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Nuclear proteasomes carry a constitutive posttranslational modification which derails SDS-PAGE (but not CTAB-PAGE)

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Abstract:

We report that subunits of human nuclear proteasomes carry a previously unrecognised, constitutive posttranslational modification. Subunits with this modification are not visualised by SDS-PAGE, which is used in almost all denaturing protein gel electrophoresis. In contrast, CTAB-PAGE readily visualises such modified subunits. Thus, under most experimental conditions, with the identical samples, SDS-PAGE yielded gel electrophoresis patterns for subunits of nuclear proteasomes which were misleading and strikingly different from those obtained with CTAB-PAGE. Initial analysis indicates a novel modification of high negative charge with some similarity to polyADP-ribose, possibly explaining compatibility with (positively-charged) CTAB-PAGE but not (negatively-charged) SDS-PAGE and providing a mechanism for how nuclear proteasomes may interact with chromatin, DNA and other nuclear components.

Keywords:

CTAB-PAGE, proteasome, ADP-ribose, ubiquitin, nuclear biology, apoptosis

1.1 Introduction

Most denaturing protein gel electrophoresis is done using SDS-PAGE, discontinuous Sodium Dodecyl Sulphate PolyAcrylamide Gel Electrophoresis,[1] due to its ease-of-use and reliability[2]. Although some posttranslational modifications may affect the migration behaviour of proteins on SDS-PAGE, e.g. N-linked glycosylation, SDS-PAGE is believed to, and generally does, faithfully represent the composition of complex protein mixtures[2]. However, we now report a novel modification of nuclear proteasomes that prevents SDS-PAGE from visualising the proteasomal subunits. By way of further background, proteasomes[3-4] are 2.5 MDa protease complexes, located in the cytoplasm and nucleus, which degrade ubiquitin-marked protein substrates. There is an alternative to SDS-PAGE, the much less used CTAB(Cetyl Trimethyl Ammonium Bromide)-PAGE system[5-6]. We report that, unlike SDS-PAGE, CTAB-PAGE *does* visualise these modified subunits of nuclear proteasomes. Thus, for nuclear proteins, combined use of SDS-PAGE and CTAB-PAGE may be required to capture sample complexity. Initial analysis into the nature of the posttranslational modification indicates that the modification shares several characteristics with, but also is distinct from, classical ADP-ribose chains.

2.1 Materials and Methods

2.1.1 Antibodies, Chemicals, Enzymes, Western Blotting, Tissue Culture. List of commercial antibodies: Enzo Lifesciences: α -Rpn10/S5a (MoAb S5a-18, BML-PW9250), α -Rpn12/S14 (PAb, BML-PW8815), α -20S α 7 (MoAb LN43, BML-PW8110), α -20S α 6 (MoAb MCP20, BML-PW8100), α -Rpt1/S7 (MoAb MSS1-104, BML-PW8825), α -Rpt2/S4 (PAb, BML-PW8305), α -Rpt3/S6b (PAb, BML-PW8175), α -Rpt4/S10b (MoAb p42-23, PW8830), α -Rpt5/S6a (MoAb TBP1-19, PW8770), α -‘core’ CP subunits (PAb, PW8155), α - β 5i/LMP7 (PAb, PW8355), α -ubiquitin (FK2, PW8810), α -poly(ADP-ribose) (PAb 96-10-04, ALX-210-890-R100). α -Rpn1/S2: Calbiochem (539166). α -Lamin B2: Abcam (MoAb, ab8983). α -SMARCB1/Ini1: Santa Cruz (PAb, sc-13055). α -Ubiquitin1: Invitrogen (MoAb, 377700).

