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The 2 PAN ATPases from Halobacterium display N-ter heterogeneity and form labile complexes with the 20S proteasome.

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Running Title: Cellular dynamics of two archaeal proteasome regulators

Abbreviations: AMC, 7-amino-4-methylcoumarin; CHAPS 3-(3-cholamidopropyl-dimethylammonio)-1-propanesulfonate; CP, catalase peroxidase; DTT, dithiothreitol; IEF, isoelectric focusing; IPTG, isopropyl- β -D-thiogalactopyranoside; MalDH, malate dehydrogenase; P45, salt shock chaperone 45; PNK, polynucleotide kinase; TF55, thermosome; TCA, trichloroacetic acid; TET, tetraedral aminopeptidase.

ABSTRACT:

The PAN proteins from Archaea represent homologs of the eukaryotic 26S proteasome regulatory ATPases. *In vitro*, the PAN complex has been previously shown to have a stimulatory effect on the peptidase activities of the 20S core. By using gradient ultracentrifugation experiments we found that, in cellular extracts, the 2 PAN proteins from *Halobacterium* do not form stable high molecular weight complexes. Only PAN B was found to associate transiently with the 20S proteasome, thus suggesting that the 2 PAN proteins are not functionally redundant. The PAN B-20S proteasome complexes associate in an ATP-dependent manner and are stabilized upon nucleotide binding. The 2 PAN were immunodetected in cellular extracts as N-ter truncated polypeptides. RNA mapping experiments and sequence analysis indicated that this process involved transcript heterogeneities and dual translational initiation mechanisms. Taken together, our results suggest that PAN N-ter modifications and their intra cellular dynamics of assembly/association may constitute important determinants of proteolysis regulation.

INTRODUCTION:

In all cell types, intracellular protein degradation is a highly controlled process. It specifically defines the half-lives of proteins and it is also important to rapidly eliminate disabled proteins that could not be rescued by the chaperone system in order to prevent their intracellular aggregation [1, 2]. A selective proteolysis also occurs to provide an irreversible way of inactivating, at the appropriate times, the regulatory molecules involved in cell cycle progression or in metabolic switches [3, 4].

In eukaryotes, the ATP-dependent Ubiquitine/26S proteasome pathway degrades the majority of cytosolic proteins [5, 6]. The 26S proteasome is a giant 2,600 kDa complex containing 62 protein subunits [7]. It can be separated in two sub-particles: the 20S self-compartmentalized peptidase core and the 19S dependent regulatory particle (RP) [8].

The yeast RP is the best characterized and consists of at least 17 proteins [9]. Six regulatory ATPases (named Rpt), all essential for cell viability, form the base of the RP [10]. These proteins belong to the AAA ATPases family (Atpases Associated with a variety of cellular Activities) [11]. The AAA complexes recognize specific proteins as targets and function as energy dependent disassembly machines involved in many different cellular functions [12]. In vitro, the eukaryotic AAA Rpts stimulate the 20S proteasome peptidase and caseinase activities and it is hypothesized that they associate into a hexameric ring complex at the base of the 19S particle [9]. In yeast, mutagenesis studies have revealed an unexpected diversity of non-redundant functions among the 6 different Rpt proteasomal ATPase [10]. The exact biological role performed in vivo by this class of AAA proteins in the regulation of the proteasome activity is therefore a matter of debate.

The ancestral proteasome system present in archaeobacteria is much simpler in its composition and structure than that found in eukaryotes and it is recognized as a paradigm to understand basic aspects of the cytosolic proteolytic function [13]: i) the Ubiquitin-like tagging machinery is apparently absent in Archaea, ii) the 20S proteasome core is made of only one or two types of alpha subunits and one catalytic beta subunit, and iii) the energy dependent regulatory complex apparently consists in a single type of Rpt-like proteins that are considered as the evolutionary precursor of the eukaryotic 19S [13]. The archaeal Rpt-like protein was named PAN (*Proteasome Activating Nucleotidase*) [14]. In *Methanocaldococcus jannaschii*, a methanogenic hyperthermophilic archaea, the recombinant PAN protein was found to stimulate the 20S proteasome hydrolytic activities on protein substrates such as casein and GFP-Ssra [14, 15]. *In vitro* experiments showed that substrate binding activates PAN ATPase activity, promotes substrate unfolding and the opening of the axial gate of the peptidase 20S core [16, 17].

The analysis of the 3 sequenced haloarchaeal genomes: *Halobacterium sp.*, *Haloferax volcanii* and *Haloarcula marismortui* revealed that they all possess 2 highly similar PAN proteins that differ significantly only in their N- and C-terminal regions [18]. In their Rpt eukaryotic homologues, the C-terminal regions are believed to be responsible for binding to the alpha subunits of the 20S proteasome, while the motifs present in the N-terminal regions have been proposed to be involved in the interaction with substrate

proteins [19-21]. These observations led to the idea that the two PANs from halophilic Archaea could play a non-redundant role in cell physiology [18]. In this paper we have addressed this question by studying, in cellular extracts, the respective biophysical states and 20S binding properties of the 2 PAN proteins from *Halobacterium*, PAN A and PAN B. The aim was also to get insights into the intracellular dynamics of the proteolytical AAA-ATPases, an aspect of proteolysis control that remains poorly documented in all three kingdoms of life. The results show that the PAN regulatory proteins accumulate as low molecular weight assemblies and do not form a tight complex with the 20S proteasome, or other partners, within the cell. In addition, both PAN proteins are prone to N-ter modifications *in vivo*. We show that this modification process is controlled at the transcript levels and we suggest that the truncated polypeptides arose from the use of alternative translational initiation sites. Finally, the 2 PAN complexes displayed differences in their oligomerization and proteasome binding properties, thus strengthening the idea that they are not functionally equivalents. These properties make PANs good candidates to be global regulatory factors and indicate that the AAA-ATPase oligomerization/association state can play an important role in the control of intracellular proteolysis.

EXPERIMENTAL

Cell culture conditions

Cells were grown in medium containing 40 % NaCl, at 37°C with agitation as described before[22]. In the case of treatment with puromycin, cells were grown to an O.D₆₀₀ of 0.6, divided into two portions and incubated with or without puromycin (50 mM final concentration). Aliquots were taken at different times and the cells were rapidly harvested by centrifugation (13000g, 3 min), frozen in liquid nitrogen and kept at -20°C until use.

SDS-PAGE and Immunodetections

To prepare *Halobacterium* protein extracts, cell pellets were resuspended in lysis buffer (50 mM Tris-HCl pH 8.3, 0,1% Triton-X) by repeated passage through a 0.2 mm diameter needle. Cells debris were removed from the homogenate by centrifugation (13000 g, 3 min). Protein concentrations were determined by using a Bio-Rad assay with bovine serum albumin as the standard. In sucrose gradient experiments, the

proteins were precipitated from the fractions with trichloroacetic acid (15% final concentration), washed with cold acetone, dried, and resuspended in ice cold 20 mM Tris-Base. Sodium Dodecyl Sulfate (SDS) loading buffer was then added to the samples (50mM Tris-HCl pH 6.8, 100 mM DTT), 2% SDS, 0,1% bromophenol blue, 10% glycerol). Before electrophoretic separation, the samples were heat-denatured at 95°C for 5 min, and then separated on 12% SDS-polyacrylamide gels. The proteins were transferred onto PVDF-membrane (Amersham) and probed with specific rabbit antibodies. Immunoreactive bands were visualized by chemiluminescence as detailed by the supplier (ECL detection kit; Amersham). Primary antibodies against MalDH, the P45 chaperone and catalase peroxidase were raised as described [23]. The antibodies against the thermosome alpha subunit, PAN A and PAN B were raised against the following synthetic peptides chosen in the primary sequences: LSEDSQRTSGEDAQSC (anti-thermosome), QDSEPAAATDVSRTFA (anti C-ter PAN A) and GDTESGPRYPYSYIQ (anti C-ter PAN B). The anti-PANA and anti-PANB immunoglobulins were purified by using peptide affinity columns (Amersham). All anti-sera were used in a 1/10 000 dilution.

