function in some cases (Gadsby, 2009; Kew and Davies, 2010). The findings that both hGAAP and vGAAP can oligomerize, and possibly form dimeric or tetrameric structures are consistent with the findings that GAAP is an ion channel or an ion exchanger. The most abundant oligomeric form of GAAP found in SDS-PAGE
analysis, even when the protein is crosslinked, is the dimer. Although tetrameric GAAP is also found, it represents only a small fraction of the total protein and the physiological relevance of each of GAAP’s oligomeric forms is still not fully understood. However, this may be a consequence of limitations in the SDS-PAGE technique.

The observation that the levels of oligomeric GAAP (dimers, trimers and tetramers) increased at higher pH suggest a possible regulation mechanism equivalent to that proposed for human BI-1 (Kim et al., 2008) and for other ion exchangers such as the Helicobacter pylori HPNhaA (Taglicht et al., 1991), in which an increase in pH would allow GAAP to oligomerize and form a functional ion channel or a more efficient ion exchanger. So far no other regulation mechanism has been proposed for GAAP. Moreover purified vGAAP and AtGAAP3 were shown to be constitutively active when an artificial membrane potential was applied in artificial lipid bilayer ion channel activity experiments because neither required any agonist to allow the channel to open. The measurement of ion channel activity, the Ca$^{2+}$ content of intracellular stores and anti-apoptotic activity of monomeric vGAAP (with both cysteine 9 and 60 mutated to serines) would show if GAAP dimerization/oligomerization is required for its known functions and activities. These two cysteine mutations combined render vGAAP unable to form dimers or other higher oligomeric forms in all the techniques used to assess dimerization and oligomerization, so if GAAP function is highly dependent on oligomerization it should be greatly affected by the loss of oligomerization ability. These data could also provide valuable evidence to determine if GAAP is an ion channel or an ion exchanger because a monomeric protein is unlikely to be an ion channel while a monomeric ion exchanger might be just less efficient in conducting ions across the membrane than the natural oligomer.

vGAAP, but not hGAAP dimerization was shown to be dependent on disulphide bounds which confirmed that the N-terminal region in vGAAP is important for oligomerization. We can speculate that the different oligomerization mechanisms found in hGAAP and vGAAP could be related to a functional regulation because a more stable vGAAP dimer could mean that the protein is more likely to be active, even in the context of viral infection when numerous cellular processes are altered.

The fact that tetramers are visible when the protein is crosslinked and analysed by non-reducing SDS-PAGE suggests that this could be GAAPs physiological
oligomeric state, but further evidence is required to confirm this hypothesis because this oligomeric form represents only a small fraction of the total protein when analysed by non-reducing SDS-PAGE. We are currently using single molecule photobleaching of GFP-tagged GAAP to assess the protein stoichiometry more accurately. This technique was used successfully to solve the stoichiometry of other channels such as the CRAC channel (Penna et al., 2008).

The presence of trimers when the protein is crosslinked does not support the dimer-of-dimers mechanism for tetramerization that is common in ion channels (Demuro et al., 2011; Tichelaar et al., 2004; Tu and Deutsch, 1999). However, the presence of putative trimeric GAAP in non-reducing SDS-PAGE analysis could be due to an artefact of the technique; further experiments, such as the single molecule photobleaching of GFP-tagged GAAP, are required to confirm and explore the physiological relevance of this GAAP form.

**7.4 hGAAP increases cell adhesion**

Simple measurements of the ability of cells either overexpressing or knocked down for hGAAP to adhere to a solid support, to remain attached during EDTA treatment, and to spread showed that hGAAP overexpression led to an increased cell ability to adhere to the substrate. Conversely, hGAAP knockdown reduced cell adherence. Increased adherence was associated with a reduced size and increased number of focal adhesions when hGAAP was overexpressed, and the converse following hGAAP knockdown. These observations were further supported and explained by the fact that hGAAP overexpression lead to an increase in focal adhesion sites turnover rate while hGAAP knockdown reduced turnover rate and decreased the levels of FAK activation. Collectively these data suggest a hypothesis in which hGAAP overexpression drives focal adhesion disassembly. The observation that hGAAP overexpression increased the cell migration rate in a wound healing assay are difficult to explain in this model. This result needs further confirmation with other cell migration assays where one can analyse single cell random migration velocity because a wound healing assay can only measure unidirectional migration of the cells. Random migration
assays and chemotaxis assays will be required to determine accurately the ability of the cells to migrate and to respond to directional cues, respectively.

Considering the role of hGAAP in Ca\(^{2+}\) modulation, and in light of the results that suggest that GAAPs are ion channels, two possible consequences for Ca\(^{2+}\) can be suggested. Because there is less Ca\(^{2+}\) in the intracellular stores of a cell overexpressing GAAP, as shown by de Mattia et al. (2009), it is conceivable that there could be less Ca\(^{2+}\) available to be released to activate effectors in the cytoplasm upon a stimulus. Alternatively, the reduced levels of Ca\(^{2+}\) in the intracellular stores would lead to the activation of store operated Ca\(^{2+}\) entry, that might lead to a localized increase of Ca\(^{2+}\) concentration near the plasma membrane from the extracellular medium. Interestingly, it was shown that store operated Ca\(^{2+}\) entry plays an important role in breast tumour cell migration and metastasis (Yang et al., 2009).