Bortezomib: Millennium/Takeda. Epoxomicin, Ada-K(Biot)-Ahx3-L3-VS: Enzo Lifesciences. Doxorubicin, vinblastin, cycloheximide, epigallocatechin gallate (EGCG): Sigma-Aldrich. Micrococcal nuclease, RNaseA/T1, RNaseH and RNase1: Fermentas/Thermo. Antibodies against yeast proteasome: Dr Daniel Finley, Harvard Medical School. α -Dbf11 antibody: Dr Christian Speck, CSC-MRC London. We generated β -glucanase (*O. xanthineolytica*) using an *E. coli* strain gifted by Dr Randy Schekman, UC Berkeley. PDE1 (from *Crotalus Adamanteus* venom): Sigma-Aldrich. PARG, ARH3 and ARH1: Enzo Life Sciences (ALX-202-045-UC01, ALX-201-290-C010, ALX-201-293-C010). We cloned the two variants of ARH2 into pET23a(+) using a HeLa cDNA library (Gibco). PAR enzyme incubations were overnight at 37°C. Extra Ca^{++} or Mg^{++} was supplied where necessary. Caspase Inhibitor Set III: Enzo, ALX-850-227-KI01, 1:500 dil.=4 μM . Antibodies were diluted in PBS+0.5% Tween20 for Western blotting, PVDF membranes were re-probed multiple times [7]. NCI-H929, KMS12-BM, RPMI-8226, OPM2, DG75 and Jurkat cells were grown in RPMI (Sigma), supplemented with 10% v/v FBS, and 1% v/v Pen/strep. SAOS2 and HEK293T cells were grown in DMEM (Sigma) with 10% v/v FBS, and 1% v/v Pen/strep.

2.1.2 CTAB-PAGE, and isoelectric focusing. CTAB-PAGE as published [6]. In short, 6% resolving gel (2.4 ml 40% acrylamide, 4 ml 1.5 M Tricine-NaOH [pH 8.0], 9.4 ml water, 160 µl 10% APS, 16 µl TEMED) was used to pour the separating gel. For the stacking gel, 30 mg agarose was mixed with 1.25 ml 0.5 M Tricine-NaOH [pH 10], 3.7 ml water, and 50 µl 10% CTAB solution (this solution has limited shelf life; discard and re-make after one month). The CTAB-PAGE gel was run in CTAB running buffer (25 mM Tricine, 13.75 mM CTAB, 75 mM arginine; using 5x stock). Protein samples were mixed with equal amount of 2×CTAB sample buffer (10 mM Tricine-NaOH [pH 8.8], 1% w/v CTAB, 10% v/v glycerol, 10 µl/ml saturated aqueous solution of crystal violet, 2% v/v β-mercaptoethanol). Samples were heated up at 94°C for 3-4 min, and 20 µl of sample were loaded in the wells. CTAB gels were run at 100 Volts, with leads reversed. After clearing the stacking gel, voltage was increased to 150V. Proteins were transferred to PVDF membrane using a semi-dry transfer system (Biorad: transfer time 20 minutes; set at 15 V, with a limit of 400 mA). Isoelectric focusing was done with the IPG-ZOOM system (Invitrogen), using a linear pH3-10 (ZM0018) or pH4-7 (ZM0012) gradient immobilized on strip according to manual's protocol, and followed by a second-dimension SDS-PAGE step.

2.1.3 Cell fractionation: cytosolic and nuclear protein extraction. Cytosolic and nuclear protein extractions were prepared from approximately 2×10^6 NCI-H929 or KMS12-BM Myeloma cells, harvested by centrifugation at 1,500 rpm for 5 min. All steps below were carried out on ice or with pre-chilled reagents and equipments. Cell pellets were washed once with 1 ml PBS and harvested by centrifugation at 5000xg for 5 min, 4°C. The pellet was resuspended in 110 µl cytosol (C) extraction buffer (25 mM Tris-HCl [pH 7.8], 5 mM MgCl₂/EDTA, 1 mM ATP/ADP, 2 mM DTT, 150 mM NaCl, 0.1% NP-40/IGEPAL) by pipetting up and down 20 times, and then spun down at 5000xg for 5 min at 4°C. 100 µl of the supernatant (the cytosolic extract: C) was transferred to new Eppendorf tubes and mixed with equal amount of 2×SDS/CTAB sample buffer. The remaining liquid was discarded. Next, the pellets were re-suspended in 110 µl nuclear extract (NE) buffer[8] (20 mM HEPES [pH 7.4], 420 mM NaCl, 0.5 mM EDTA, 0.5 mM EGTA, 1 mM DTT, 1 tablet of inhibitor cocktail (Roche) per 50 ml) by pipetting up and down 20 times, and then left on ice for 20 min. After vortexing 10 seconds, the mixtures were spun down at 10,000xg for 20 min at 4 °C. 100 µl of the supernatants (the nuclear extracts: NE) were transferred to new 1.5-ml microcentrifuge tubes and mixed with equal amount of 2×SDS/CTAB sample buffer. The remainder of the supernatant was discarded. Next, 110 µl of nuclear extract buffer was added to the pellets (the nuclear pellet fraction: NP), and mixed with equal amount of 2×SDS/CTAB sample buffer. When using the NE for enzyme treatment, 2 volumes of H₂O were added to 1 volume NE to reduce the NaCl concentration to physiological levels.

