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Proteins interacting with the 26S proteasome

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Abstract. The 26S proteasome is the multi-protein protease that recognizes and degrades ubiquitinylated substrates targeted for destruction by the ubiquitin pathway. In addition to the well-documented subunit organization of the 26S holoenzyme, it is clear that a number of other proteins transiently associate with the 26S complex. These transiently associated proteins confer a number of different roles such as substrate presentation, cleavage of the multi-ubiquitin chain from the protein substrate and turnover of misfolded proteins. Such activities are essential for the 26S proteasome to efficiently fulfill its intracellular function in protein degradation.

Key words. Proteolysis; 26S proteasome; ubiquitin; multi-ubiquitin binding proteins; UBA domain; UIM domain; UBL domain.

Introduction

The 26S proteasome is the multi-protein protease that is responsible for degradation of intracellular proteins of which most have been targeted by the addition of a multi-ubiquitin chain to a lysine residue. In vitro experiments have demonstrated that for efficient recognition of the protein substrate the multi-ubiquitin chain must be made up of at least four ubiquitin moieties. The 26S proteasome is composed of two stable multi-protein complexes called the 20S proteasome and the 19S regulatory complex [1, 2]. In this review the Rpn/Rpt budding yeast nomenclature is used first, followed by the fission yeast names and lastly the human names of the different proteins [3].

The 20S proteasome is the catalytic complex. All the peptidase activities for proteolytic cleavage of the protein reside in this structure. The complex is made up of 14 different proteins, with each subunit represented twice. These are classified as either α subunits or β subunits based on their similarities to the two subunits found in the simpler version of the 20S proteasome found in the archaebacterium *Thermoplasma acidophilum*. The α and β subunits form four seven-membered rings lying on top of each other to form a barrel structure. The two α rings are located at either end of the structure while the two β rings are positioned internally, yielding a conformation $7\alpha7\beta7\beta7\alpha$. The crystal structure has been solved for the 20S complexes found in *Thermoplasma*, budding yeast and mammals allowing a detailed insight into how protein substrates are cleaved. The crystal structure demonstrated that the 20S proteasome contains three internal chambers, and degradation occurs inside the central inner chamber, consistent with the observation that three of the β subunits, $\beta1$, $\beta2$ and $\beta5$, harbor the three known peptidase activities of the complex [4-6]. Like the large chaperone complexes such as GroEL, the 20S proteasome can be thought of as an example of a protein organelle where the activity of the complex occurs internally protected from the outside cytosol. Although, the structures worked out for the 20S complex from archaebacterium, yeast and mammals were very similar, they differed in one important aspect. The *Themoplasma* complex appeared to have a narrow pore 1.3 nm wide in the α rings at each end of the complex, while in the eukaryotic 20S proteasome this pore was absent and the complex appeared closed with no apparent access to the internal chambers of the complex. The solution to this paradox described below is that after binding to the 19S regulatory complex to form the mature 26S proteasome, an allosteric interaction is thought to occur, resulting in the opening of a narrow pore in the external α rings of the 20S complex. This function of the 19S complex is referred to as the 'gating function'. One prediction from the size of the narrow pore that forms in the α rings is

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that only an unfolded polypeptide will be allowed entry into the 20S complex. Therefore, the protein substrate must first be fully unfolded before being translocated into the 20S complex for degradation [7, 8].

