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To Degrade or not to Degrade: Protein Handling by the Proteasome

Christian Heinen

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From THE DEPARTMENT OF CELL AND MOLECULAR BIOLOGY
Karolinska Institutet, Stockholm, Sweden

**TO DEGRADE OR NOT TO DEGRADE:
PROTEIN HANDLING BY THE PROTEASOME**

Christian Heinen



**Karolinska
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ABSTRACT

The proteasome, a large multi-subunit protease that degrades proteins, must be able to distinguish between substrates deemed for destruction and accessory proteins that should be spared. The shuttle factor Rad23 operates in close proximity to the proteasome in order to deliver proteins for their degradation. While its cargo is efficiently degraded, the shuttle factor itself remains unharmed. Rad23 is protected by its C-terminal ubiquitin-associated (UBA)-2 domain that has been identified to be a *cis*-acting stabilization signal. A major aim of the work presented in this dissertation has been to decipher the mode of action responsible for this protective effect.

Another protein that is targeted to the proteasome but resists degradation was studied in **Paper I**. UBB+1 is an aberrant product of the ubiquitin precursor gene *UBB* and comprises ubiquitin and a 19 amino acid-long C-terminal extension. Even though UBB+1 structurally resembles a ubiquitin fusion degradation (UFD) substrate, a class of proteins that is targeted for degradation by a non-cleavable N-terminal ubiquitin moiety, it is poorly degraded. We found that designed UFD substrates with equally short C-terminal extensions were stable. Extending their C-termini to 25 amino acids converted them into short-lived proteins, suggesting that the proteasome requires an unstructured polypeptide of a minimum length from which unfolding of the substrate may be initiated.

In **Paper II** we further investigated the impact of unstructured polypeptide sequences on proteasomal degradation. We found that C-terminal initiation sites for proteasomal unfolding rendered the degradation of UFD substrates independent of the ubiquitin-binding chaperone Cdc48 and polyubiquitylation. This observation suggests that substrates bypassing the unfoldase activity of Cdc48 require an unstructured initiation site for their efficient degradation and implies that the chaperone complex acting upstream of the proteasome can be the primary determinant for the polyubiquitin-dependency of proteasomal degradation. Consistently, we found that the ubiquitin-like (Ubl) domains of Rad23 and Dsk2, which interact with the proteasome in a polyubiquitin-independent manner, only sufficed to target proteins carrying initiation sites for degradation.

The impact of C-terminal unstructured polypeptide sequences on proteasomal degradation prompted us to investigate the role of structural integrity in UBA-mediated protection in **Paper III**. To this end, we used the C-terminal UBA domain of the human protein p62 and took advantage of naturally occurring mutant domains that are

linked to Paget's disease of bone. We found that not only the wild-type UBA domain could delay degradation of reporter substrates in yeast but also mutant domains that were thermally stable but impaired in their ubiquitin binding. This suggests that the UBA-mediated protective effect depends rather on the structural integrity than ubiquitin binding capability.

Our finding that proteins targeted to the proteasome through a UbL domain require an unstructured initiation site for efficient degradation turned out to be of significance for understanding the molecular mechanism behind Rad23 protection and encouraged us to study in **Paper IV** whether the protective UBA domains interfered with unfolding. Strikingly, introduction of C-terminal unstructured polypeptides turned the shuttle factor Rad23 into an efficiently degraded proteasome substrate. Positioning the UBA2 domain C-terminally to an adjacent unstructured polypeptide inhibited degradation, whereas non-protective UBA domains were able to function as initiation sites themselves.

In summary, we provide evidence that the protective effect of UBA domains is mediated by preventing initiation of degradation by the proteasome. These molecular insights help explain how proteasomes decide which proteins to degrade and which proteins to spare and thus how shuttle factors can deliver substrates to the proteasome without themselves becoming subject to degradation.

LIST OF PUBLICATIONS

This thesis is based on the following publications, which are referred to in the text by the Roman numerals:

- I. Verhoef LGGC, **Heinen C**, Selivanova A, Salomons FA and Dantuma NP. Minimal length requirement for proteasomal degradation of ubiquitin-dependent substrates. *FASEB J.* 2009, 23: 123-33.
- II. **Heinen C** and Dantuma NP. Cdc48-mediated unfolding as a critical determinant for the need of polyubiquitin chains in proteasomal degradation. *Submitted manuscript.*
- III. **Heinen C**, Garner TP, Long J, Böttcher C, Ralston SH, Cavey JR, Searle MS, Layfield R and Dantuma NP. Mutant p62/SQSTM1 UBA domains linked to Paget's disease of bone differ in their abilities to function as stabilization signals. *FEBS Lett.* 2010, 584:1585-90.
- IV. **Heinen C**, Ács K, Hoogstraten D and Dantuma NP. UBA-mediated inhibition of protein unfolding prevents proteasomal degradation. *Submitted manuscript.*

RELATED PUBLICATION

Publication by the author that is not included in the thesis:

Dantuma NP, **Heinen C** and Hoogstraten D. The ubiquitin receptor Rad23: at the crossroads of nucleotide excision repair and proteasomal degradation. Review. *DNA Repair (Amst.)*. 2009, 8: 449-60.

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LIST OF ABBREVIATIONS

aa	Amino acid
ATP	Adenosine triphosphate
C	Carboxy
DUB	Deubiquitylation enzyme
E1	Ubiquitin activating enzyme
E2	Ubiquitin conjugating enzyme
E3	Ubiquitin ligase
E4	Ubiquitin chain elongation factor
ERAD	Endoplasmic reticulum-associated degradation
GFP	Green fluorescent protein
N	Amino
PDB	Paget's disease of bone
S	Svedberg sedimentation coefficient
UBA	Ubiquitin-associated
UBB	Ubiquitin B
UBD	Ubiquitin-binding domain
UbL	Ubiquitin-like domain
UBX	Ubiquitin-regulatory X
UFD	Ubiquitin fusion degradation
UIM	Ubiquitin-interacting motif

1 PREFACE

The Nobel Prize in Chemistry in 2004 was awarded for the discovery of a small ubiquitous protein coined ubiquitin that is responsible for regulated protein destruction in cells. Ubiquitin is conserved from yeast to mammals and it has been found to play important roles in numerous cellular processes including protein quality control, DNA repair, transcription, and the immune response (Hershko and Ciechanover, 1998).