2D gel analysis

Cells from mid-exponential phase were centrifuged at 13000 g for 10 min. The pellets were resuspended in 10 volumes of lysis buffer containing 50 mM Tris-HCl pH 8.0, 0.1% triton and 0.04% of protease cocktail inhibitor and centrifuged at 13000 g for 5 min to eliminate cell debris. Protein concentration was determined at this step using the Bradford protein assay (Biorad). Four hundred microliters of protein sample were mixed with an equal amount of lysis buffer Samples and then desalted using a 3 KDa cut-off column (Millipore). This step was repeated four times to ensure a complete desalting as salt interferes with isoelectric focusing (IEF). Proteins were then precipitated by a 10 time dilution in 10% TCA rapidly followed by a centrifugation at 13000 g for 10 min. Protein pellets were then washed with 10% cold acetone. The sample was spun again, and precipitated proteins were air-dried and then solubilized in rehydration buffer containing 8 M urea, 4% CHAPS, 60 mM DTT, 0.5% IPG ampholytes, and a trace of Bromophenol Blue. A brief centrifugation was carried out before the rehydration step to eliminate insoluble proteins. IPG strips (Bio-Rad) with a pH range of 3.9-5.1 were used for IEF or the first dimension. One hundred micrograms of protein samples were used

for the passive rehydration for 12 h at 20°C. Isoelectric focusing was then carried out using a protean IEF cell system (Bio-Rad) with the following conditions: 1h at 10V, 1h at 100V, 1h at 1000V and 6h for a total of 6400V. IPG strips were stored at –20°C overnight or at –80°C for a few days until use. Before the second dimension, IPG strips were washed in equilibration buffer (6 M urea, 2% SDS, 0,375 M Tris-HCL pH 8.8, 20% glycerol) for 2 x 15 min. The first wash was supplemented with 130 mM DTT and the second wash with 135 mM iodoacetamide. The second dimension was performed on 12% polyacrylamide gels and immunoblotting was then performed as described previously.

Density-gradient sedimentation analysis

For oligomerization and association profiles, 2 grams of cells (OD=0.9) were pelleted and resuspended in 15 mL of ice cold buffer A containing 3.2 M KCl, 60 mM MgOAc and 50 mM Tris-HCl pH 7.6. After sonication (6 X 10 sec), 50 µg of DNase1 (Roche) were added. This treatment led to complete cell lysis and the membranes were eliminated by a 45 min centrifugation at 30000g at 4°C. The supernatants (S30) were incubated for 30 min at room temperature in the presence or absence of 10 mM ATP or 1mM ATPγS or 50 µg of pure halo archaeal 20S proteasome prepared as described [24]. 0.5 ml of the S30 supernatants were then layered on top of 10 mL 5–20% sucrose gradients made in buffer A (containing 2 mM ATP when ATP was added to the S30 extract).

Different halophilic protein complexes were purified to homogeneity to calibrate the gradient ultracentrifugation experiments. The recombinant *Haloarcula marismortui* malate dehydrogenase was purified as described [25]. The native chaperone Ssp45, 20S proteasome, TET aminopeptidase and the catalase peroxidase were purified from halophilic cells following the protocols described previously [23, 24, 26, 27]. Centrifugation of the gradients was performed in a Beckman SW41 rotor for 20 h at 35 000 rpm and 10 °C. The gradients were fractionated from the top into 750-µL aliquots. All experiments were repeated at least 3 times.

Halophilic 20S proteasome activity

To assess for the distribution of the 20S proteasome in the sucrose gradients, 50 µL of each fraction were added to a 300 µL reaction mixture containing 2 M KCl, MgOAc

100 mM, 60 mM Tris-HCl, pH 7.6, and 0.5 mM of the Suc-LLVY-AMC peptide (Bachem), a typical proteasomal substrate. To assess for the specificity of the activities that were detected, 10 μ M of NLVS (Affinity), a proteasome-specific inhibitor, were added in parallel assays. The quantity of AMC liberated was measured in a Victor 1420 multilabel counter (Wallac). Fluorescence was measured using excitation and emission wavelengths of 335 and 460 nm respectively. The reaction mixture was incubated at 40°C and the hydrolysis of substrate was monitored after different incubation times. The total amount of proteins in the sucrose gradient fractions was determined by using the Bradford assays from Bio-Rad. The values plotted in figure 6 were situated in the linear part of the activity curves and are expressed as fluorescence units liberated per mg of total protein. All experiments were done in triplicates.

General molecular biology techniques

General molecular biology techniques were performed according to Sambrook *et al.* Restriction enzymes and other DNA modification enzymes were purchased from Roche or from Ambion. Oligonucleotides used for probe construction and for primer extension reaction were purchased from Eurogentec. The PCR conditions used to amplify the different gene fragments from *Halobacterium* genomic DNA are available on request. The identity of all constructs was confirmed by DNA sequencing.

Cloning and expression of PAN A and PAN B proteins in *E.coli*

The *Halobacterium* sp genomic DNA was prepared as described in the “Halohandbook” (available on line at <http://www.microbiol.unimelb.edu.au/people/dyallsmith/resources>). The genes of PAN A and PAN B were amplified by PCR from the genomic DNA using the following sets of oligonucleotides: 5'-GGA ATT CCA TAT GAC CGA TAC CGT CGA GGA-3' and 5'-CGG GAT CCC GCT ACG CGA ACG TCC GGG AGA C-3' for PAN A; 5'-GGA ATT CCA TAT GTC GCG TAG CCC CTC ATT G-3' and 5'-CGG GAT CCC GTT ACT GAA TGT AGC TCG GGT-3' for PAN B. The termini were designed to be compatible with the *NdeI* and *BamHI* restriction sites for insertion into expression vectors. The PCR products were purified and cloned into the vector Topos2.1 purchased (Invitrogen) according to the supplier's protocol. Using the insertion sites *BamHI* and *NdeI*, the inserts were then cloned into pet11a (Novagene). The identity of all constructs was confirmed by DNA sequencing. *E.coli* BL21 (DE3) strains were transformed with the pet11a-PAN A or pet11a-PAN B vectors and were

grown at 37°C for 3 hours. The cells were then induced with 0.5 mM IPTG at an O.D₆₀₀ = 0.6. The culture was allowed to grow for 3 h after addition of IPTG. The cells were collected by centrifugation (6000g, 5 min) and were used as a control lane for the protein in *Halobacterium* extracts.

Isolation of RNA from *Halobacterium*.

For RNA extraction, 1 mL of exponentially growing cells (O.D = 0,6) were centrifuged at 13 000 g for 5 min at room temperature. The pellet was resuspended in 500 µl of the RNAwiz extraction buffer (Ambion). After homogenization and 5 min incubation, 100 µl of chloroform were added and the sample was incubated for 10 min at room temperature. The suspension was then centrifuged at 13000 g, 15 min, 4°C, and the aqueous phase was transferred into another tube containing 250 µL of H₂O and 500 µL of isopropanol. After centrifugation (13 000 rpm for 15 min, at 4°C), the RNA pellets were washed twice with 100 % and then 60 % ethanol. The pellets were dried and resuspended in an appropriate RNase-free water volume.

RNA mapping experiments.

For primer extension analysis, oligonucleotides (5'-CAA CAA ATA CCA GCA GAA GC-3' and 5'-GAG AAC GAG ACG CTG AAA ACG-3') complementary to the RNA sequences situated downstream of the translation initiation sites of PAN A and PAN B were synthesized. Twentyfive nanograms of each oligonucleotide were ³²P labeled using T4 PNK in a 10 µl reaction mixture containing 1 µl of ³²PγATP (3000 Ci/mol, 3.3 pmoles). One microliter of the labeled oligonucleotide was then incubated with 20 µg of total RNA extracted from *Halobacterium*. The mixture was ethanol precipitated and resuspended in 10 µl of 80 mM KCl, 20 mM Tris-HCl (pH 8.5) and 0.5 mM EDTA. For hybridization, the mixture was heated at 65°C for 5 min and cooled down till 37°C for 1 hour. The extension reaction was then performed at 37°C for 15 min by adding 10 µL of the following mix: 10 mM MgCl₂, 1 mM dNTP, and 10 mM β-mercaptoethanol, containing 5 Units of RNA guard (Amersham). The reverse transcription reaction was performed with 50 Units of reverse transcriptase from Moloney murine leukemia virus (Roche). The extension products were ethanol precipitated and resuspended in 2.5 µL

H₂O and 2,5 µL of formaldehyde loading dye (Ambion). Extension products were size fractionated on a 7 M Urea/6% polyacrylamide gel in 1X TBE. A sequencing reaction of the PAN A promoter region was migrated along with the primer extension products. The reaction used the oligonucleotide that served for the primer extension reaction and was performed with the T7 sequencing kit (Amersham). The gels were dried and the signals corresponding to the 5' ends were developed by autoradiography after 12 h of exposure.