The 19S regulatory complex is made up of 17 or 18 subunits. Under certain conditions, such as high salt concentrations, the complex breaks down into two sub-complexes called the lid and the base. The base is made up of 10 subunits. Six of the subunits are ATPases of the AAA family of ATPases. These ATPases forms a six-membered ring and interact directly with the α ring of the 20S proteasome [8–12]. According to in vitro cross-linking studies one of the ATPases, Rpt5/S6a, is able to bind to multi-ubiquitin chains, implying that this subunit forms part of the mechanism by which ubiquitinylated substrates are recognized by the 26S proteasome [13]. In addition, the ATPase ring has been shown to possess two additional biochemical activities essential for the successful degradation of protein substrates. First, once bound to the α ring of the 20S complex, the ATPase ring is responsible for the formation of a narrow pore to allow the polypeptide to enter the catalytic complex, the so-called gating function alluded to above. Second, the ATPases are at least partly involved in the anti-chaperone activity required to unfold the protein to allow entry into the catalytic complex [7, 8]. The other four subunits of the base subcomplex are all non-ATPases. One, Rpn10/Pus1/S5a, contains a UIM (Ubiquitin Interacting Motif) domain which binds to ubiquitin chains in vitro but has little affinity for mono-ubiquitin [14, 15]. Another subunit, Uch2/UCH37, has sequence homology to ubiquitin hydrolases [9, 16]. Surprisingly, this subunit appears not to be present in the budding yeast. In addition, a null mutant in the fission yeast UCH37 ubiquitin hydrolase orthologue, *uch2*$^+$, was viable, demonstrating that the function of this ubiquitin hydrolase was not essential for ubiquitin recycling [16]. At present the role that this ubiquitin hydrolase plays in ubiquitin dynamics remains elusive. The other two subunits, Rpn1/Mts4/S2 and Rpn2/S1, seem to play a central role in the structure of the 19S complex in that they provide a link between the ATPase ring of the base sub-complex and the lid sub-complex [17]. In addition, these subunits seem to play an important role in acting as a platform to facilitate the transient interaction of a number of non-proteasomal proteins (see below).

The lid sub-complex is made up of eight non-ATPase subunits. One of the subunits, Rpn11/Pad1/S13, has recently been to shown to contain a novel metalloprotease domain and plays a key role in the recycling of ubiquitin by cleaving the ubiquitin chain from the protein substrate [18]. The function of the other subunits is at present unknown. However, the lid complex shows a remarkable conservation in its overall structure/subunit composition with two other protein complexes, the COP9/Signalosome and the eIF3 complex [19, 20].

The above subunits are taken to compose the holoenzyme of the 26S proteasome. However, recent studies from budding yeast have implicated three additional subunits, the deubiquitinating enzyme, Ubp6, the HECT ubiquitin ligase, Hul5 and the previously uncharacterized protein, Ecm29, to be present in the 26S complex. These proteins were identified from affinity-purified proteasomes where Rpn11 or Rpt1 or Pre1 subunits were epitope tagged with the TEV-Protein A epitope. The probable reason why these proteins were not previously identified in 26S proteasome preparations was that all three were found to dissociate under conditions of high salt concentrations. Such salt conditions were used routinely in the chromatographic methods used to purify the 26S proteasome to homogeneity. Biochemical studies indicated that the Ecm29 protein appeared to be involved the interaction between the 20S proteasome and the regulatory complex as the mature 26S proteasome appeared to be much more stable in the presence of the Ecm29 protein. In addition, a strain in which the *ECM29* gene had been deleted, although viable, was more sensitive than wild-type cells to the amino acid analogue canavanine. Such a phenotype is common to mutants in the ubiquitin/proteasome system, implying that the Ecm29 protein was an authentic subunit of the 26S proteasome [21].

The proteins described above represent the components of the holoenzyme of the 26S proteasome. However, a growing body of work has indicated that a number of additional proteins interact transiently to aid in the turnover of proteins by this complex. The remaining part of this review will describe these proteins and their potential roles in the efficient recognition and turnover of proteins by the 26S proteasome.

Multi-ubiquitin chain binding proteins

Studies, mainly from fission and budding yeast, have shown that a number of proteins seem to act as adapters to present ubiquitinated proteins for destruction by the 26S proteasome. Three types of proteins seem to be involved in this process.

The first is the Rpn10/Pus1/S5a protein. In most organisms this protein exists in two forms. The Rpn10/Pus1/S5a protein exists as a subunit of the 26S proteasome (see above) and as a free subunit outside the proteasome [22–25]. The Rpn10/Pus1/S5a protein has the properties expected for a multi-ubiquitin receptor in that it is able to bind to multi-ubiquitin chains but shows little affinity for mono-ubiquitin [14]. The multi-ubiquitin binding domain has been identified by deletion analysis to reside in the C-terminal UIM. The UIM domain is 20 amino acids long and is present in a number of different proteins in addition to the Rpn10/Pus1/S5a proteasomal subunit [15].