Given hGAAP’s role in Ca\(^{2+}\) modulation (de Mattia et al., 2009) and the effects observed in focal adhesion turnover upon hGAAP overexpression and knockdown, we began to investigate calpain2 as a possible link between Ca\(^{2+}\) modulation and cell adhesion. Calpain2 is dependent on Ca\(^{2+}\) for its activation and its proteolytic activity is required for focal adhesion disassembly (Franco and Huttenlocher, 2005). To investigate if hGAAP was affecting calpain2 activity the levels of cleaved talin following hGAAP overexpression and knockdown were measured as a proxy for calpain2 activity at the focal adhesion complexes. Talin is the best described and characterized calpain2 substrate and the functional consequences of this proteolytic cleavage are essential for adhesion site disassembly and the promotion of adhesion site turnover (Franco et al., 2004b). Initial attempts to determine the cleavage levels of this protein suggest a possible role for hGAAP in activating calpain2 activity because when hGAAP was knocked down the levels of cleaved talin were reduced, while when hGAAP was overexpressed slightly higher levels of cleaved talin were detected. More proteolytic targets of calpain2 need investigation and specific calpain2 activity assays need to be explored, but these preliminary results point to a possible link between hGAAP and calpain2 activation as shown in Figure 7.1.
Figure 7.1 – Factors affecting adhesion assembly and disassembly in a migrating cell – hypothetical model for the role of hGAAP in cell adhesion. At the leading edge new adhesions are formed and matured, and this is modulated mainly by integrin binding to the ECM proteins and secondarily by small Rho GTPases. At the rear of the cell the mature adhesions are disassembled to allow the cell to migrate forward. This process is regulated by a series of cellular factors including calpain2 cleavage of adhesion components such as talin or FAK. We hypothesise that hGAAP might be involved in the activation of calpain2 leading to an increase in adhesion turnover by a mechanism still unknown. Adapted from Frame et al. (2002).

Although calpain2 seems an attractive link between modulation of Ca$^{2+}$ and cell adhesion by hGAAP, other routes are possible. Many links between adhesion/migration and Ca$^{2+}$ have been established and were introduced in some detail in section 1.6.3 of this thesis. To explore the mechanism by which hGAAP regulates cell adhesion thoroughly some of these links must be investigated. hGAAP, neo and hGAAP Cmut U2OS cell lines have been engineered to express Lifeact-GFP, a GFP-tagged peptide that binds and acts as a marker of F-actin in live cells without affecting its assembly and disassembly dynamics (Riedl et al., 2008), to explore GAAPs possible impact on actin dynamics. Another group of important molecules that regulate the focal adhesion turnover process are the small GTPases and these are also regulated directly and
indirectly by \( \text{Ca}^{2+} \) (introduced in section 1.6.3.2) and are responsible for establishing a link between mechanosensitive ion channels at the plasma membrane, integrin activation, ER \( \text{Ca}^{2+} \) release, calpain activation and adhesion (McHugh et al., 2010).

Given the role of hGAAP on adhesion and previously published data that hGAAP mRNA levels are upregulated in some human cancers (van 't Veer et al., 2002), it is possible that hGAAP might play a role in metastatic cells, which show an increased adhesion to the extracellular matrix (Klein, 2008). Moreover another member of the TMBIM family, BI-1, can enhance cancer metastasis in a mouse model (Lee et al., 2010b) and cell adhesion (Lee et al., 2010a). Cell adhesion control represents a very important and attractive target for the development of drugs that aim to control cancer spread and metastasis formation (Ko et al., 2010; Li and Feng, 2011). In this context, a more detailed knowledge of hGAAP role in cancer and metastasis could provide a new putative target for drug development.

## 7.5 Open questions on GAAP

Many aspects of GAAP function and its relevance for both cellular and viral processes are still not fully understood. The degree of amino acid sequence conservation found in GAAPs from all eukaryotes identified so far points to a possible highly conserved and essential function for these proteins. The fact that hGAAP was shown to be an essential protein without which cells die (Gubser et al., 2007) further supports this theory. Also it was found in our lab that some prokaryotic proteins shared some amino acid similarity with hGAAP (Benjamin Johnson, unpublished data) adding to the growing body of data showing that GAAPs are indeed very ancient and important proteins.

There are still many unanswered questions about the role of GAAPs in cellular and viral processes but the most important task at this stage is to determine if the ion channel activity of GAAP is related to its ability to modulate the \( \text{Ca}^{2+} \) content of intracellular stores, and if this function is directly linked to cell protection from apoptotic stimuli by GAAP. Other secondary questions on virus-induced cell motility or in cancer development are still unanswered and will be crucial to fully understand the role of both cellular and viral GAAPs.
7.6 Summary

This study has increased knowledge of the GAAPs (TMBIM4) function and biochemical characteristics. Using yeast to express and produce several GAAPs enough purified protein was produced for basic biochemical characterization and for functional assays. The finding that vGAAP and AtGAAP3 can show ion channel-like activity in artificial lipid bilayers suggests that this might be a conserved function among the GAAP family. Furthermore characterization of the oligomeric state of GAAP suggests the formation of tetramers that is consistent with ion channel-like activity for GAAP. vGAAP, but not hGAAP, oligomerization is dependent on cysteine residues located at the N terminus of the protein. hGAAP was also shown to have a role in cell adhesion, although the mechanism by which hGAAP leads to an increased cell adhesion and increased adhesion turnover rate is still unclear and requires further investigation.

To understand more fully how both vGAAP and hGAAP function, and their relevance for viral and cellular processes, the possible links between anti-apoptotic, Ca^{2+} modulation, ion channel-like activity and cell adhesion, need to be explored to understand if these functions are interdependent or not (Figure 7.2). The high degree of conservation of this protein family throughout evolution and the various cellular processes modulated by hGAAP and vGAAP make these valuable tools to explore new possible links between apoptosis, Ca^{2+} modulation and cell adhesion.
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