The best-studied function of ubiquitin is its role in proteolysis, a process that proceeds in a tightly regulated manner and is responsible for degradation of the vast majority of intracellular proteins. Ubiquitin plays the role of a small modifier that is conjugated to proteins deemed for destruction in the form of a long chain consisting of multiple ubiquitin molecules. This polyubiquitin-tag targets the protein to the proteasome, a large multi-subunit protease, where it is eventually degraded (Baumeister et al., 1998).

The first breakthrough in the research field of protein degradation came in 1942, when Rudolph Schönheimer postulated in his book 'The Dynamic State of Body Constituents' that proteins are constantly build up and broken down (Schoenheimer, 1942). Previously, only little attention had been devoted to protein destruction and this insight into continual regeneration of the cell led to a new view of metabolism. However, it took several decades before the pioneering work by Aaron Ciechanover, Avram Hershko, and Irwin Rose discovered that regulated proteolysis of proteins is an energy-dependent process mediated by ubiquitin (Ciechanover et al., 1980; Ciechanover et al., 1978; Hershko et al., 1980; Hershko et al., 1979). Later, an adenosine triphosphate (ATP)-driven protease was identified that is responsible for degrading the ubiquitin conjugates (Hough et al., 1987; Tanaka et al., 1986). Today, this large protease complex is known as the 26S proteasome. Since then a growing number of physiological substrates and components that contribute to ubiquitin-mediated proteolysis have been identified. However, we are still at an early stage in the functional analysis of ubiquitin-driven proteolysis. One of the major tasks will be to understand the overwhelming functional complexity in the coordination of the degradation process.

2 INTRODUCTION

The ubiquitin/proteasome system is best known for its function in regulated protein turnover. Being extremely specific in a temporal and spatial manner it plays decisive roles in many cellular events (Hershko and Ciechanover, 1998).

Ubiquitin-mediated proteolysis can be roughly divided into two distinct steps: Ubiquitylation and proteasomal degradation (Figure 1). The small 76 amino acid-long protein ubiquitin is conjugated specifically to a target protein designated for degradation (Pickart, 2001). Notably, ubiquitin is a protein that itself can function as an acceptor for another ubiquitin and thus repetitive conjugations to the previous attached ubiquitin lead to a polyubiquitin signal that ensures the localization of the substrate to the protein degradation machinery (Pickart, 2001). Responsible for the degradation of proteins that carry polyubiquitin chains is a large multi-subunit protease complex of 2.5 megadaltons (Coux et al., 1996). It is composed of two sub-complexes: The 20S core particle and the 19S regulatory particle, that together form the 26S proteasome (Voges et al., 1999).

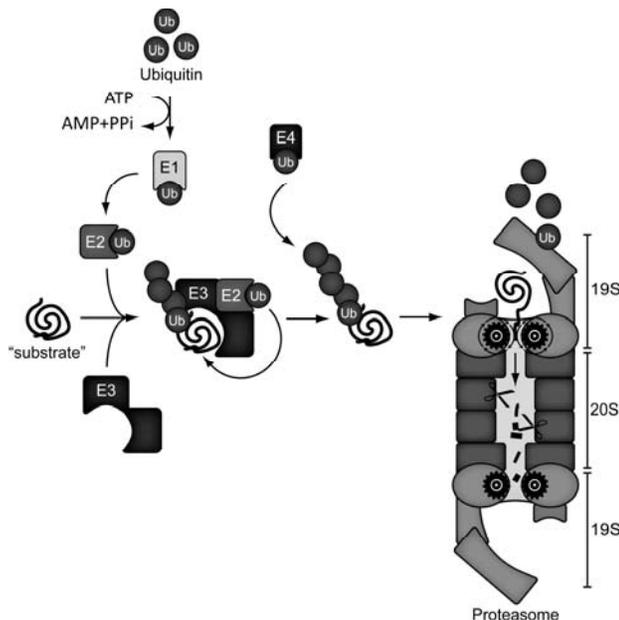


Figure 1 Schematic representation of the ubiquitin/proteasome system. The ubiquitin/proteasome system can be divided in ubiquitylation and proteasomal degradation. Proteins harboring a degradation signal are ubiquitylated by an enzymatic cascade (see text for more details). The polyubiquitylated protein is then targeted to the proteasome, where it is deubiquitylated, unfolded, and degraded.

2.1 THE UBIQUITIN TAG, A SMALL MODIFYING PROTEIN

2.1.1 Ubiquitylation

Ubiquitylation is a posttranslational modification that results in the formation of an isopeptide bond between the carboxy (C)-terminal glycine (G) of ubiquitin (Figure 2) and the ϵ -amino group of a lysine residue in the substrate, or less common to the amino (N)-terminus of proteins (Pickart, 2001). Responsible for this reaction is a cascade of three different enzymes: (i) A cysteine residue in the catalytic site of a ubiquitin activating enzyme (E1) forms a thiol ester with the carboxyl group of G76 at the C terminus of ubiquitin to activate ubiquitin for a nucleophilic attack. This reaction requires ATP. (ii) Next, a ubiquitin conjugating enzyme (E2) transiently takes over the activated ubiquitin by forming another thiol ester linkage, before (iii) a ubiquitin ligase (E3) transfers the activated ubiquitin from the E2 to a lysine residue in the substrate (Hershko et al., 1983). While there is only one E1 enzyme about a dozen E2 enzymes have been identified in yeast. The vast majority of ubiquitylation enzymes is presented by E3 enzymes, being responsible for substrate specificity (Pickart, 2001). So far three different classes of E3 ligases have been identified: RING (Really Interesting New Gene), U-box and HECT (Homologous with E6-associated protein C-Terminus) domain ligases. Sometimes efficient polyubiquitylation needs an additional ubiquitylation factor (E4) that binds to and further extends existing polyubiquitin chains in conjunction with E1, E2, and E3 (Koeagl et al., 1999).