The Rpn10/Pus1/S5a protein is able to interact with the proteasome via its N-terminus. The Rpn10/Pus1/S5a subunit is able to interact with the non-ATPase base subunits, Rpn1/Mts4/S2 and Rpn2/S1. In each case the Rpn10/Pus1/S5a subunit interacts with the leucine-rich repeat PC domain found in both subunits. Interestingly, a PC domain is also found in the APC/cyclosome complex subunit Apc1/Cut4/Tsg24. Consistent with this observation in vitro studies in fission yeast have shown that the Pus1/Rpn10 protein can interact directly with the Mts4/Rpn1, SpRpn2 and Cut4 proteins. In addition, a *pus1/rpn10* deletion strain is synthetically lethal, with a *cut4-533* strain indicating a possible interaction in vivo [24, 26].

The second family of proteins that appear to be involved in recognition of ubiquitinylated substrates are the Dsk2/Dph1, Rad23/Rhp23 and the Ddi1 proteins. Each of these proteins contains two conserved domains intimately involved in the presentation of substrates, the UBL domain and the UBA domain. The UBA domain is a 50-amino acid domain originally identified by sequence comparison [27]. The structure of the UBA domain has been solved by nuclear magnetic resonance (NMR) spectroscopy and is composed of three α helices exposing a hydrophobic patch that is thought to act as a ubiquitin interacting domain [28, 29]. Although the UBA domain can bind mono-ubiquitin, it has a much higher affinity for multi-ubiquitin chains [30–33]. In addition to the UBA multi-ubiquitin binding domain each of the above proteins also contains a second domain at the N-terminus called a UBL domain. The Ubiquitin Like domain is so called because it bears homology to ubiquitin. Typically, UBL domains have ~20–30% identity with ubiquitin. The UBL domain has been shown to be a proteasome interacting domain (see below).

The two sets of multi-ubiquitin binding proteins share similar biochemical properties. The Rpn10/Pus1 and the UBL/UBA proteins were able to bind to the 26S proteasome via their N-terminus, and both were also able to interact with multi-ubiquitin. Therefore, it was not surprising that genetic experiments in budding and fission yeast have demonstrated that the different multi-ubiquitin binding proteins share overlapping functions in vivo. In fission yeast deletions in the *pus1*$^+$, *rhp23*$^+$ or the *dph1*$^+$ genes are all viable, while the triple mutant strain is lethal. It has been proposed that the Rpn10/Pus1 and the UBL/UBA domain containing proteins function as adapter proteins to present substrates to the 26S proteasome for efficient degradation [30, 34–36] (see fig.1).

Two observations have placed some doubt that the simple model shown in figure 1 will turn out to be an accurate reflection of what is happening in vivo. The first is that the UBA containing proteins have been shown to have the ability to dimerize. In budding yeast the UBA domains were responsible for homodimerization of Rad23 and heterodimerization of Rad23 with Ddi1, but surprisingly not for the homodimeriziation of Ddi1 [37]. Presumably the heterodimerization between different UBA containing proteins will turn out to have some regulatory role. However, the work of Bertolaet et al. demonstrates that the UBL/UBA proteins will not function as a monomer but as part of a complex. The second observation is that in an in vitro system the efficiency of degradation of a test substrate did not increase when the concentration of the Rad23 protein was increased, a prediction of the simple model shown in figure 1. In fact the opposite was observed; degradation of the test substrate was inhibited. This implied that the Rad23 protein was not functioning as an adapter protein [33].

If the proteins with both a UBL and UBA domain are acting as adapter proteins, then an important question is what subunit interacts with the UBL domain. A number of recent reports have addressed the question. Using a cross-linking assay, Saeki et al. identified Rpn1 and Rpn2 as the UBL interacting subunits [38]. In contrast, further studies in both fission and budding yeast have implicated