For ribonuclease protection assays, a 594 bp DNA fragment encompassing the promoter region of PAN B was amplified from genomic DNA using the following oligonucleotides: 5'-GCA TCC CGC ATT TCT ACT ACA A-3' and 5'-CGT TTT CAG CGT CTC TCG TTC T-3'. The PCR fragment was cloned into the PCRII-TOPO vector from Invitrogen. The plasmid containing the DNA insert was linearized with XbaI and used as a template to transcribed the antisens riboprobe using the T7 maxiscript kit from Ambion according to the manufacturer's instructions. The probe was radioactively labeled by ³²P –UTP. The RNA was then purified on a 7M Urea/5% polyacrylamide gel as described in Sambrook *et al* [28]. Six hundred picograms of the radiolabeled probe were mixed with 0,6 µg of total *Halobacterium* RNA or the same amount of total RNA from yeast for negative control. The ribonuclease protection assays were then conducted using a kit from Ambion following the manufacturer's protocols. An RNase A/RNase T1 mixture was used for the digestion of the unprotected RNA molecules. The protected RNA fragments were separated on a 7M Urea/6% polyacrylamide gel that was run in TBE buffer. RNA fragments of known sizes were 3'-end labeled using γ³²P-ATP and run in parallel to assess for the lengths of the protected RNA fragments. The gel was dried and the radioactive bands were detected by autoradiography.

RESULTS

The PAN A and PAN B proteins accumulate *in cellulo* as 2 polypeptides that differ in their N-terminal length

The two *Halobacterium salinarium* open reading frames that encode for the proteasome regulatory subunits ATPase, OE1765 and OE3805, were retrieved from the Halolex

web site of the Max Planck Institute of Biochemistry (<http://Halolex.mpg.de>). OE1765 encodes for a 44.94 kDa protein (PAN A) that shares 60% sequence identity and 78% similarity with the 45.280 kDa product of OE3805 (PAN B). Sequence alignment revealed that the two *Halobacterium sp* PAN proteins mainly differ in their N and C-termini (Fig. 1). We used therefore synthetic peptides derived from the C-terminal regions to obtain specific antibodies against PAN A and PAN B proteins (Fig. 1). The antibodies were purified on peptide affinity columns. Western blot analyses of *Halobacterium* total protein extracts revealed that both PAN A and PAN B native proteins migrate as two forms on SDS-PAGE gels (Fig. 2). The apparent molecular weights are 54 and 47 kDa for PAN A and 56 and 49 kDa for PAN B. Note that, due to their high acidity, halophilic proteins usually migrate at higher apparent molecular weight on SDS-PAGE [29]. The two PAN polypeptides were detected in all protein extracts, independently from the growth stage or the physiological state of the cells (data not shown). To establish if the PAN additional bands arise from post-translational modifications or from truncated form of the *pan* gene products, the *pan A* and *pan B* genes were cloned into expression vectors and the corresponding recombinant proteins were produced in *E. coli*. The PAN B full-length gene was translated in *E. coli* as a unique polypeptide that migrated at the same position than the PAN B upper band that we detected in the *Halobacterium* extracts (Fig. 2). Since the antibodies were raised against the C-terminal end of the protein, our experiments indicated that the short product that accumulates in *Halobacterium* cells corresponded to a PAN B isoform truncated in its N-terminal region. Similarly, when PAN A was expressed in *E. coli*, the most abundant translation product was found to corresponds to the upper band detected in archaeal extracts. Since, as for PAN B, the antibodies were raised against the C-terminal extremity of the protein, the short band detected in *Halobacterium* should also arise from a N-terminal truncation of the native protein. An additional PAN A smaller band was detected in lower abundance in the *E. coli* recombinant extracts. This short product had the same size than the truncated PAN A detected in the archaeal extracts. This observation indicated that the mechanism responsible for the production of the PAN A truncated protein in *Halobacterium* cells can also occur in *E. coli*. The presence of 2 shorter isoforms for PAN A and PAN B in *Halobacterium* protein extracts was confirmed by 2D gel electrophoresis (Fig. 2). The electrophoretic mobilities of the PAN proteins indicated a 7 kDa difference in size between the two products (about 40 amino acids). The mobility shift of the short PAN A protein observed on the 2D gel indicates a

considerable difference in charge between the two PAN A proteins, the short polypeptide being more basic than the full length product. From these experiments, we estimated the pI of the short PAN A protein to be 4.8. This value is consistent with the theoretical value calculated for a 40 AA N-ter deletion in the PAN A sequence. In the case of PAN B, The 2D gel electrophoretic behavior was also in accordance with a 7 KDa N-ter deletion since the modification does not affect drastically the protein charge. Taken together these experiments indicated that, in *Halobacterium* cells, N-terminal shortening is a common modification for the two PAN regulators. We named the truncated versions of the PAN A and PAN B proteins Δ PAN A and Δ PAN B respectively.

The truncated PAN isoforms do not arise from post-translational processing events.

To examine whether the N-ter truncated PAN versions are produced co-translationally or from a post-translational processing mechanism, puromycin a drug that blocks protein synthesis was added to *Halobacterium* cultures. The drug treatment was found to block completely the cell growth (not shown). The cellular accumulation of the two PAN isoforms was followed kinetically in presence or absence of puromycin (Fig. 3). If Δ PAN A and Δ PAN B were produced post-translationally from full length precursors one would expect a diminution of the upper band over time associated with an increase in the lower band intensity. As shown in Fig 3, the ratio between the two PAN versions remained unaffected in the puromycin samples, even after 12 hours of treatment. These experiments indicate that PAN N-ter heterogeneities are not regulated at the post-translational level.

Transcriptional control mediates the production of the PAN N-truncated versions.

The examination of the PAN A and PAN B primary sequences in the 3 known haloarchaeal genomes revealed that a second possible translation initiation codon is situated inside the reading frames. In the case of PAN A, an ATG codon is located in frame, 117 bases downstream from the first initiation codon and a Shine-Dalgarno consensus sequence is appropriately located at position – 10 (Fig. 4A). The calculated

molecular weight of the corresponding polypeptide is 40.616 KDa with a pI of 4.79. These values are consistent with those measured experimentally when Δ PAN A was immunodetected in 2D Gels (Fig. 1A). Interestingly, when the PAN A ORF was expressed in *E.coli*, the same Δ PAN A product was also detected (Fig. 2). This suggests that Δ PAN A originates from an internal ribosome binding site that is also recognized by the *E. coli* translational machinery. In the case of PAN B, a GTG codon, that in Archaea as in Bacteria often serves as an initiation start [30, 31], is located at position +108 from the first methionine. As for PAN A, this second putative initiation codon (encoding for a valine in the translated sequences) is conserved in all known PAN B sequences from halo archaea (Fig. 4A). We could not detect a second PAN B product when the ORF was expressed in *E. coli*. This could be linked to the absence of ribosome binding like consensus in the PAN B primary sequence.

We used mRNA mapping experiments to check if the use of internal start codons for PAN expression arises from internal translational initiations or from shorter messenger RNAs devoid of the first start codon. Primer extension results revealed the existence of shorter PAN A and PAN B transcripts starting in between the first methionine codons and the second putative translation starts (Fig 4B). In PAN A, the 5' end of the short transcript was located at position - 85 upstream from the second ATG start codon. In PAN B, the extension product ended 3 nucleotides upstream the GTG initiation codon. We confirmed the existence of this second short PAN B transcript by RNase protection assay (Fig 4C). The molecular weight of the protected RNA fragment, calculated with respect to RNA molecular weight markers that were run alongside, is consistent with a leaderless transcript starting a few nucleotides upstream from the GTG initiator codon. These data suggested that a transcriptional control mediates the production of the PAN N-truncated versions. Taken together, these observations suggested that dual translational initiation is a mechanism conserved in all halophilic Archaea, used to produce truncated versions of the 2 PAN proteins.