2.1.4 Purification of yeast proteasomes, and of nuclear proteasomes from primary human leukocytes or OPM2 myeloma cells. Yeast proteasomes were affinity-purified using strain sMK-50 as published [9]. C-terminally His₆-tagged ubiquitin-like (Ubl) domain of hPLIC2 was used to capture human proteasomes (construct #4615, [10]), except for Figures 2A, 2B, S2 and S3 where GST-Ubl (construct #4602; [11]) and GST-Uba (construct #4607; [11]) were used. Bacterial expression and affinity-purification (Sigma, His Select HF Nickel Affinity Gel, H0537) was described previously [10]. Ubl-His₆ protein from one litre of bacterial culture was loaded onto 600 µl of resin. Human leukocytes from anonymised donor leukaphoresis collections (released for research with donor consent) were thawed, washed twice in PBS and centrifuged at low speed at 4°C to obtain a dry cell pellet which was resuspended in 6 ml of cytosol extraction buffer (25 mM Tris-HCl [7.6], 5

mM EDTA, 1 mM ADP, 2 mM DTT, 150 mM NaCl, 0.1% v/v NP40/IGEPAL) by pipetting up/down several times, and spun down again at 3,000 rpm for 7 minutes at 4°C in a Beckman J-M6 JA4.2 rotor). Next, supernatant was removed, and cells were resuspended in 5 ml of nuclear extract buffer (see section above) by carefully pipetting several times using a 25-ml volume pipette. The suspension was incubated on ice for 20 minutes, then spun down for 20 minutes at 3000 rpm at 4°C (Beckman J-M6 JA4.2 rotor). Supernatant, i.e. nuclear extract, was recovered and diluted with 2 volumes of ddH₂O. The 600 µl of Ubl-His₆-loaded Ni⁺⁺NTA resin was added to the diluted nuclear extract, and rotated at 4°C for 1 hr. Next, the beads were washed several times in 25 mM Tris-HCl [7.6], 1 mM ADP, 2 mM DTT, 100 mM NaCl, 0.1% NP40/IGEPAL, 1 mM EDTA. Ubl-His₆ proteins carrying captured proteasomes were eluted by incubation with a 75% wash buffer/25% 1M imidazole solution. Eluate was concentrated in YM-50 Centricon, and applied to a Superose6 PC-3.2/30 column. The FPLC running buffer was 25 mM Tris-HCl [7.6], 2 mM DTT, 150 mM NaCl, 0.1% NP40/IGEPAL, 5 mM EDTA, 0.1 mM ATP. 100 µl fractions were collected, and analysed by both CTAB-PAGE and SDS-PAGE. For Figures 1F, 2A, 2B, S2 and S3, the cytosolic extract was also used for further proteasome purification steps. For Figures 2A, 2B, S2 and S3, myeloma OPM2 cells were used.

3.1 Results and Discussion

When comparing the *same* human cell lysate sample side-by-side on SDS-PAGE and CTAB-PAGE, we noticed unexpected differences in the resulting gels. In more detail: Western Blot analysis with a panel of proteasome antibodies showed the expected distinct separate band or bands on SDS-PAGE. Unexpectedly, only CTAB-PAGE showed the presence of multiple additional high-molecular weight species for individual proteasome subunits (Figure1A). CTAB-PAGE is not inherently prone to smearing: (a) CTAB-PAGE performed equally well as SDS-PAGE in separating a mixture of defined purified proteins (FigureS1), in line with published reports[5-6]. (b) Our standard conditions with β-mercaptoethanol and sample boiling completely denatured multimeric protein complexes β-amylase and alcohol dehydrogenase into their monomeric subunits with both CTAB-PAGE and SDS-PAGE (FigureS1). (c) Not all proteins we looked at migrated on CTAB-PAGE as multiple species, e.g. not LaminB2, Ini1, or Rpt3, Figures 1A, 1D. (d) CTAB-PAGE-unique bands were not the result of combining CTAB-PAGE with any particular research reagent: we gathered and tested a large number of antibodies against different proteasome subunits, and observed CTAB-PAGE-unique bands for most of these (Figure1D, 1E).