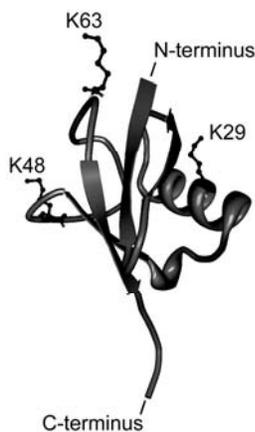


Figure 2 Crystal structure of ubiquitin (PDB file: 1UBQ). The image was generated using Protein Workshop. Lysine (K)29, K48, and K63 are highlighted.

As mentioned above ubiquitin can serve itself as a substrate and function as an acceptor for additional ubiquitin molecules leading to the formation of a polyubiquitin chain (Hoppe, 2005). Seven lysine (K) residues (K6, K11, K27, K29, K33, K48, and K63) within the ubiquitin sequence can lead to different chain conformations and K48- and K11-linked chains have been found to be the most abundant chain types in cells (Xu et al., 2009). Polyubiquitin chains of four K48-linked ubiquitin molecules (chains where all ubiquitins are conjugated to the lysine residue at position 48 of the previously attached ubiquitin) have been identified as the minimal proteasomal degradation targeting signal (Thrower et al., 2000). Despite being as abundant as K48 in yeast, there are only few reports devoted to K11-linked chains. It has been shown that K11-linked chains can serve as proteasomal degradation signals for a subset of substrates in the endoplasmic reticulum-associated degradation (ERAD) pathway in yeast (Xu et al., 2009). Another commonly observed polyubiquitin modification is the K63-linked chain, a chain type that is primarily involved in non-degradative functions such as activation of nuclear factor- κ B signaling (Wu et al., 2006) and regulation of DNA repair (Hoege et al., 2002; Stelter and Ulrich, 2003). Interestingly, ubiquitin chains linked through each of the lysine residues were identified in cells (Xu et al., 2009). In addition, branched ubiquitin chains have been detected conjugated to substrates *in vitro* (Kim et al., 2007b) and *in vivo* (Peng et al., 2003).

A polyubiquitin chain is not always the outcome of an ubiquitylation reaction. Proteins can be modified with a single ubiquitin on a single lysine residue (monoubiquitylation) or on multiple lysine residues (multi-monoubiquitylation). Monoubiquitylation is mainly found to be involved in membrane trafficking, histone function, transcription regulation, DNA repair, and DNA replication (Sigismund et al., 2004).

2.1.2 Deubiquitylation

As many other protein modifications, ubiquitylation is a reversible process. For this purpose, cells contain a number of proteases that can cleave the isopeptide bond connecting the ubiquitin molecule, leading to deubiquitylation of proteins (Reyes-Turcu et al., 2009). While the vast majority of deubiquitylation enzymes (DUBs) are not associated with the proteasome, a few of these proteases are intrinsic subunits of the 26S proteasome. For example, Rpn11, a subunit of the 19S regulatory particle, removes

whole polyubiquitin chains of substrates before degradation by hydrolyzing the isopeptide bond between the substrate's lysine and the proximal ubiquitin molecule of the chain (Verma et al., 2002; Yao and Cohen, 2002). Notably, deubiquitylation by Rpn11 and degradation are coupled events and thus only substrates that are fully committed for degradation are processed (Verma et al., 2002).

Two other DUBs associated with the proteasome can trim the polyubiquitin chain of substrates. In yeast, Uch37 and Ubp6 cleave single ubiquitin molecules or di-/triubiquitin chains, respectively, from the distal end of a polyubiquitin chain (Hanna et al., 2006; Lam et al., 1997). By progressively shortening instead of removing entire chains, these DUBs are most likely responsible for reducing the affinity of substrates for the proteasome. A likely model is that if degradation is not initiated before the entire chain has been removed, the protein will escape degradation and thus Uch37 and Ubp6 can negatively influence proteasomal degradation. However, Hul5, another proteasome-associated protein, is counteracting the DUB activity (Crosas et al., 2006; Kraut et al., 2007). Hul5 is an E4 elongation factor responsible for further ubiquitylation of existing polyubiquitin chains on substrates, whereby it most likely, in concert with Uch37 and Ubp6, fine-tunes the selectivity of the degradation by the proteasome.

2.1.3 Ubiquitin-binding domains

Ubiquitin-binding domains (UBDs) are usually small motifs consisting of 20-150 amino acids (aa) in length that non-covalently bind to ubiquitin. As described earlier, ubiquitylation of proteins can trigger specific outcomes. To this end, ubiquitin-dependent processes are mediated by various effector proteins that harbor UBDs to bind to the ubiquitin modification and initiate the appropriate downstream event (Hicke et al., 2005). Several families of UBDs have been described during the last years.