Characterization of PAN A and PAN B oligomerization and association states in cell lysates

There is little information about the *in vivo* biophysical state of the AAA ATPase proteins. As for other members of the AAA ATPase protein family the *in vitro* chaperone and unfoldase activities are dependent upon their oligomerization state while

the stimulation of the 20S proteasome activity depends upon their capacity to associate with the proteasome outer alpha rings, probably as hexameric rings [12]. To study the PAN oligomerisation/association states in close to physiological conditions, post membrane *Halobacterium* extracts (S30) were fractionated through a 5-20% non-denaturing sucrose density gradient. The experiments were performed in the high salt conditions corresponding to the physiological ionic strength of *Halobacterium* cytosol [32]. To calibrate the sedimentation experiments, several protein complexes purified from halophilic Archaea, were run on the gradients. The proteins used as molecular weight markers were malate dehydrogenase (130 KDa), catalase peroxidase (180 KDa), TET aminopeptidase (504 KDa) and the 20S proteasome (720 KDa). To be sure that our experimental conditions corresponded to the native ones, the sedimentations of 4 halophilic proteins known to form quaternary structures of different sizes, were monitored by Western-blot analysis in the fractionated S30 extracts (Fig. 6). Among these proteins, P45 is a molecular chaperone that was previously characterized *in vitro* as a 12.5 S oligomer (450 KDa) by analytical centrifugation [23]. The sedimentation data showed that the P45 chaperone is indeed fully assembled as stable oligomers in the S30 *Halobacterium* cellular extracts. The Archaeal thermosome is a type II chaperonin (Hsp60) made of a double ring of 7 alpha and 7 beta subunits. The alpha subunits were detected in the gradients at the expected position for a stable, tightly associated 1 MDa double ring complex. In conclusion, all the halophilic protein complexes that we tested behaved in the total cellular extracts as homogeneous populations of stable oligomers, devoid of lower molecular weight oligomeric forms. Having this set of calibration and control experiments in hand, it was possible to assess for the biophysical states of the various PAN species in the archaeal extracts.

The PAN hexamers have a predicted molecular weight of 270 KDa and, according to our calibration profile, are expected to be present in fraction 6 of the sucrose gradients (Fig. 5). Unexpectedly, most of the 2 PAN proteins were detected in fractions 2-3 with sedimentations coefficients that are lower than that of the malate dehydrogenase. This shows that PAN A and PAN B accumulated as dimers or trimers in the cells and not as the stable hexamers that would correspond to their activated states. However, in all our experiments, we reproducibly found that a significant proportion of the N-ter truncated PAN B sedimented further in the density gradients, thus suggesting a self-association as hexamers and/or weak interactions with other large molecular assemblies. A significant

part of Δ PAN B was also found to sediment in fraction 15, thus indicating a propensity to associate with large cellular structures such as polysomes, or to form aggregates. These features were not observed with PAN A thus indicating that the two PAN proteins are not functionally equivalent regarding their oligomerisation/association properties and that, in the case of PAN B, the N-ter deletion seems to increase the rate of interaction compared to the full length version.

Regarding the PAN association states, the fractionation experiments demonstrated that the majority of PAN A and PAN B proteins accumulate in a proteasome-free state in post-cellular extracts. Interestingly, only Δ PAN B was found to sediment into the gradients as high molecular weight species up to fraction 10. The 20S peptidase particles were detected in the S30 fractionation experiments by using a specific activity test (Fig. 6). The 20S peptidase activity was found in fraction 9-10-11, while the purified 20S particle was found to sediment in fraction 8-9. One could therefore hypothesize that part of the 20S proteasome is associated with regulatory proteins such as Δ PAN B in the S30 extracts. *In vitro*, the *M. janaaschi* PAN-20S proteasome association was found to require nucleotide binding and ATP hydrolysis inhibits complex formation [33]. Therefore, to demonstrate that the Δ PAN B indeed corresponds to a labile association with the 20S proteasome, we tested the effect of ATP binding or hydrolysis on the sedimentation behavior of the PAN complexes (Fig. 6). Our data show that the physical states of the PAN complexes are clearly modified in the presence of hydrolysable ATP: less oligomeric forms were detected for PAN A and PAN B and the presence of Δ PAN B in the proteasome-containing fractions disappeared. On the contrary, when the cellular extracts were incubated in the presence of non-hydrolyzable ATP (ATP γ S), we observed a stabilization of the Δ PAN B associated state. To further demonstrate the capacity of PAN proteins to interact with the 20S proteasome we added an excess of purified 20S proteasome complexes in the S30 extracts. After incubation the extracts were fractionated on the sucrose gradient. The results showed that, compared to the control experiments, a larger proportion of the PAN B proteins run further into the gradient, indicating an interaction between the PAN protein and the 20S proteasome. Interestingly, in these conditions, even part of the full-length PAN B proteins assembled as high molecular weight species. We confirmed the effect of ATP on the PAN-proteasome interactions: when hydrolyzable ATP was added to the extracts,

less PAN-proteasome complexes were formed while non-hydrolyzable ATP had a stabilization effect on these complexes. In conclusion, our results showed that the PAN B-20S proteasome is a labile complex in cellular extracts and suggested that the truncated forms of PAN B interact more efficiently with the proteasome alpha rings than the full length form. Another important conclusion is that, under all the experimental conditions that we tested, we could never detect an interaction between PAN A and the 20S proteasome. Once again, these observations suggest that PAN A and PAN B are not functionally redundant in haloarchaeal cells.

DISCUSSION

Despite their importance for the regulation of intracellular proteolysis, there is little information about the *in vivo* physical states and the possible regulatory action of the proteolytic regulatory AAA ATPases, in all three kingdoms of life. The archaeal PAN systems may represent a paradigm to address these questions. In this study, we showed that a significant fraction of the PAN gene products accumulated in *Halobacterium* cells as N-terminal truncated polypeptides, 6 KDa shorter than the full length PANs. Due to an internal ribosome entry site, the recombinant PAN protein from *M. jannaschii* was found to be partially synthesized in *E. coli* as a derivative that had a deletion of 73 residues [15]. However, unlike the PANs from *Halobacterium*, no truncated proteins were detected in the *M. jannaschii* total extract. Interestingly, the ClpA and ClpX bacterial proteolytic regulatory AAA-ATPases that assemble co-axially with the ClpP peptidase, were also detected *in vivo* as two isoforms [34]. Internal translational initiation mechanisms were demonstrated for ClpA unfoldase as well as for the ClpB chaperone [35-37]. In the case of *Halobacterium* PAN A and PAN B, the primary sequences analysis and the kinetic studies of the gene product accumulation within translationally inactive cells suggest that the short molecules are also derived from the use of alternative translation starts that are conserved in all halophilic PAN genes. These observations indicate that N-terminal heterogeneity and the use of a second translational initiation site represent a widespread regulatory strategy for the prokaryotic ATPases implicated in intracellular proteolysis.

The molecular mechanisms responsible for the use of a second translation start in bacterial AAA ATPases are not understood. In this work, we showed that, in the case of the Archaeal PANs, a second transcript start is situated upstream to a second putative

translational start codon that is conserved in all haloarchaeal PAN sequences. This observation indicates that, in halophilic Archaea, transcriptional control is involved in the production of truncated AAA ATPase proteins. The archaeal translational apparatus is a mosaic of bacterial and eukaryal features [38, 39]. While the PAN A short transcript contain a bacterial-like SD motif, the PAN B gene is transcribed as a leaderless messenger RNA starting 3 bases upstream from a GUG codon (valine). The most common start codon in Archaea is AUG but, as in bacteria, GUG are also frequently used [30, 31]. Computer studies showed that, in archaea, many mRNAs are expected to be leaderless and, in *Halobacterium*, it has been shown that protein synthesis can be initiated efficiently with a GUG codon from a leaderless transcript [39]. The archaeal leaderless initiation mechanism involves an initiator tRNA and is reminiscent of the eukaryotic pathway [38]. Thus, it appears that the *in vivo* synthesis of the truncated PAN versions arise from a bacterial-like mechanism for PAN A and from a eukaryotic-like process for PAN B. This difference would explain why the short PAN A product is produced when the proteins were expressed in *E.coli*, while the truncated PAN B version cannot be detected in the *E.coli* extract.

In the *Halobacterium* cells the two versions of PAN A appeared to accumulate in equimolar quantities while, for PAN B the ratio between the two long and short forms varied to a greater extent. Several molecular mechanisms could be suggested to explain this. In the case of PANB, the ratio between the two PAN versions appeared to be consistent with the respective abundances of the two mRNA 5' ends detected by primer extension experiments. In the case of PANA, the long transcripts are more abundant than the short transcripts while the immunodetection experiments show that the short version of PANA is as abundant as the long ones. This discrepancy could be explained by the fact that the 5' end of the truncated transcripts is recognized more efficiently by the translational initiation complex than in the non truncated transcripts. Another possibility being that the Archaeal ribosomes have the capability to synthesize the short PANB transcript from the unprocessed mRNA by internal initiation or RNA scanning processes.