We predicted that, if CTAB-PAGE-unique bands represented distinct species of proteasome subunits, they would persist *in isolation* on CTAB-PAGE. To test this, we attempted to purify the CTAB-PAGE-unique bands away from main-band material in order to run both fractions in separate lanes on CTAB-PAGE. We found that, using sub-cellular fractionation[12] (Figure1B-1D), and irrespective of cell type used (Figure 1A,1B), CTAB-PAGE-unique material could be separated from main-band material and that it persisted *in isolation*. Most of the main-band material was NP40-extractable (cytosolic), whereas the CTAB-PAGE-unique bands were found in the nuclear pellet and could be separated further from chromatin and nuclear architecture using a nuclear extraction step (Figure1D)[8]. EDTA in the fractionation buffer improved separation of the main band from the collection of high-molecular-weight bands, bands which were then stable and immune even to prolonged incubation at 37°C (Figure1C). A subsequent high-salt nuclear extraction step removed about half of the proteasomes from the nuclear pellet; we used the

chromatin remodelling complex component SMARCB1/Ini1 as positive control and Lamin B2 as negative control (Figure1D). Note that it was *not* the nuclear environment in which proteasomes reside at the moment of encountering CTAB in the lysis buffer that determined their subunits' migration behaviour on CTAB-PAGE: CTAB-PAGE-unique bands persisted when proteasomes were extracted away from chromatin and nuclear architecture prior to exposure to CTAB (Figure1D) and, in the case of yeast *S. cerevisiae*, nuclear and cytosolic proteasome subunits migrated identically on CTAB-PAGE (below, Figure S4C).

Additional validation that CTAB-PAGE-unique bands corresponded to true protein species came from identifying experimental conditions under which *also* SDS-PAGE could visualise the CTAB-PAGE-unique bands, thus uncoupling visualisation of protein species from use of a particular PAGE system. While Figure1D's sub-cellular fractionation experiment still revealed a striking discrepancy between the *same* samples' SDS-PAGE and CTAB-PAGE patterns, further purification steps led to convergence of proteasome subunit pattern on both PAGE systems. Importantly, with increased purification, the SDS-PAGE pattern started to resemble the CTAB-PAGE pattern, rather than the other way around (Figure1E, 1F). Thus, on SDS-PAGE, nuclear proteasome subunits *also* migrated as collections of multiple species, on condition that these proteasomes were sufficiently purified. Briefly, proteasomes were captured from primary human leukocytes using a resin-immobilised ubiquitin-like (Ubl) domain of hPLIC2/Ubiquilin2[10, 13-14], then further purified using FPLC size-exclusion chromatography (Figure1E). CTAB-PAGE analysis of purified nuclear proteasomes showed extensive modification of the Regulatory Particle (RP) subunits Rpn12 and Rpt2, and the β 5i Core Particle (CP) subunit, as before; by contrast, the CP subunit α 7 migrated as a single band (see also Figure2C,2D). Surprisingly, unlike in the experiments described above, analysis of *purified* nuclear proteasomes by SDS-PAGE gave virtually identical results to those seen with CTAB-PAGE (Figure1E). One interpretation why SDS-PAGE -with increasing sample purification- starts to yield CTAB-PAGE patterns, is that modified nuclear proteasomes interact with other components of the nuclear extract and that this interaction prevents SDS-PAGE from visualising proteasomes. Consistent with this interpretation, when analysing samples taken over the course of a single purification procedure, modified proteasomes were only visible on SDS-PAGE in samples taken *after* the Ubl-capturing step (Figure1F), indicating that sample complexity is 'masked' in unpurified lysate or extract mixtures. Interestingly, in Figure 1F, the Ubl domain [10, 13] did not capture unmodified Rpt2 from the cytoplasmic extract; we found this to be the result of the particular construct [10-11] and buffer conditions used (Figure S2). In sum, CTAB-PAGE detected human proteasomes that are located in a distinct sub-cellular location, most likely in the nucleus (although a tightly-bound cytosolic or cytoskeletal location could not formally be excluded, and relative distribution of proteasomes between fractions may depend to some extent on fractionation procedure), and that carry constitutive modifications which are largely incompatible with SDS-PAGE analysis.