Most UBDs use α -helical structures to interact with a hydrophobic patch on the surface of ubiquitin comprising residues leucine 8, isoleucine (I)44, and valine 70. These α -helical binding motifs can either consist of a single helix or a repeat of discontinued helices. For example, the UIM (ubiquitin-interacting motif), inverted UIM (IUIM) also known as MIU (motif interacting with ubiquitin), and the ubiquitin-binding zinc finger (UBZ) domain are single α -helix binding motifs (Bomar et al., 2007; Hirano et al., 2006a; Lee et al., 2006; Penengo et al., 2006; Swanson et al., 2003;

Introduction

Wang et al., 2005). In contrast, ubiquitin-associated (UBA) domains and CUE (coupling of ubiquitin to ERAD) domains are bundles of discontinued, packed helices (Chang et al., 2006; Kang et al., 2003; Ohno et al., 2005; Swanson et al., 2006). The UBA domains were originally discovered by computational analysis as a small conserved domain that was commonly found in proteins involved in ubiquitylation (Hofmann and Bucher, 1996). Although the function of these domains was unknown at that time, they were dubbed UBA domains in reference to their tight connection to protein ubiquitylation (Hofmann and Bucher, 1996). A few years later, it was reported that these domains were able to bind ubiquitin, providing an explanation for the presence of these domains in ubiquitylation enzymes and other proteins linked to ubiquitin-dependent protein degradation (Wilkinson et al., 2001).

In addition to α -helical binding motifs, β -sheet-based UBDs exist, which bind preferentially to monoubiquitin. The ubiquitin-conjugating (Ubc)-like domain, GLUE (GRAM-like ubiquitin-binding in EAP45) and PRU (PH receptor for ubiquitin) domain are examples for β -sheet-based binding domains that, like the α -helical binding motifs, interact with the hydrophobic patch on ubiquitin (Alam et al., 2006; Hirano et al., 2006b; Husnjak et al., 2008; Schreiner et al., 2008).

As the α -helical and β -sheet-based binding motifs, zinc finger (ZnF) ubiquitin binding motifs, like the nuclear protein localization 4ZnF (NZF) domain and the ubiquitin-binding ZnF (UBZ) domain bind to the hydrophobic patch containing I44 of ubiquitin (Alam et al., 2004; Bienko et al., 2005). However, not all UBDs bind to the hydrophobic patch surrounding I44. Exceptions are variants of a ZnF motif that have been described to bind ubiquitin in a different manner. The A20-type ZnF of the protein RABEX5 interacts with a polar surface that is surrounding aspartic acid 58 (Lee et al., 2006) and the ZnF domain of the IsoT deubiquitylation enzyme binds to the C-terminus of ubiquitin (Reyes-Turcu et al., 2006).

As described earlier, polyubiquitin chains can form a variety of different linkages and interestingly most UBDs have been found to be able to specifically recognize certain linkages. However, since most of the ubiquitin-binding domain surfaces interact with a single hydrophobic patch on the ubiquitin surface, it is hard to imagine how UBDs can discriminate between different polyubiquitin chain linkages.

Insights into the selective recognition of different polyubiquitin chains come from the UBA2 domain of the human homolog Rad23 (hHR23)-A protein, which shows a preference for K48-linked chains. Structural analysis of the UBA domain in

complex with diubiquitin revealed that the UBA domain is sandwiched between two ubiquitin moieties leading to a larger binding surface compared to binding to monoubiquitin (Varadan et al., 2005). The distal ubiquitin moiety binds to the major interaction surface composed of the first and third helical bundle, whereas the proximal ubiquitin associates with the second helical bundle located at the back of the UBA domain. Thus, the specific closed conformation of the K48 linkage allows the high affinity binding of diubiquitin to all helices of the UBA domain. While this insight is giving a possible explanation for the preferential binding of hHR23A's UBA2 domain to K48-linked polyubiquitin chains, it does not explain why other UBA domains show different preferences or only little distinction between various polyubiquitin linkages (Raasi et al., 2005).

Another mechanism for selective recognition of polyubiquitin chains has been proposed for repetitive multiple UBDs that can be found in many proteins. For example the human DNA damage response protein RAP80 binds with its N-terminal tandem UIM repeat specifically to K63-linked polyubiquitin chains (Kim et al., 2007a; Sobhian et al., 2007; Yan et al., 2007). It has been proposed that the linker sequence between the UIMs specifically positions the two domains for efficient and selective K63-linked polyubiquitin binding (Sims and Cohen, 2009). Thus, tandem UBDs can be spatially arranged in proteins, to favor a specific ubiquitin linkage and to disregard other types of chains.

2.1.4 Ubiquitin-like domains

The concept of using ubiquitin or related structures as binding modules appears to have been a very successful approach as evidenced by the large number of polypeptides that resemble ubiquitin in eukaryotes (Jentsch and Pyrowolakis, 2000). The first class of proteins that are related to ubiquitin are the so-called ubiquitin-like modifiers, such as Smt3/SUMO and Rub1/Nedd8, which are, analogous to ubiquitin covalently conjugated to internal lysine residues of target proteins (Welchman et al., 2005). The second class of structures that resemble ubiquitin are internal domains (Buchberger, 2002). Unlike the ubiquitin-like modifiers, ubiquitin resembling domains are integrated in larger proteins and cannot be conjugated to other proteins and are not processed by DUBs that normally cleave linear ubiquitin fusions (Wilkinson, 2000). Internal domains that relate to ubiquitin can be divided into ubiquitin-like (UbL) domains and

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ubiquitin regulatory X (UBX) domains (Buchberger, 2002). Both types of domains are approximately 80 amino acids long and adopt a typical ubiquitin fold, even though the sequence homology with ubiquitin is limited (Walters et al., 2002). Notably, interactions between both types of ubiquitin-like domains and UBDs have been reported underscoring the fact that these domains structurally resemble ubiquitin (Ryu et al., 2003). At the same time, UBX and UbL domains appear to have adopted divergent binding specificities. Whereas UbL domains interact with proteasome subunits that are not known to interact with ubiquitin (Elsasser et al., 2002; Saeki et al., 2002), UBX domains appear to primarily facilitate binding to the AAA-ATPase Cdc48, or its human homolog p97 (Alexandru et al., 2008; Neuber et al., 2005; Schubert and Buchberger, 2005), which in complex with the Ufd1/Npl4 ubiquitin binding co-factors is implicated in proteasomal degradation of a subclass of proteasome substrates as well as non-proteolytic functions (Halawani and Latterich, 2006). Interestingly, UBX domains are often located close the C-termini of proteins whereas UbL domains are in most cases located at the extreme N-termini (Buchberger, 2002).