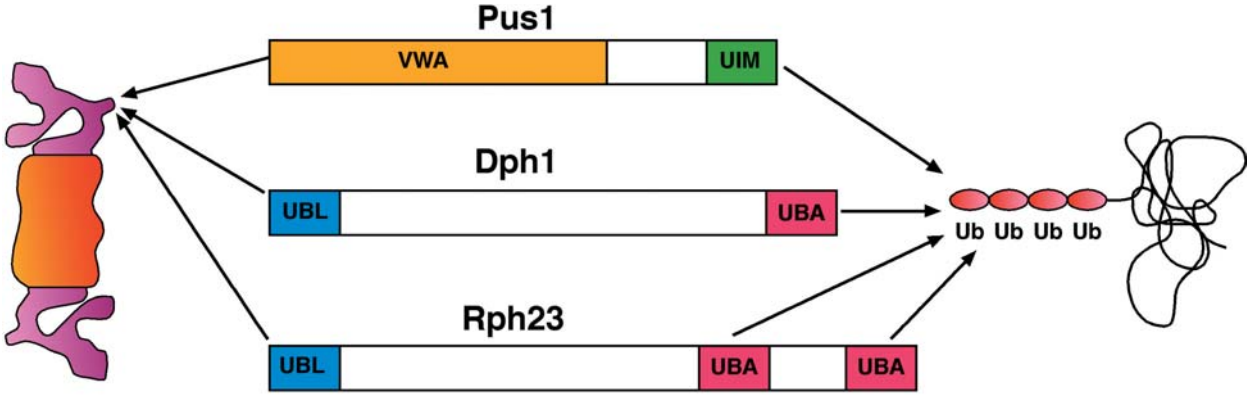

Figure 1. Presentation of ubiquitinated substrates to the 26S proteasome. Rpn10/Pus1/S5a, Dsk2/Dph1 and Rad23/Rhp23 have redundant roles in the presentation of substrates to the 26S proteasome. All three bind to the 26S proteasome via their N-terminus and to ubiquitinated substrates via their C-terminal UIM domain (Rpn10/Pus1/S5a) or UBA domain (Dsk2/Dph1 and Rad23/Rhp23).

only the Rpn1/Mts4 subunit as the UBL binding subunit, both laboratories finding no evidence for interaction with the Rpn2 subunit. Interestingly, both laboratories found that the Rpn1/Mts4 protein showed no affinity for ubiquitin. However, deletion analysis carried out by both studies defined different regions of the Rpn1/Mts4 protein to be responsible for the UBL interaction. Further experiments will have to be carried out to resolve this apparent paradox [26, 39].

The Rpn1/Mts4 and the Rpn2 subunits seem therefore to play multiple roles in the normal function of the 26S proteasome. They provide a link between the lid sub-complex and the ATPase ring of the base. In addition, they also seem to act as a binding platform for the multi-ubiquitin binding proteins. The Pus1/Rpn10 protein is in contact with both Rpn1/Mts4 and Rpn2 via the respective PC domains. The UBL/UBA containing proteins bind to Rpn1/Mts4. As can be seen from figure 2, the ubiquitinylated substrates therefore bind to a region between the lid sub-complex and the ATPase ring. This will probably position the substrate ready for threading through the base sub-complex and into the reaction chamber of the proteasome barrel.

Recently it has been demonstrated in vitro that multi-ubiquitin binding proteins protect ubquitinylated substrates from disassembly by ubiquitin hydrolases. This protection was dependent on the ability of the proteins to bind to the ubiquitin chains. The mechanism of protection of disassembly appeared to be that once the multi-ubiquitin chain binding proteins bind, they no longer allow access of the ubiquitin hydrolases to cleave the chains. Therefore, it has been speculated that the multi-ubiquitin chain binding proteins promote degradation by presenting substrates to the 26S proteasome and by protecting the ubiquitinylated substrates from deubiquitination [33, 40].

A third protein potentially involved in substrate presentation to the 26S proteasome is the Cdc48/Vasolin containing protein (Vcp). Studies in mammalian cells and budding yeast have demonstrated that the Cdc48 protein can bind to multi-ubiquitin chains. Deletion analysis identified the N-terminal region of the Cdc48 protein as being critical for multi-ubiquitin chain binding. Cdc48/Vcp protein also copurifies with the 26S proteasome, implying that the Cdc48/Vcp protein could also function as adaptor protein [41, 42].