In order to understand why CTAB- but not SDS-PAGE visualised proteins modified in this manner, we sought to identify the nature of the posttranslational modification. We used a GST-Ubl [11] construct to capture proteasomes from either cytoplasmic or subsequent nuclear extract (Figure S2), and compared these by two-dimensional isoelectric focusing (IEF)/SDS-PAGE. The posttranslational modification substantially changed focusing of proteasomal subunits, even in a wide-range pH3-10 gradient (Figure 2A, S3). The modification imparted a complex focusing phenotype, including (1) a 'striated' rather than punctuate focusing pattern (even in a narrow pH

range, Figure 2B, and with steady-state focusing achieved), and (2) diagonal 'slanting' towards the acidic pole (figure 2B). The pronounced effects of the posttranslational modification on the pI of proteasomal subunits strongly suggests it carries charge (and, in the case of the population slanting towards the acidic pole, negative charge) [15]. It was not DNA or RNA (Figure2C). We found that the modification of nuclear proteasomes shared several characteristics with, but also is distinct from, classical ADP-ribose chains (poly(ADP-ribosyl)–PAR–chains)[16-17]. PAR is a nuclear modification of high negative charge, which could explain relative incompatibility with (negatively-charged) SDS-PAGE. Characteristics shared with PAR include: (1) Sensitivity to the PAR hydrolytic enzymes PARG, ARH1, ARH2 and ARH3. Incubation with these enzymes, alone or in combination, altered the Western blot patterns of several proteasome subunits (Figure2D, 2E). Most of the changes reflected activity of PARG, which hydrolyses ribose–ribose linkages between coupled ADP-ribose units in PAR chains (Figure2D). Note that, even though we used standard SDS-PAGE rather than CTAB-PAGE (as was possible with *purified* nuclear proteasomes as substrate), PAR-hydrolysing enzymes had unusual effects on migration of proteasome subunits. These include apparent upward migration and increased signal intensity, in part due to PARG-facilitated entry of modified proteasome subunits into the SDS-PAGE gel (Figure1F). A direct comparison on SDS-PAGE of cytosolic *versus* (purified) nuclear Rpt2 showed that nuclear Rpt2 migrated with an apparent molecular weight range below that of unmodified, cytosolic Rpt2, as may be expected from a highly negatively-charged protein modification (Figure1F,2B). (2) Sensitivity to venom phosphodiesterase I (PDE1). PDE1 hydrolyses pyrophosphate bonds of ADP-ribose units in PAR, and also changed Rpt2 pattern (Figure2F)[18]. (3) α -PAR antibodies immunoprecipitated proteasomal antigens and, *vice versa*, proteasome antibodies immunoprecipitated PAR-reactive antigens (FigureS4B). We found that an alternative approach, using biotin-NAD⁺ for metabolic labelling, was not feasible, because living cells did not take up biotin-NAD⁺ nor could we use digitonin-permeabilised cells for our constitutively-present modifications. (4) Yeast proteasomes, which lack PAR[19], were unmodified on CTAB-PAGE (FigureS4C). However, the modification of human nuclear proteasomes also differs from PAR in several respects: (1) Complete resolution of modified nuclear proteasomes into single subunit bands using PAR enzymes was never achieved (FigureS4A), even when combined and under a wide variety of experimental conditions. (2) The nuclear proteasome modification is *constitutively* present, whereas standard PAR chains on substrates are generally transient, short-lived and synthesised in response to stress at sites of e.g. DNA damage[16-17]. Interestingly, the constitutive modification of nuclear proteasomes we report here expands on what is already known, namely that *standard* PAR transiently activates nuclear proteasomes to degrade oxidatively damaged histones[8, 17, 20] and regulates proteasome assembly in the nucleus via modification of assembly factor PI31[21]. Because PDE1/PARG enzyme treatment was only partially effective against the proteasome modification (FigureS4A), it has not yet been possible, despite various attempts, to obtain a mass spectrometry signature.