Rad23 was the first UbL domain-encoding gene to be identified (Watkins et al., 1993). Presently, a number of UbL domains have been identified and members of this family include proteins such as Dsk2 and Ddi1, which share with Rad23 the characteristic that they harbor besides the UbL domain also ubiquitin binding UBA domains, a feature that is often present in UBX- or UbL-containing proteins (Buchberger, 2002).

Interestingly, substituting the UbL domain of Rad23 with an N-terminal ubiquitin moiety resulted in a protein that was fully functional (Lambertson et al., 2003). This demonstrated that Rad23's UbL can be functionally replaced by an alternative proteasome interaction module suggesting that the primary function of this domain is to accommodate interaction with the proteasome. It has become clear that UbL-containing proteins interact differently with the proteasome as compared to polyubiquitylated proteasome substrates. In yeast at least two proteasome subunits that are both located in the 19S regulator, Rpn10 and Rpn13, directly interact with K48-linked polyubiquitylated proteins (Husnjak et al., 2008; Schreiner et al., 2008; van Nocker et al., 1996). However, the UbL domain of Rad23 binds to the Rpn1 subunit in the base of the 19S regulatory particle of the proteasome (Elsasser et al., 2002) suggesting that UbL domains must be intrinsically different from ubiquitin. Notably, Rpn10 is able to bind UbL domains, since it does bind the UbL domain of Dsk2 (Ishii

et al., 2006), suggesting that there are subtle differences between the UbL domains of different proteins that determine their binding specificity.

It is noteworthy that, even though UbL domains have been most extensively studied in their interaction with the proteasome, these domains also mediate interactions with other proteins. For example, the ubiquitin chain elongation-factor Ufd2 (Kim et al., 2004) and the peptidyl transfer RNA hydrolase 2 (Pth2) (Ishii et al., 2006), interact with the UbL domain of Rad23. Interestingly, both proteins compete with the proteasome for binding to the UbL domain. Whereas the interaction with the ubiquitylation enzyme Ufd2 appears to be important for Rad23's role in facilitating proteasomal degradation (Kim et al., 2004), binding to Pth2, on the contrary, impedes ubiquitin-dependent proteolysis possibly by preventing interaction of Rad23 with the proteasome (Ishii et al., 2006). Thus, it appears that various proteins involved in proteasomal degradation are competing for binding to Rad23's UbL domain, which may be of functional significance for the regulation of ubiquitin-dependent proteolysis (Goh et al., 2008).

2.2 THE PROTEASOME, A LARGE MULTI-SUBUNIT PROTEASE

The proteasome is responsible for the degradation of proteins deemed for degradation and it is composed of different multi-subunit particles (Figure 3). The 19S regulatory particle is responsible for binding the polyubiquitylated proteins, releasing the ubiquitin chain, and the regulation of the substrate entry into the catalytic chamber that resides within the 20S core particle (Baumeister et al., 1998).

2.2.1 The 20S core particle

The 20S core particle is a barrel-shaped structure that is composed of four stacked rings. The two outer rings consist each of seven unique α subunits and the two inner rings are composed each of seven unique β subunits. These rings form a hollow cylinder, with the catalytic active sites of the β 1, β 2, and β 5 subunits facing towards the inside of the 20S core particle (Groll et al., 1997). β 1 is a protease with caspase-like activity that prefers to cleave C-terminally of an acidic amino acid residue. The β 2-site has trypsin-like activity, cutting after tryptic residues and β 5 is a protease with chymotrypsin-like activity that preferentially cleaves after hydrophobic amino acids.

Introduction

The combination of three different active sites is necessary to allow degradation of a broad variety of peptide sequences and ensures high efficiency in protein degradation (Borissenko and Groll, 2007).

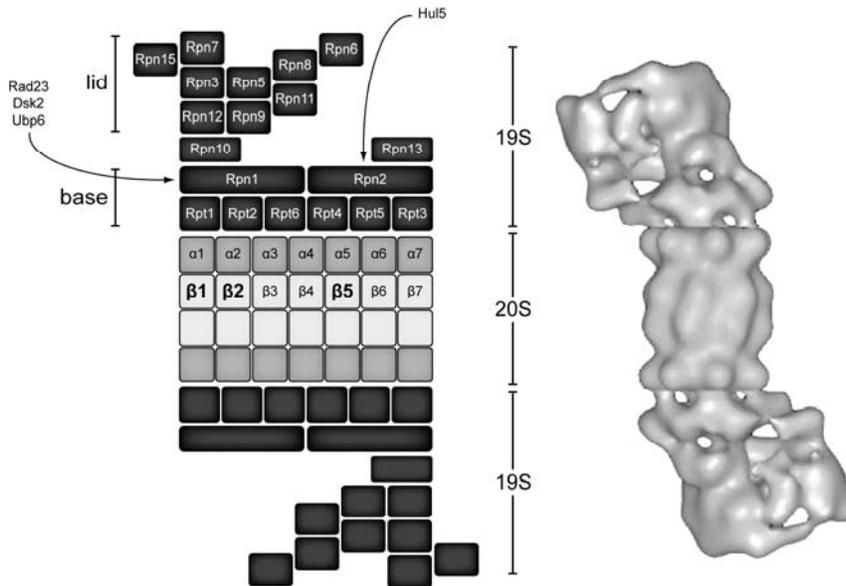


Figure 3 The subunit composition and structure of the 26S proteasome. The proteasome consists of the 20S core particle and one or two 19S regulatory particles. The 20S is built up by two outer α rings and two inner β rings, each ring comprising seven subunits. The proteolytic active sites are depicted in bold. The 19S is divided into two subcomplexes: the base and the lid complex. Examples of proteasome-associated proteins that interact transiently with specific proteasomal subunits are marked by an arrow. Positions of the individual subunits do not necessarily reflect the quaternary structure of the 26S. The holoenzyme structure shows a composite model of electron microscopic structures of the 19S combined with a low-pass filtered 20S. The structure was taken with permission from the publisher (Walz et al., 1998).