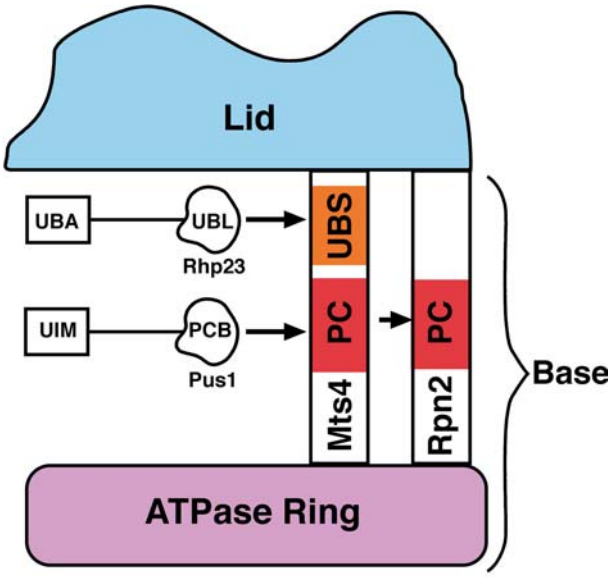

Figure 2. Interaction of the multi-ubiquitin binding proteins with the fission yeast proteasome. The Pus1 protein binds to the PC domains of Mts4/Rpn1 and Rpn2 proteins via its PC binding domain (PCB) located at he N-terminus of the Pus1 protein. The Rhp23 protein binds to the UBL binding sequence (UBS) of the Mts4/Rpn1 protein. Ubiquitinated protein substrates are thus brought between the Lid sub-complex and the ATPase ring via binding to the UIM and UBA multi-ubiquitin binding domains.

Other UBL domain containing proteins

The UBL domain has been identified in the N-terminus of a number of additional proteins. Some of these proteins are shown in figure 2. In three cases, the mammalian Bag1 protein, the Ubp6/Ubp14 protein and the fission yeast Udp7 protein, the UBL domain has been shown to be functional, allowing the protein to interact efficiently with the 26S proteasome. Two, the Ubp6/Ubp14 and the Udp7 proteins, have also been shown to interact specifically with the Rpn1/Mts4/S2 proteasome subunit [21, 26, 43]. This adds to the growing amount of data that all the UBL containing proteins will be able to interact with a common binding domain on the Rpn1/Mts4/S2 subunit. Presumably, access to this domain by the different UBL containing proteins would be subject to strict regulation. However, this simple model of UBL/proteasome interaction appears to be more complicated based on studies in human cells. Work from several laboratories has detected an interaction between the UBL domains of the Parkin, Plic1 (a human orthologue of the budding yeast *DSK2* and fission yeast *dph1*$^{+}$ genes) and the hRad23 proteins with the human Rpn10 orthologue S5a. When the binding site on the S5a protein was mapped by deletion analysis, it was shown to be in a region that is not found in the simpler orthologues of the S5a protein seen in either budding or fission yeast. This implies that there are two different binding sites on the mammalian 26S proteasome for the UBL domain. This adds another layer of regulation for the binding of UBL containing proteins to the proteasome which does not appear to be present in yeast [44–46].

Some of the UBL-containing proteins have additional biochemical activities known to be intimately involved in the ubiquitin/proteasome system. The human parkin gene

has a RING finger motif and has been shown biochemically to function as an E3 ligase. Recent work has shown that Parkin constructs which have had the UBL domain removed appear to be more stable, implying that binding to the proteasome is involved in efficient turnover of the full-length protein [47]. The UBL containing protein Ubp6 is a ubiquitin hydrolase. One hypothesis is that the Ubp6/USP14 protein is involved in the recycling of the ubiquitin chains, which are not degraded by the proteasome [21, 48].

The Bag1 protein has a BAG domain, whose function is to bind to the ATPase domain of Hsp70 proteins and regulate its chaperone activity. Interaction of the Bag1 protein with the proteasome could provide a link between the switch from trying to refold a misfolded protein, via the chaperone pathway, to targeting it for degradation by the 26S proteasome [49, 50]. The fission yeast Udp7 protein contains a putative Zn protease domain. Orthologues of *udp7* exist in budding yeast and plants but not in mammalian cells. For other UBL containing proteins, such as the microtubule assembly co-factor Alp11, it is not obvious why it would be necessary to interact with the 26S proteasome [51]. Figure 3 shows the domain organization of the UBL containing proteins found in fission yeast.

A critical question which still remains unresolved is, Do all the different UBL containing proteins bind the same domain on the Mts4 protein? If the UBL binding domain is restricted to Rpn1/Mts4 and its orthologues, then presumably there would be competition for binding and it would have to be tightly regulated. Experiments carried out in budding yeast shows that the Rad23 protein can compete for binding to the proteasome with the Dsk2 protein, implying that both proteins do indeed bind to a common domain on the proteasome [39].