What is the functional significance of the N-terminally truncated variant of the haloarchaeal PAN proteins? Structural studies on other proteolytical ATPases such as the archaeal P97/VAT or the bacterial ClpA and HslU complexes showed that the N-

parts of the proteins correspond to flexible domains of about 150 residues located outside the hexameric rings when the complexes are assembled [21, 40]. These N-ter domains form smaller outer rings that have been observed in EM images of PAN-Proteasome complexes [21]. Many different functional studies have demonstrated that these N-terminal domains play an important role in modulating the nucleotidase activity and the unfolding rates of protein substrates [41-45]. The N-ter regions were also found to be the principal means of interaction with adaptator molecules which activate the recognition of specific substrates [46, 47]. Like all other known proteolysis regulatory ATPases, PAN possesses a predicted coiled-coil motif at its N-termini [14, 48]. In various chaperones, this motif was proposed to be involved in disaggregation activity and is also responsible for the recognition of substrate proteins [43]. Consequently, a 7 KDa shortening of the PAN A and PAN B N-ter parts is likely to confer different functional and physical properties to the AAA proteins. For example, in the case of the bacterial ClpA regulator, the 7 kDa-truncated protein derived from the internal translational initiation showed a reduced ATPase activity and inhibited the ClpP proteolytic activity [37]. The *in vivo* N-ter truncation may therefore represent a way to modify the biochemical activities of the PAN proteolytical regulators as well as their substrate specificity. The biochemical differences between the different versions of PAN A and PAN B could not be experimentally tested *in vitro*: the corresponding recombinant halophilic polypeptides were found to be insoluble when produced in *E.coli* and the native PAN proteins could not be purified from *Halobacterium* cell paste. However, cellular fractionation by gradient ultracentrifugation has showed that the truncated PAN B exhibits a better propensity to oligomerize and to interact with the 20S proteasome. These results suggest that the deleted PAN B has a higher affinity toward the 20S alpha rings compared to the full length polypeptides, probably due to a higher rate of oligomerization.

The AAA ATPases need to be assembled to perform their mechanical actions on substrate proteins and to associate with the peptidase complexes [12]. Most AAA+ modules or proteins have been crystallized as stable ring shaped oligomers. The recombinant PAN proteins from *M. jannaschii* were purified as large aggregates corresponding to 6 or 12 subunits [14]. There is limited information about the proteolysis regulatory AAA ATPases physical state, *in vivo*. Here, we report that, in cellular extracts, the vast majority of the PAN A and PAN B proteins accumulate as

dimers or trimers. These observations demonstrate that the PAN complexes do not exist as stable ring shaped edifices within the cytosol. Thus, *in vivo*, the formation of unfoldase-competent and proteolytically-active PAN particles must be a dynamic process driven by external factors such as ATP hydrolysis, substrate binding or their association with the 20S proteasome. *In vitro*, ATP binding and hydrolysis was found to control the oligomerization rates and the activity of many AAA ATPases, including PAN [17]. The present studies on PAN oligomeric states within cellular extracts indicate that ATP hydrolysis destabilizes the PAN low molecular weight oligomers, while ATP binding has a stabilization effect.

The association state between the proteolytical-activating complexes and their corresponding peptidase particles remains poorly documented in all 3 kingdoms of life. In the cytosol, the proteasome regulatory particles such as the 19S complex in Eukarya and PAN in Archaea could be constitutively docked on top of the 20S proteasome barrels (Fig. 7). However, a stable association between PAN and the 20S peptidase core was found to be difficult to obtain *in vitro* and it has been suggested that PAN stimulates proteolysis by altering the substrates and does not interact directly with the 20S proteasome [49]. Recently, surface plasmon resonance and electron microscopy experiments demonstrated that the *M. Jannaaschii* PAN oligomers can indeed interact *in vitro* to the 20S proteasome from *Thermoplasma acidophilum* under specific conditions [33]. In cellular extracts, our sedimentation experiments showed the existence of a PAN–20S proteasome interaction. However, the assemblies with the 20S proteasome appear to be labile and restricted to PAN B, with a better affinity for the N-ter truncated form of the protein toward the 20S proteasome. The fractionation experiments also demonstrated that the PAN proteins do not exist as stable and free active hexamers in the cytosol. In fact, the association dynamics of Δ PAN B appears to be a labile equilibrium between dimers/trimers and the proteasome-PAN complex. This short lived association can be displaced by adding an excess of 20S proteasome and is stabilized by ATP binding as demonstrated by the use of a slowly-hydrolysable ATP analogue. The presence of ATP weakens this association, probably due to ADP production. This ATP-dependent association is in accordance with the *in vitro* data that showed that ATP binding to PAN stabilizes its association with the 20S proteasome and that ATP hydrolysis triggers rapid dissociation of the 19S particles from the 26S proteasome [33,

50]. Together, these findings are consistent with a “dynamic” model that explains the protein degradation by the PAN-20S complex differently from those proposed previously (Fig. 7).

Although the two PAN proteins from *Halobacterium* are very similar, we have observed a striking difference between PAN A and PAN B regarding their respective abilities to oligomerize and to associate in the cellular extracts with the 20S proteasome core. In all cellular extracts that we have tested, only PAN B has been found to sediment as high molecular weights particles and to pellet as large aggregates in the polysomal fraction. It has been reported that the association of the PA28, 11S and 19S eukaryotic regulatory complex as well as the *M. jannaschii* PAN with the proteasome α -rings requires their C-terminal sequences [21, 49, 51]. This process involves a 7-residue peptide composed of a hydrophobic-sequence that has been recognized as an α -ring opening motif [21]. The PAN B C-ter sequence contains such a highly hydrophobic stretch (RYPSYIQ) while the PAN A C-ter extremity is clearly less hydrophobic. This difference could explain why PAN B can better interact with the 20S proteasome in the fractionated cell extract. A differential growth regulation was previously reported between the *Haloferax* PAN A and PAN B proteins, indicating that these proteins may not be functionally redundant [18]. The distinct sedimentation behaviors of the 2 PAN AAA-ATPases from *Halobacterium* strengthen this idea. An attractive hypothesis can be that the two PAN proteins have different substrate specificities: PAN B could be a “housekeeping” proteolytical activator while PAN A would bind regulatory factors or a distinct class of proteins only present in specific physiological conditions.

It is now clear that protein degradation by the PAN-proteasome system is a multi step mechanism that offers many possible levels of regulation: substrate recognition, ATP dependent unfolding, translocation and opening of the gated channel in the 20S proteasome [17, 33, 52]. Our findings suggest that, in addition to these processes, PAN N-terminal modifications and PAN intra cellular dynamics of assembly may constitute general determinants of proteolysis regulation. In the future it would be interesting to assess if PAN oligomerization rates and proteasome binding activity are regulated *in vivo* by global mechanisms and/or if specific substrate binding triggers the whole activation process.

Legends to figures :

Figure 1: Sequence alignment of *Halobacterium salinarium* PAN A and PAN B amino acids sequences (Hs) with the PAN sequence from *Methanocaldococcus janaashi* (Mj) and the Human 19S complex ATPase Rpt4. The two Hs open reading frames were retrieved from <http://Halolex.mpg.de>. The C-terminal peptides used to obtain PAN A and PAN B specific antibodies are underlined.

Figure 2: PAN A and PAN B accumulate as N-truncated polypeptides in *Halobacterium* cells. Proteins were resolved on 1D (Left) or 2D (Right) SDS-PAGE gels. T, *Halobacterium* extracts. R, protein extracts from *E. coli* cells expressing recombinant PAN polypeptides. The different isoforms of PAN polypeptides were detected by Western blotting with corresponding C-ter specific antisera. The bands, or the spots, situated at the level indicated by a star on the left correspond to N-ter truncated form of PAN A and PAN B.

Figure 3: Accumulation Kinetics of PAN A and PAN B polypeptides within *Halobacterium* after puromycin treatment. Total proteins were separated and detected by Western blotting with PAN A and PAN B C-ter specific antibodies. The positions of the N-ter truncated polypeptides are indicated by arrows.