2.2.2 The 19S regulatory particle

The 19S regulatory particle is composed of at least 19 subunits and can be divided into the base and lid sub-complex (Finley, 2009).

The base of the 19S, situated proximal to the 20S core particle, comprises a ring of six AAA (ATPase associated with a variety of cellular activities)-ATPases, named Rpt1-6 (Regulatory particle triple A) in yeast, and accordingly degradation of proteasomal substrates is strictly depending on ATP (Kleijnen et al., 2007; Liu et al., 2006; Rubin et al., 1998; Verma et al., 2002; Yao and Cohen, 2002). Before a protein can reach the catalytic sites, a critical step is the unfolding of the protein since the

entrance to the proteolytic chamber is narrow (Groll et al., 2000). Responsible for the unfolding step is the ring of ATPases that unwinds the substrate and thereby assists in the translocation of the substrate into the catalytic chamber (Navon and Goldberg, 2001). In addition, the Rpt subunits also open a channel into the catalytic core (Rabl et al., 2008). For this purpose the C-termini of the Rpt subunits dock into specific α pockets of the core particle and open a channel into the 20S core (Figure 4) (Gillette et al., 2008; Rabl et al., 2008; Smith et al., 2007).

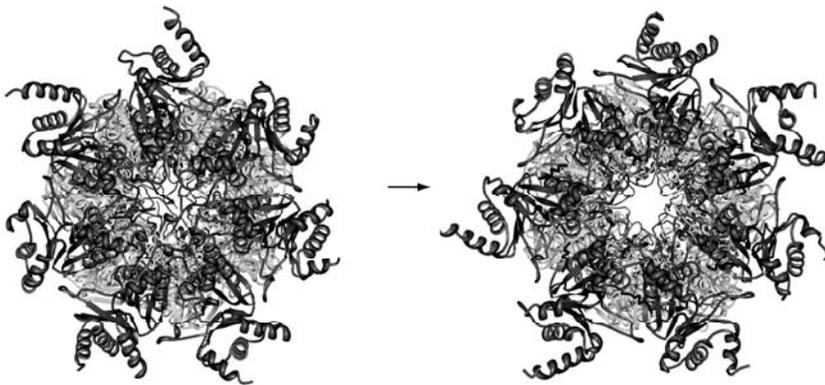


Figure 4 Structures of the proteasome core particle. Left panel: topview of a 20S core particle with closed channel (PDB file: 1RYP). Right panel: topview of a 20S core particle with open channel. C-termini of a proteasome activator (black) bind to pockets in the α -ring and open the channel into the proteolytic chamber (PDB file: 1FNT). The images were generated using Protein Workshop.

Two additional subunits of the 19S base are Rpn1 and Rpn2 (Regulatory particle non-ATPase). These subunits were predicted to function as a central scaffold or platform unit that mediates binding of a variety of proteasomal co-factors important for proteasome activity (Finley, 2009). As such, the intrinsic ubiquitin receptor Rpn13 binds via Rpn2 to the proteasome. Rpn13 binds ubiquitin via a PRU domain (Husnjak et al., 2008; Schreiner et al., 2008). Ubiquitin receptors, like Rad23, Dsk2, and Ddi1 have N-terminal UbL domains that can bind to the proteasome and UBA domains that interact with polyubiquitylated proteins (Schauber et al., 1998; Wilkinson et al., 2001). These UbL/UBA proteins transiently interact with the Rpn1 subunit (Elsasser et al., 2002) and thus it has been proposed that they function as shuttles that selectively deliver substrates to the proteasome (Hartmann-Petersen et al., 2003; Verma et al.,

2004). Whether the delivered substrates are handed over from these shuttles to intrinsic ubiquitin receptors prior to degradation is presently unclear.

Another intrinsic proteasomal ubiquitin receptor is the Rpn10 subunit, which binds selectively polyubiquitylated substrates via a UIM domain (Kang et al., 2007; Mayor et al., 2007; Wang et al., 2005; Young et al., 1998). Thus, five ubiquitin receptors have been identified so far: two intrinsic proteasome subunits (Rpn10 and Rpn13) and three so called shuttle factors (Rad23, Dsk2, and Ddi1). Surprisingly, none of these receptors is essential in yeast (Elsasser and Finley, 2005; Husnjak et al., 2008) and proteasomes that lack all of them are still able to degrade polyubiquitylated proteins albeit with reduced efficiency suggesting that more ubiquitin binding factors might be involved in substrate recruitment.

Not only ubiquitin receptors bind to the proteasome. It has been shown that the DUB Ubp6 binds the proteasome via Rpn1 (Leggett et al., 2002; Stone et al., 2004) and the E3 ligase Hul5 binds through the subunit Rpn2 to the proteasome (Crosas et al., 2006). Another DUB, named Uch37, is linked via Rpn13 to Rpn2 (Hamazaki et al., 2006; Schreiner et al., 2008; Yao et al., 2006).

Even though the proteasome lid contains nine subunits, the only subunit with a known catalytic activity is Rpn11. Rpn11 has DUB activity and deubiquitylates substrates prior to degradation, a function that has been shown to be important for efficient proteasomal degradation (Gallery et al., 2007; Maytal-Kivity et al., 2002; Verma et al., 2002; Yao and Cohen, 2002).