Finally, we identified a physiological process which induces changes in CTAB-PAGE detectable proteasome modifications. It has been reported that, during apoptosis, activated caspases cut once in each of three proteasome subunits. These cleavage events were visualised with SDS-PAGE[22-23]. However, we now report, changes to proteasome structure during apoptosis are far more dramatic: CTAB-PAGE-unique subunit species of multiple proteasome subunits changed dramatically during apoptosis, as observed when treating cells with a lethal dose of Bortezomib versus control- or sub-IC50-treated cells (Figure 3A). The same was observed when using apoptosis-inducing compounds which are not proteasome inhibitors (Figure 3C, S6A), or

proteasome inhibitors other than Bortezomib (Figure S5). Most, but not all, of these changes were caspase-dependent, because caspase inhibitors prevented many, though not all, of these changes (Figures 3B). Interestingly, by comparing eight human cell lines on CTAB-PAGE, we found that proteasome modification patterns can vary to some extent (Figure S6B). We are currently studying how this modification affects proteasome function.

In sum, we found that nuclear proteasome subunits carry a posttranslational modification which makes those subunits invisible to SDS-PAGE. We identified an alternative system that overcomes this problem (CTAB-PAGE), and gained insight into the nature of the modification leading to a possible explanation for observed PAGE compatibilities. Finally, we identified the first example of specific biology involving CTAB-PAGE detectable protein modifications.

Even though the constitutive modifications of nuclear proteasomes differ from classical PAR chains, their sharing of some characteristics suggests a possible function for these modifications in proteasome biology. Nuclear proteasomes are known to have a variety of nuclear functions [24-28]; they must interact with chromatin, DNA and other nuclear components, but little is known about how this is accomplished. In general, when PAR chains are present, they recruit e.g. PAR-binding repair proteins to DNA and chromosomes [16-17]. In analogy, we hypothesise that proteins in nuclear processes capture the (constitutive) CTAB-PAGE-detectable modifications on nuclear proteasomes, thereby recruiting proteasome function to e.g. gene expression, even under non-stress conditions.

Our identification of an SDS-PAGE-incompatible protein modification may also explain the difficulty in resolving the controversy as to whether RNA species, which early studies suggested are associated with proteasomes (then called *prosomes*)[29], are bona fide components of proteasomes [30]. These modifications may account for the reported RNP-like density of prosomes/proteasomes, and our finding that SDS-PAGE fails to detect modified proteasomal subunits may explain the difficulty in resolving the controversy. Our data suggest that CTAB-PAGE should be used routinely in nuclear biology to ascertain whether or not a protein of interest is similarly modified.

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6.1 Figure Legends

Figure 1. A proteasome modification that correlates with subcellular localization.

(A) Lysate from KMS12-BM myeloma cells was split and run on SDS and CTAB PAGE systems, before being blotted for proteasome subunits. Red brackets indicate CTAB-PAGE unique signal. Note that CTAB-PAGE is not inherently prone to smearing: not all proteins we tested migrated as a smear (e.g. LaminB2, Figure 1A; Ini1, Rpt3, Figure 1D), and lanes with a CTAB-PAGE-unique smear also contained sharply focused bands. (B) NCI-H929 myeloma cells were subjected to subcellular fractionation, and fractions were analysed by Western blotting against proteasome subunits using CTAB-PAGE. Red brackets indicate CTAB-PAGE unique signal. (C) Presence of divalent cations in fractionation buffer affects CTAB-PAGE pattern. NCI-H929 myeloma cells were fractionated using a cytosol extraction buffer containing either $MgCl_2$ or EDTA. Fractions were kept on ice or incubated at $37^\circ C$ for one hour, after which they were analysed using CTAB-PAGE and Western blotting with an α -Rpn12/S14 proteasome subunit antibody. (D) Cell fractionation of NCI-H929 cells was extended with a nuclear extraction step. All fractions were split and subjected to CTAB-PAGE and SDS-PAGE, and analysed by Western blotting against proteasome subunits. P = Pellet remaining after nuclear extraction, C = cytosolic extract, NE = nuclear extract. Notice that although the same protein was loaded on both systems, SDS-PAGE doesn't display all of the species observed in CTAB-PAGE. The fractionation protocol (Materials and Methods, section 2.1.3) normalizes for cell number: lanes contain the amount of protein extracted or remaining in pellet per equal number of cells. In addition, by employing a very efficient stripping procedure [7], the same membrane is used for all antibodies, thus eliminating pipetting variation between panels. (E) Nuclear human proteasomes were affinity-captured from nuclear extract with a ubiquitin-like proteasome binding domain (Ubl-His₆), released, and subjected to Superose6 FPLC fractionation. FPLC fractions were analysed on both CTAB-PAGE and SDS-PAGE by Western blotting against proteasome subunits. Arrows indicate Blue Dextran FPLC marker band. (F) Protein samples were taken from different stages of a large-scale nuclear human proteasome purification experiment, and immunoblotted with an α -Rpt2 proteasome antibody. Unmodified Rpt2 is indicated by an arrow, modified nuclear Rpt2 species are indicated with a red bracket.