Figure 4: Mapping of the PAN transcripts **A** : alignments of the N-ter regions of the 2 PAN proteins from *Haloferax volcanii* (Hv), *Haloarcula marismortui* (Hm) and *Halobacterium sp* (Hs). The conserved initiator methionines and their corresponding codons in the nucleotide sequences from the Hs PAN promoter regions (translated as a valine in the case of the second *PanB* initiation site) are boxed in grey. A star indicates the second internal codons. A potential Shine Dalgarno sequence situated upstream from the second *PanA* methionine is underlined. The positions of the second 5'-RNA starts (S2, in the mapping experiments presented in B and C are indicated by broken arrows. **B** : determination of PAN A and PAN B mRNA 5'-ends by primer extension analysis. PAN A and PAN B specific primers were 5'-end-labelled with $\gamma^{32}\text{P}$ -ATP and hybridized to total mRNA from *Halobacterium*. The hybrids were then extended using

reverse transcriptase and the lengths of the radioactive extension products were precisely determined by comparing their respective mobilities with DNA sequencing products in a 6% polyacrylamide-urea denaturing gel. Lanes T, G, C, A indicate the dideoxy termination lanes from sequencing reactions. Lane 1: primer extension products from PAN A. Lane 2: primer extension products from PAN B. The two main 5' transcripts ends (S1 and S2) are indicated by arrows. **C.** RNase protection mapping of the Pan B mRNA 5' end. An RNA radioactive antisense probe covering the PanB promoter region was synthesized and hybridized with total mRNA from *Halobacterium*. After RNase digestions, the protected RNA fragments were separated on a 7 M Urea/6% polyacrylamide gel. Lane P: untreated Probe-mRNA sample. Lane A, RNase protection assay. Lane M, RNA molecular weight markers. The RNA sizes are indicated in number of nucleotides. The two protected fragments, S1 and S2, are indicated by arrows. The length of the S2 fragment corresponds precisely to the second 5' ends determined by the primer extension experiment.

Figure 5. Determination of PAN A and PAN B oligomerization and association states by sucrose gradient ultracentrifugations. Post-membrane cellular lysates (S30) from exponentially growing *Halobacterium* were subjected to sucrose density ultracentrifugation (5-20% sucrose). Gradients were fractionated from the top (fraction 1) to bottom (fraction 15) for subsequent analysis. The pellets containing the polyribosomes were resuspended and pooled with fractions 15 (15+P). The proteins from each fraction were TCA precipitated and separated by SDS-12% PAGE. **A :** Coomassie Blue staining pattern of the fractionated cellular extract. The fractions numbers are indicated on top and the arrow represent the sedimentation orientation. **B.** Distribution of specific *Halobacterium* complexes in the gradients. Proteins were transferred onto membranes and probed with the indicated antibodies (Western blots): MDH, anti-malate dehydrogenase; CP, anti-catalase peroxidase; P45, anti-salt shock protein P45; TF55, anti-alpha subunit of the thermosome, PAN A, anti PAN A-Cter; PAN B, anti PAN B C-ter. The numbers on top correspond to experimentally-determined positions of different halophilic high molecular weight complexes that were purified to homogeneity and run on 5-20% sucrose gradients in conditions identical to the S30. 130 KDa: malate dehydrogenase, 180 KDa: catalase peroxidase, 420 KDa: TET

aminopeptidase and 720 kDa: 20S proteasome. The position of the N-ter truncated PAN polypeptides (Δ PAN A and Δ PAN B) are indicated by an arrow.

Figure 6. Sedimentation behaviors of PAN A and PAN B in the presence of ATP and the 20S proteasome. S30 extracts were treated with ATP, ATP γ S, and/or supplemented with purified 20S proteasome. Proteins were resolved by sucrose density gradient ultracentrifugation and the distribution of PAN A and PAN B was analyzed by immunodetection as described. The 20S proteasome was followed in the fractions collected from untreated gradient by measuring the hydrolysis of the fluorogenic peptide substrate Suc-LLVY-AMC, in the absence (filled circle) and in the presence (open circles) of NLVS, a 20S proteasome-specific inhibitor. The resulting hydrolytic activity profiles (fluorescent units liberated per min and per mg of protein) are reported on the lower panel.

Figure 7. Models explaining the *in vivo* functioning of the PAN-proteasome system. According to the studies presented in this paper, the two first mechanisms, presented on the left, are unlikely to occur within cells and the boxed “dynamic model”, on the right, can be proposed. **1.** “Static” model: the PAN hexamers are constitutively docked on top of the proteasome, the proteins to be eliminated are recognized, and unfolded at the level of already assembled PAN-20S proteasome complexes. **2.** “Pull down” model: the PAN proteins exist as stable hexamers in the cells that are competent to recognize and to target substrate proteins to the 20S proteasome where they will be unfolded and translocated into the peptidase catalytic cavity for proteolysis. **3.** “Dynamic” model: The PAN proteins accumulate as a pool of low molecular weights species (dimers or trimers) that oligomerize and assemble transiently with the 20S proteasome as indicated by the brackets. ATP hydrolysis weakens these interactions while ATP binding, and possibly protein-binding, stabilize the PAN-proteasome assembly and/or the hexamerization of the PAN proteins. In *Halobacterium*, it appeared that only the N-ter truncated form of PAN B (Δ PAN B) displayed significant interactions with the 20S proteasome. No interactions have been detected with PAN A in the physiological conditions tested.

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Figure 1

Figure 1 displays the amino acid sequence alignment of the PANA-Hs, PANB-Hs, PAN-MJ, and Rpt4-Human proteins. The sequences are shown in blocks, with positions 1 to 50, 60 to 100, 110 to 170, 180 to 240, 250 to 310, 320 to 380, and 390 to 400 indicated. The sequences are aligned to show conserved regions and differences between the four proteins.

Sequence 1 (Positions 1-50):

```

PANA-Hs  . . . . . M T D T . . . V E D V E L P Y D D S A S Q Q D K L E A L E E Q L S T L E E E N E . . . E M R D R L L D A N A E N
PANB-Hs  . . . . . M S R S P S L P D R P T L D V D P E S T P A E R L N A L Q D H Y V D I V A V N G . . . E L Q A Q L D D V E A R R
PAN-MJ    . . . . . M V F E E F I S T E L K K K K K A P T E E F K E E K E I N D N S N L K N D L L K E L Q E K A R I A E L E S R I L K L E L E K
Rpt4-Human M G Q S Q S G G H G P G G G K K D D K D K K K K Y E P P V P T R V G K K K K K T K G P D A A S K L P L V T P . H T Q C R L K L K L E R I K

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Sequence 2 (Positions 60-100):

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PANA-Hs  N . . . . . K Y Q Q K L E R S S H E N K K L K . . . . . Q S P L F I A T V Q E L T D E G . A V I K Q H G N N Q E A L T E V T D E
PANB-Hs  E . . . . . E L R E E V N R L Q R E N E T L K . . . . . T A S L Y L A T V E D L P E D G S A V I K Q H G N N Q E V L T E L S P R
PAN-MJ    K . . . . . E L E R E N L Q M K E N E I L R R E L D R M R V P P L I V G T V V D K V G E R K V V K S S . T G S F L V N V S H F
Rpt4-Human D Y L L M E E E F I R N Q E Q M K P L E E K Q E E E R S K V D D L R G T P M S V G T L E E I I D D N H A I V S T S . V G S E H Y V S I L S F

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Sequence 3 (Positions 110-170):

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PANA-Hs  L R . A T L E P G S R V A V N . N S L S V V R O L D D E A D V R A R V M E V D D S P D V G Y E D I G G L D D Q L R E V R S T V E L P M K D P
PANB-Hs  L A . D T L E V G D R V A I N . D S F S V Q R V L D D E T D A R A Q A M E V D E S P S V T Y A D I G G L D D Q L R E V R E A V E D P L V N P
PAN-MJ    V N P D D L A P G K R V C L N Q O T L T V V D V L P E N K D Y R A K A M E V D E R P N V R Y E D I G G L E K Q M Q E I R V V E L P L K H P
Rpt4-Human V D K D L L E P G C S V L L N H K V H A V I G V L M D D T D P L V T V M K V E K A P O E T Y A D I G G L D N Q I Q E I K S V E L P L T H P

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Sequence 4 (Positions 180-240):

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PANA-Hs  G L F E T V G I N P P S G V L L H G P P G T G K T L M A K A V A S Q T D A S F I K M A G S E L V H K F I G E G A K L V R D L F O V A R D H E
PANB-Hs  E K F D A V G I E P P S G V L L H G P P G T G K T L M A K A V A N Q T D A S F I K M A G S E L V R K F I G E G S R L V R D L F E L A E Q K D
PAN-MJ    E L F E K V G I E P P K G I L L Y G P P G T G K T L L A K A V A T E T N A T E I R V V G S E L V K K F I G E G A S L V K D I F K L A E K A
Rpt4-Human E Y Y E E M G I K P P K G V I L Y G P P G T G K T L L A K A V A N Q T S A T F L R V V G S E L I Q K Y L G D G P K L V R E L F R V A E H A

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Sequence 5 (Positions 250-310):

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PANA-Hs  P A V V F I D E I D A I A S K R T D S K T S G D A E V O R T M N Q L L S E M D G F D E R G D I R I T A A T N R F D M L D R A I L R P G R F D
PANB-Hs  P A I I F I D E I D A V A A K R T D S K T S G D A E V O R T M N Q L L S E M D G F D E R G D I R I T A A T N R F D M L D S A I L R P G R F D
PAN-MJ    P S I I F I D E I D A I A A K R T D A L T G D R E V O R T L M Q L L A E M D G F D A R G D V K I I G A T N R F D I L D P A I L R P G R F D
Rpt4-Human P S I V F I D E I D A I G T K R Y D S N S G G E R E I Q R T M L E L L N Q L D G F D S R G D V K V I M A T N R I E T L D P A I L R P G R I D

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Sequence 6 (Positions 320-380):

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PANA-Hs  R L I E V P H P N V G G R E K I F R I H T R A M N V A D S V D F G E L A A D T G D L S G A D V K A I C T E A C M F A I R D D R T E V R M Q D
PANB-Hs  R L I E V P N P N P D A R E R I L E T H A G E M N V A D S V D F S D L A A D T A E F S G A Q L A S L A T E A C M F A I R D D R D E V H R Q D
PAN-MJ    R L I E V P A P D E K G R L E I L K I E T R K M N L A E D V N L E E I A K M T E G C V G A E L K A I C T E A C M N A I R E L R D Y V T M D D
Rpt4-Human R K I E F P L E D E K T K K R I F Q I E T S R M T L A D V T L D D L I M A K D D L S G A D I K A I C T E A G L M A L E R R M K V T N E D

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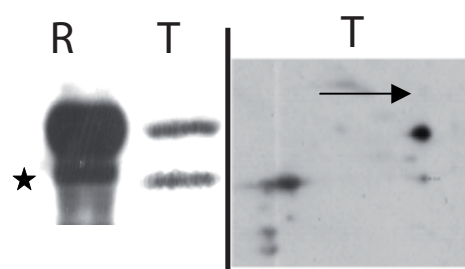
Sequence 7 (Positions 390-400):

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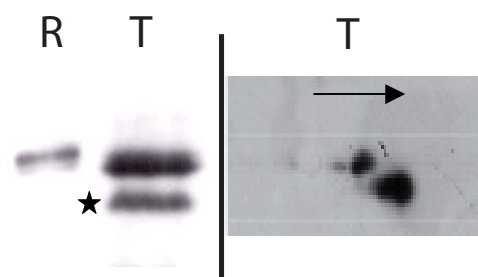
PANA-Hs  F Q S A R E K L D O D S E P A A A T D V S R T F A . .
PANB-Hs  F D D A Y E K L V A E G D T E S S G P R Y P S Y I Q .
PAN-MJ    F R K A V E K I M E K K K V K V K E P A H L D V L Y R
Rpt4-Human F K K S K E N V L Y K K Q E G T P E G L Y L . . . .