2.3 DEGRADATION SIGNALS

Degradation signals are a major determinant for a protein's lifespan (Laney and Hochstrasser, 1999). The N-end rule degron was the first degradation signal identified (Bachmair et al., 1986; Bachmair and Varshavsky, 1989; Varshavsky, 1996). Other degradation signals that have been studied in detail are the ubiquitin fusion degradation (UFD) signal (Johnson et al., 1992; Johnson et al., 1995), the PEST sequence (Rogers et al., 1986), and the destruction box (Glutzer et al., 1991). Degradation signals trigger the turnover of proteins by recruiting specific E3 ligases that conjugate K48-linked polyubiquitin chains to substrates (Pickart, 2001).

The N-end rule pathway was first described in yeast and later found to be conserved and present in mammalian cells. According to the N-end rule the nature of the N-terminal amino acid defines the half-life of a protein (Bachmair et al., 1986). Newly synthesized proteins normally contain an N-terminal methionine. To create an N-end rule degradation signal, the stabilizing Met at the N-terminus has to be removed. Endoproteolytic cleavage is a possibility that leads to proteins with non-canonical N-terminal amino acids. The E3 ligase Ubr1 is able to distinguish the different side chains of the N-terminal residues and ubiquitylates N-end rule substrates to target them for degradation. Interestingly, depending on their N-terminal amino acid, the half-lives of proteins range from a few minutes to hours (Bartel et al., 1990). N-end rule pathway enzymes have been found to be involved in chromosome stability (Nasmyth, 2002), peptide import regulation (Byrd et al., 1998), apoptosis (Ditzel et al., 2003), muscle wasting (Cao et al., 2005), and cytoplasmic protein quality control (Heck et al., 2010).

The UFD signal has been commonly used to target proteins of interest for ubiquitin-dependent proteasomal degradation. Substrates harbor a non-cleavable N-terminal ubiquitin that functions as a degradation signal itself (Johnson et al., 1992). Ufd4 and Ufd2, which are an E3 and E4 ligase, respectively, recognize the UFD signal and assemble polyubiquitin chains on K29 and K48 of the non-removable ubiquitin moiety (Johnson et al., 1995; Koegl et al., 1999). Notably, the physiological relevance of this type of substrates is less clear. The only natural UFD substrate found is the UBB+1 protein, a protein encoded by an erroneous transcript of the ubiquitin B (*UBB*) gene. It comprises a ubiquitin moiety and a 19 aa C-terminal extension. Even though it structurally resembles a UFD substrate, it is only poorly degraded. However, enzymes of the UFD pathway have been implicated in the degradation of various endogenous proteins that are unrelated to the UFD class of substrates (Hoppe et al., 2004; Janiesch et al., 2007; Neuber et al., 2005).

2.4 STABILIZATION SIGNALS

In addition to degradation signals, some proteins also harbor stabilization signals that may delay or block proteasomal degradation (Dantuma and Masucci, 2002). Examples of stabilization signals are the expanded polyglutamine repeats causative in some neurodegenerative disorders (Verhoef et al., 2002) and the glycine alanine repeat of

Introduction

Epstein-Barr virus (Levitskaya et al., 1995; Levitskaya et al., 1997), which stabilize the proteins in which they reside.

Moreover, two UBDs have been shown to protect proteins from degradation in yeast. The UBA2 domain of Rad23 functions as an intrinsic stabilization signal that protects Rad23 from proteasomal degradation (Heessen et al., 2005). Rad23 is a scaffold protein that has to interact with the proteasome to fulfill its roles in targeting polyubiquitylated proteins for proteasomal degradation (Verma et al., 2004) and DNA repair (Russell et al., 1999). Rad23 lacking the protective UBA2 domain is rapidly degraded by the proteasome causing sensitivity to various proteotoxic stress conditions as well as genotoxic stress caused by ultraviolet radiation (Heessen et al., 2005). Besides the C-terminal UBA2 domain, Rad23 also contains an internal UBA1 domain. The UBA1 domain did not inhibit proteasomal degradation suggesting that ubiquitin binding is not the sole determinant for the protective effect (Heessen et al., 2005).

The protein Met4 is a transcription factor in yeast that is activated under certain conditions of low intracellular concentrations of S-adenosylmethionine and in response to cadmium or arsenic stress (Barbey et al., 2005; Thomas and Surdin-Kerjan, 1997; Yen et al., 2005). Met4's activity is regulated by the presence of a K48-linked polyubiquitin chain that usually labels proteins for degradation. However, ubiquitylated Met4 is not targeted for proteasomal degradation (Flick et al., 2004). Interestingly, an internal UIM domain has been proposed to be responsible for the stability of Met4 (Flick et al., 2006). Thus, two unrelated UBDs regulate the stability of the proteins in which they reside by counteracting signals that would otherwise target these proteins for ubiquitin-dependent proteasomal degradation. Flick and co-workers propose that the UIM domain protects Met4 from proteasomal degradation by inhibiting chain assembly allowing only formation of polyubiquitin chains that are not sufficiently long to target Met4 for degradation (Flick et al., 2006). However, in a later study the stability of Met4 was attributed to the unusual tight interaction between Met4 and its ubiquitin ligase SCF^{Met30}, which prevented proteasomal degradation (Chandrasekaran et al., 2006). Even though these two models for Met4's stability differ at essential points, they both propose that the stability of Met4 is due to preventing binding of polyubiquitylated Met4 to the proteasome.

Although the mode of action by which the UBA2 domain protects Rad23 from proteasomal degradation remains unclear, it is likely to deviate from the models proposed for Met4 since it is well established that Rad23 has to interact with the

proteasome in order to fulfill its tasks of shuttling polyubiquitylated proteins to the proteasome and for nucleotide excision repair (Dantuma et al., 2009).