Figure 2. The constitutive modification of proteasomes shares several characteristics with –but is distinct from– poly(ADP)-ribose.

(A) Proteasomes were captured, using GST-Ubl (see also Figure S2), from both cytoplasmic extract and subsequent nuclear extract of myeloma OPM2 cells. The two samples were compared on two-dimensional isoelectric focusing/SDS-PAGE with an α -Rpt2 antibody. The red brackets indicate where nucleus-extracted, modified Rpt2 focuses in a pH 3-10 linear gradient. Care was taken to ensure that the isoelectric focusing was run to completion and that steady-state positions were reached. * indicates nonspecific signal due to the presence of a large amount of GST-Ubl (see corresponding PonceauS staining in Figure S3A). (B) Experiment as in (A), but this time samples were analyzed on a narrow-range pH 4-7 gradient. A longer exposure of the left part of the gel is shown underneath to highlight the 'striated' focusing phenotype. (C) Nuclear proteasomes were captured with Ubl-His₆ and further purified by FPLC, as in Figure 1E. Combined proteasome FPLC fractions 13-18 were incubated with nucleotide modification enzymes Micrococcal Nuclease, RNaseA/T1 mixture, RNase1 and RNaseH. Samples were run on SDS-PAGE, and analysed by

Western blot using an α -Rpt2/S4 proteasomal subunit antibody. **(D)** Pooled FPLC fractions 12-19 were incubated with combinations of PAR hydrolysing enzymes PARG, ARH1 and ARH3. Subsequently, samples were analysed by SDS-PAGE and Western blotting using several antibodies against proteasomal subunits. PAR enzyme-dependent changes in subunit pattern were observed for Rpn12, α 7 and Rpt2. Prior to re-probing, the membrane was checked after stripping [7] and found to be void of signal. **(E)** The orphan PAR enzyme ARH2, for which no substrate is known as yet, does not affect the migration behaviour of the α 7 proteasome subunit. Two isoforms of ARH2 were cloned and tested, neither of which had activity; shown here is ARH-2.2. **(F)** FPLC fractions were incubated with phosphodiesterase 1 (PDE1), ARH3 and PARG. Samples were run on SDS-PAGE, and analysed by western Blot using an α -Rpt2/S4 proteasomal subunit antibodies.

Figure 3. The constitutive modification of proteasomes changes in response to a physiological stimulus.

(A) Western blot analysis of the effects of a Bortezomib concentration range on the CTAB-PAGE patterns of several proteasome subunits. NCI-H929 myeloma cells were treated with the indicated Bortezomib concentrations for 24 hours, after which total cell lysate was analysed by CTAB-PAGE and Western Blot. (Asterisk denotes IC50 value for the NCI-H929 cell line used). The bottom panel shows that Bortezomib between 1 and 10 nM induces apoptosis in these cells, as determined by AnnexinV-7AAD staining. **(B)** Western blot analysis of the effects of Bortezomib (20 nM) and a caspase inhibitor cocktail on the CTAB-PAGE pattern of proteasome subunits. Indicated with red and black arrows are Bortezomib-induced changes in CTAB-PAGE pattern which are caspase-independent and caspase-mediated, respectively. **(C)** Myeloma OPM2 cells were treated for 29 hrs with Bortezomib (250 nM), doxorubicin (10 μ g/ml), cycloheximide (500 μ g/ml), or vinblastin (10 μ g/ml). Cell viability was assessed by AnnexinV/DAPI, and total cell lysate was analyzed by CTAB-PAGE.

Figure 1

Figure 2

Figure 3

Graphical Abstract

Highlights

- Nuclear proteasomes carry a previously unrecognized posttranslational modification
- Modified subunits of nuclear proteasomes are not visualized by SDS-PAGE
- Modified subunits of nuclear proteasomes can be observed with CTAB-PAGE
- Modification shares characteristics with, but also is distinct from, polyADP-ribose
- Modification suggests mechanism how proteasomes may execute their nuclear functions