```

Figure 2



Anti C-ter PANA



Anti C-ter PANB

Figure 3

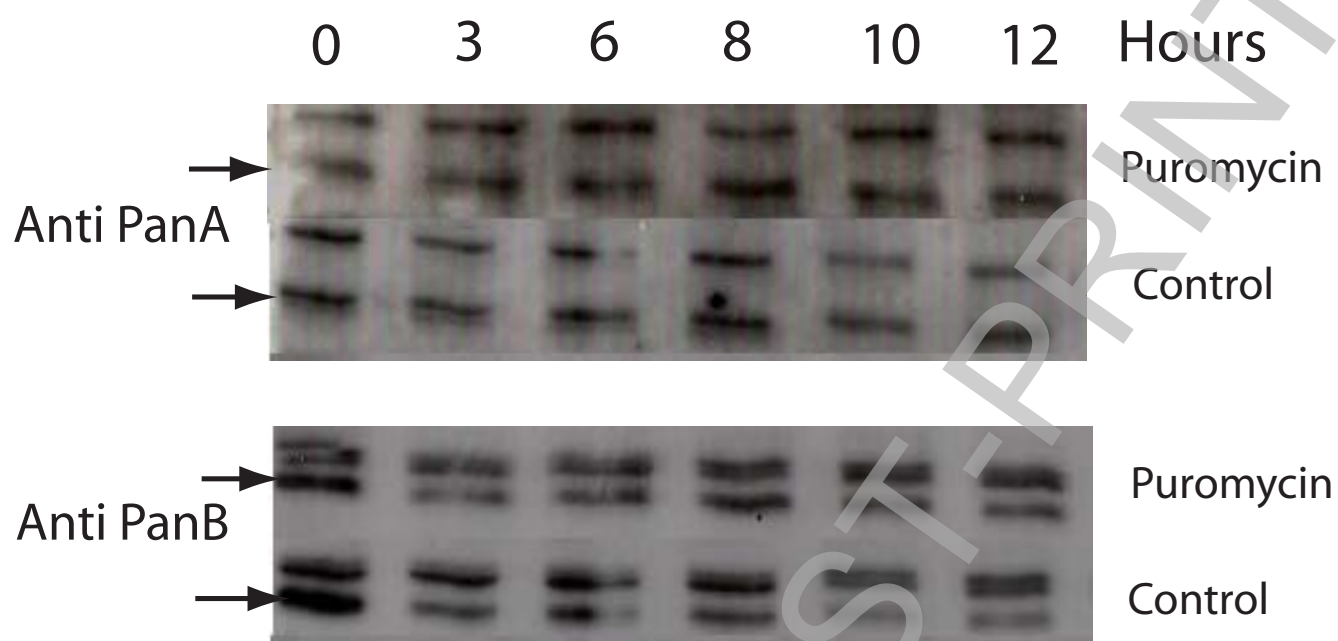


Figure 4

A

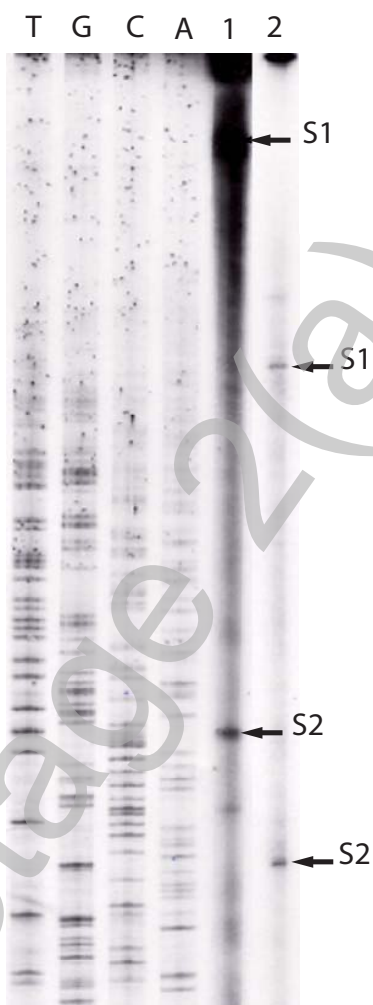
HvPanA MMTDTVDVDLPYDKDSASQQEKITALQERLEVLETQNEEMRDKLLDTNAENNKYQQKLE
HmPanA M-TDTVDEVDMPYD-DDASQQQKIEALQERLEVLETQNEEMRDKLLDANAENNKYQQKLE
HsPanA M-TDTVEDVELPYD-DSASQQDKLEALEEQLESTLEENEEMRDRLLDANAENNKYQQKLE

HspanA ATGACCGATACCGTCGAGGACGTGGAGCTCCCTACGACGACAGCGCCTCACA
M | T | D |
CAGGACAACTGGAGGCGCTAGAGGAGCAACTCTCT ACCCTCGAAGAGGAGAA
CGAGGAGATGGGAGAC..
M | R | D |

HvPanB MSRSPSLPERPHLDLPEDMSDAERLSALRQHFERMVDVNRELDQRLQNAADDRHAELVDEV
HmPanB MSRSPSLPERPRLELDPDMTPDERLEALREHFKEIVQVNEQLTEQLDAA RDRQHDLTDEV
HsPanB MSRSPSLPDRPTLDVDPESTPAERLNALQDHYVDIVAVNGELQAQLDDVEARREELREEV

HspanB ATGTCGCGTAGCCCCCTCATTGCCGACCGGCCGACGCTCGACGTCGATCCCAGT
M | S | R |
CGACGCCGGCGGAA CGCCTCAACGCGCTCCAAGACCACTACGTGGATAT CGTGG
N | G | M |
CGGTCGCGGTCAA.....