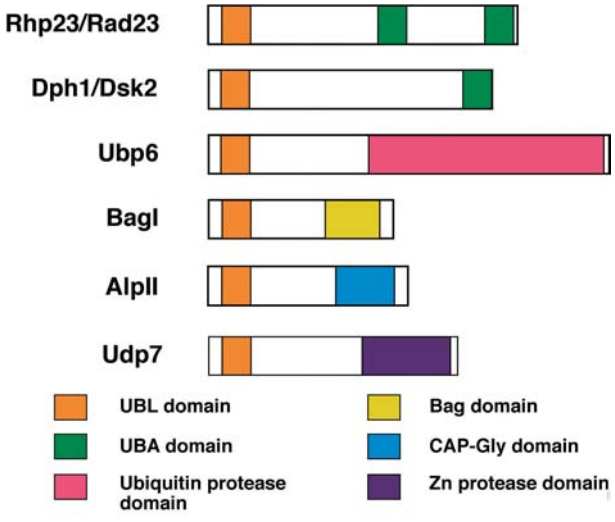

Figure 3. Domain organization of the UBL containing proteins in fission yeast.

Interaction of E2 and E3 proteins with the proteasome

A number of E2 ubiquitin conjugating enzymes and E3 ubiquitin ligase proteins have been implicated in direct interaction with the 26S proteasome in a UBL-independent manner. From studies in budding yeast a number of different E2 ubiquitin conjugating enzymes have been shown to interact with the 26S proteasome. Ubc1, Ubc2, Ubc4 and Ubc5 were all able to interact with the proteasome. In the case of the interaction with the Ubc4 E2, the interaction was investigated further. It was shown that the Ubc4/proteasome interaction increased after cells were subjected to heat shock. Interestingly, a Ubc4 null mutant has been implicated in the stress response, indicating that this increased interaction could be biologically relevant. However, this study did not address whether binding of the E2 proteins to the proteasome was direct or indirect, for example via binding to their cognate E3 ubiquitin ligases [52].

A number of different E3 ubiquitin ligases have been shown to interact with the proteasome. In human cells, the KIAA10 E3 has been shown to bind to the human S2/Rpn1 subunit. Interestingly, this is the same subunit which has been shown to bind to proteins carrying the UBL domain (see above). In budding yeast, the Ubr1 and Ufd2 ubiquitin ligases have been shown to directly interact with the proteasome. The Ubr1 protein has been shown to interact with the Rpt1, Rpt6 and Rpn2 subunits, while the Ufd4 protein interacts specifically with the Rpt6 subunit [53, 54]. In the case of the Ufd4/proteasome interaction, deletion analysis demonstrated that the N-terminal region of the Ufd4 protein was critical for binding. Interestingly, they also showed that mutant forms of the Ufd4 protein lacking the proteasome interacting region but which still contained fully competent E3 ubiquitin ligase activity were defective in the degradation of the ufd pathway test substrate Ub^{76}-V-βgal [55]. This strongly implied that the Ufd4 protein played some role in substrate delivery in an analogous manner to the multi-ubiquitin chain binding proteins described above.

The budding yeast Cic1 protein was identified by its ability to bind to the 20S α subunit Pre6 in a two-hybrid screen. In yeast protein extracts Cic1 co-fractionated with the 26S proteasome and not the 20S core particle, implying that the protein only interacted with the mature 26S proteasome. *CIC1* is an essential gene, and characterization of temperature-sensitive *cic1* alleles demonstrated that a number of different ubiquitin/proteasome pathway substrates were not stabilized in the mutants at the restrictive temperature. This demonstrated that the Cic1 protein was not required for general proteasomal degradation. Instead Cic1 was required for degradation of the Cdc4 and Grr1 F-box proteins. F-box domain containing proteins are the substrate binding recognition subunits of the SCF multi-subunit E3 ligase. The Cic1 protein appears to play

no part in the ubiquitinylation of the Cdc4 protein but seems to fulfill its role by acting as an adaptor, bringing the SCF complex to the proteasome [56]. This putative adaptor role seems to be a common function for a number of the proteasome interacting proteins described in this review.

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