B



C

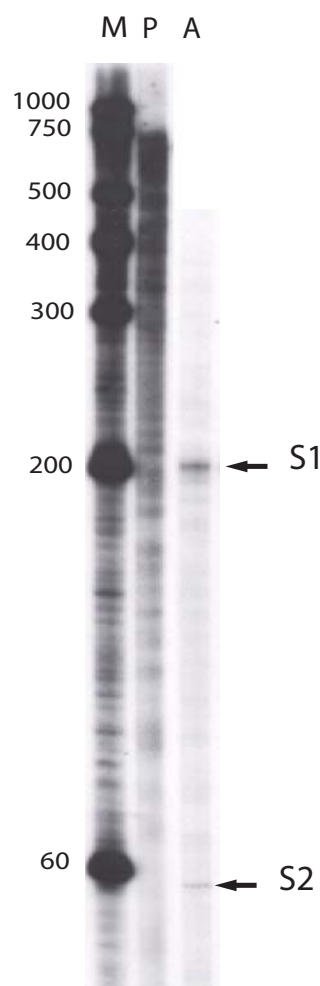


Figure 5

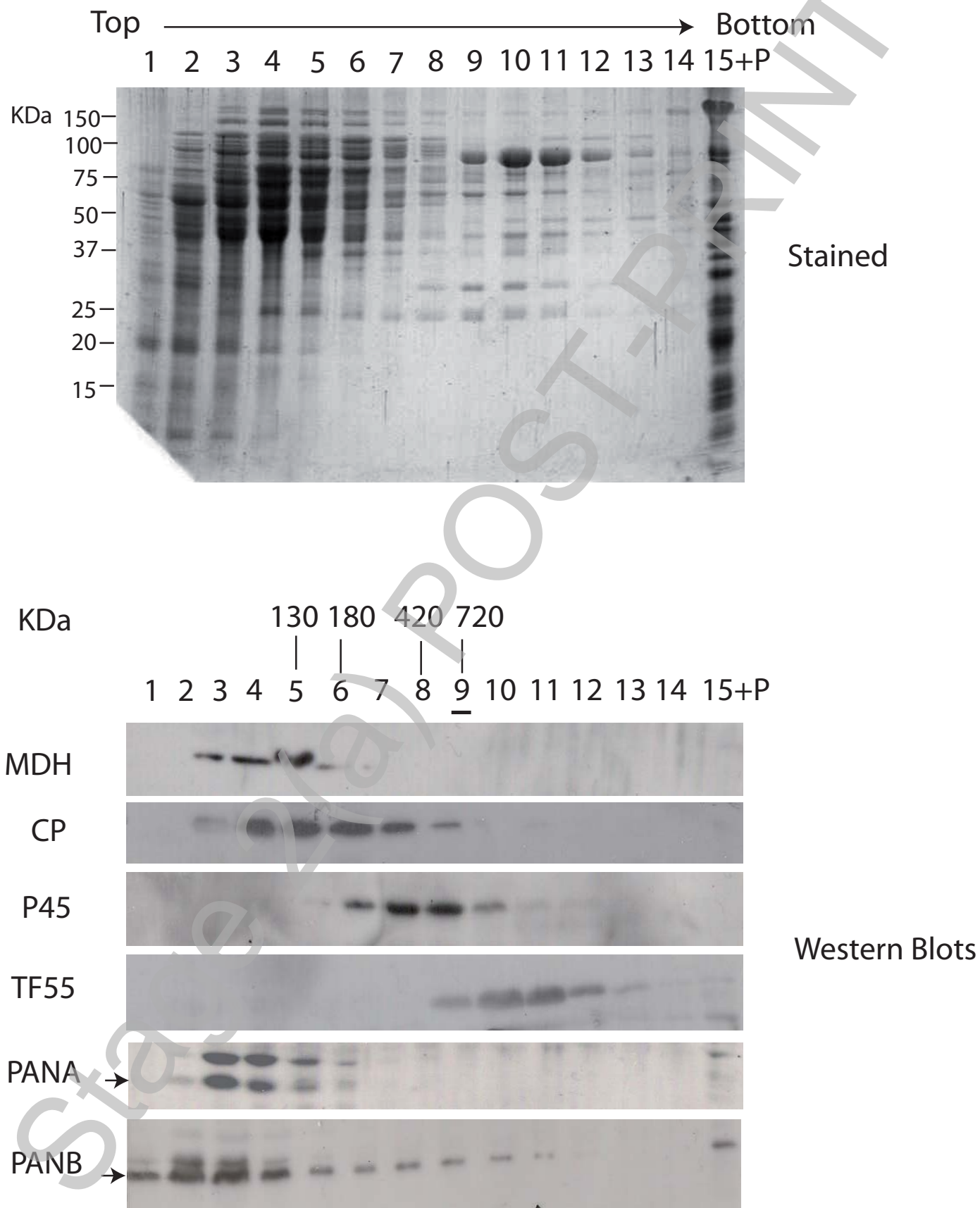


FIGURE 6

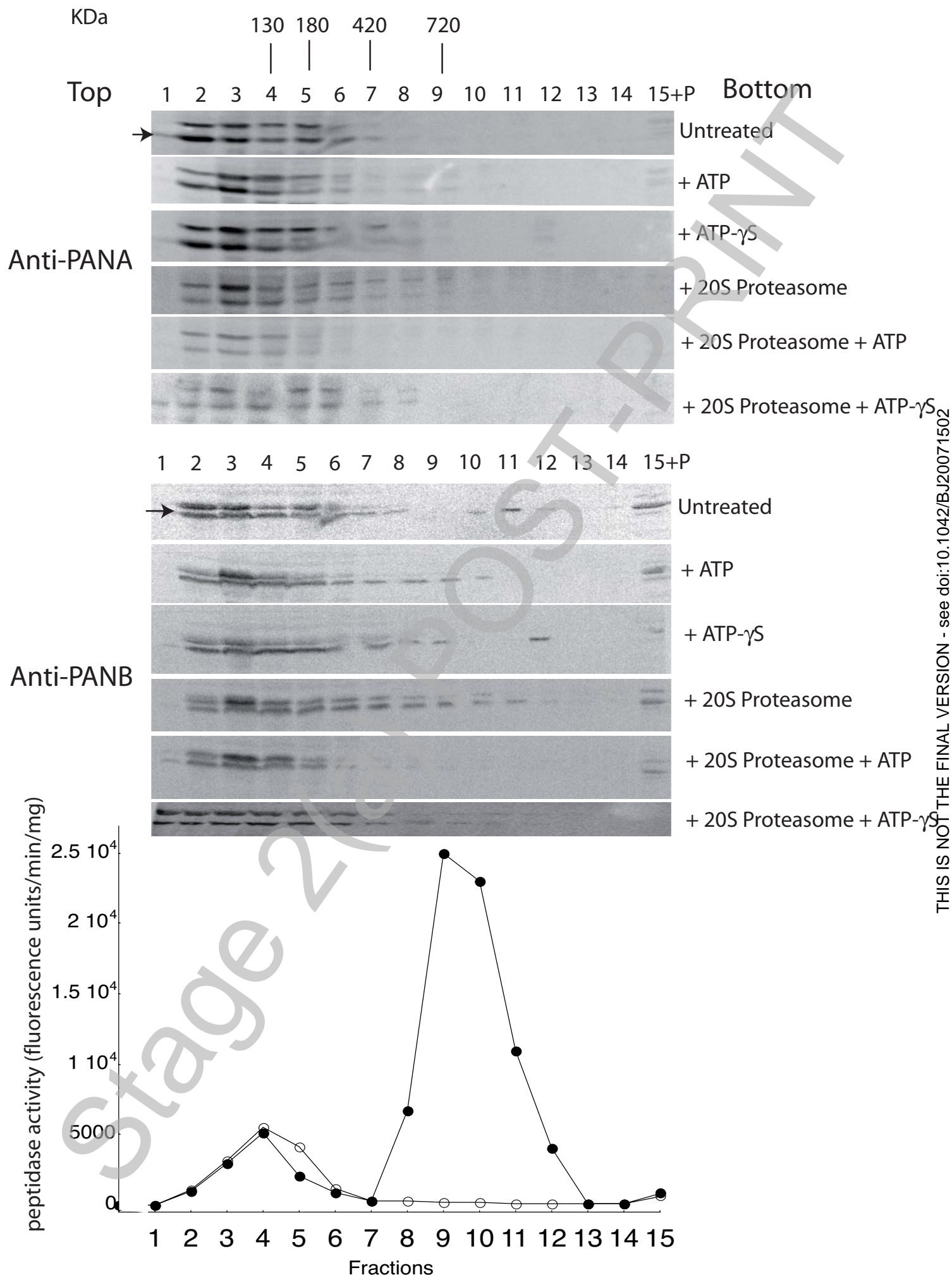


Figure 7