2.5 THE CDC48 CHAPERONE

The molecular chaperone Cdc48 (Cell division cycle 48) is a homohexameric ring-shaped AAA-ATPase (Figure 5), distantly related to the ATPase ring in the 19S regulatory particle of the proteasome (Elsasser and Finley, 2005). It is conserved in eukaryotes from yeast to mammals and plays an important role in ubiquitin/proteasome system-related processes like the UFD pathway (Ghislain et al., 1996), the processing of transcription factors (Hitchcock et al., 2001; Hoppe et al., 2000; Rape et al., 2001), and the ERAD pathway (Bays et al., 2001; Jarosch et al., 2002; Rabinovich et al., 2002; Ye et al., 2001) but also in ubiquitin/proteasome system-independent pathways like membrane fusion (Hetzer et al., 2001; Kano et al., 2005; Latterich et al., 1995; Uchiyama and Kondo, 2005; Wang et al., 2004). In addition, Cdc48 has been reported to be required for the degradation of certain cytoplasmic substrates (Cao et al., 2003; Decottignies et al., 2004; Fu et al., 2003; Ghislain et al., 1996; Hartmann-Petersen et al., 2004; Verma et al., 2004), indicating that the Rpt subunits of the proteasomal 19S regulatory particle need assistance in unfolding certain substrates. Recent evidence indeed suggests an unfoldase activity for the mammalian homolog p97 in proteasomal degradation by pre-processing of substrates prior to degradation by the 26S proteasome (Beskow et al., 2009).

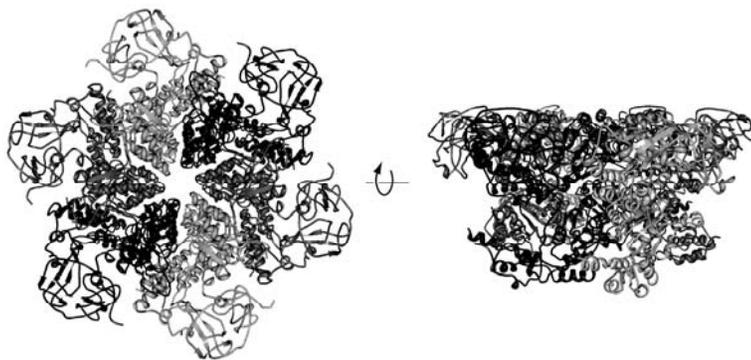


Figure 5 Structure of the mammalian homolog of Cdc48 (PDB file: 3CF1). The image was generated using Protein Workshop. Left panel: topview; right panel: sideview.

The versatile nature of Cdc48 is realized by its multiple cofactors that usually bind to the N-terminal domain of Cdc48. Co-factors that harbor ubiquitin binding domains like the heterodimeric subunit Ufd1/Npl4 are responsible for recruiting ubiquitylated substrates to Cdc48 (Rape et al., 2001; Richly et al., 2005), whereas enzymes, like the E4 Ufd2 (Koegl et al., 1999; Saeki et al., 2004) or the DUB Otu1 (Rumpf and Jentsch, 2006) can influence the length of the polyubiquitin chain attached to the substrate.

Cdc48's ATPase activity is located in two consecutive AAA-ATPase domains, named D1 and D2. ATP hydrolysis leads to a conformational change in the Cdc48 structure applying mechanical forces on bound proteins or protein complexes (Rouiller et al., 2002). The AAA-ATPase ring of Cdc48 surrounds a central channel, similar to the ATPase ring of the proteasome. However, recent structural data propose a blockage of the putative central channel by a zinc ion leaving only a broad pore region as the one possibility for substrates to enter and exit Cdc48 (DeLaBarre and Brunger, 2003).

2.6 DELIVERY OF UBIQUITYLATED SUBSTRATES TO THE PROTEASOME

The delivery of ubiquitylated proteins can occur in different ways. Five ubiquitin receptors for the proteasome have been identified so far, including the intrinsic subunits Rpn10, Rpn13 and the shuttling factors Rad23, Dsk2, and Ddi1 (Finley, 2009).

The proteasome subunit Rpn10 was the first ubiquitin receptor identified and it has been shown that it can bind certain substrates directly (Deveraux et al., 1994). The other intrinsic ubiquitin binding subunit is Rpn13 (Husnjak et al., 2008).

In addition to the *bona fide* proteasome subunits Rpn10 and Rpn13, the UbL/UBA family of proteins has been suggested to recruit polyubiquitylated proteins to the proteasome. As outlined above, the UbL/UBA proteins Rad23, Dsk2, and Ddi1 are referred to as 'shuttle factors' that bind polyubiquitylated substrates to deliver them to the proteasome for their degradation, adding an additional layer of substrate selectivity to the ubiquitin/proteasome system (Chen and Madura, 2002; Elsasser et al., 2004; Rao and Sastry, 2002; Verma et al., 2004; Wilkinson et al., 2001). The UBA domain binds ubiquitin chains (Bertolaet et al., 2001; Wilkinson et al., 2001), whereas the N-terminal UbL domain transiently interacts with the proteasome (Elsasser et al., 2002). Consistent with this hypothesis was the finding that deletion of Dsk2 in yeast

reduced ubiquitin-dependent proteasomal degradation (Funakoshi et al., 2002) and that Rad23 can promote binding of ubiquitylated proteins to the proteasome (Chen and Madura, 2002). Interestingly, a large portion of Rpn10 in yeast is not associated with the proteasome. Whether Rpn10 also shuttles proteins to the proteasome, similar to the function of Rad23, Dsk2, or Ddi1, in addition to its function as an intrinsic ubiquitin receptor remains to be clarified. However, free Rpn10 has been demonstrated to bind Dsk2's UbL domain via its UIM motif suggesting that the free pool of Rpn10 may regulate the binding of Dsk2 to the proteasome (Matiuhin et al., 2008).

Notably, initial studies on the function of UbL/UBA proteins suggested rather an inhibitory role in proteasomal degradation. Early *in vitro* and *in vivo* experiments showed that Rad23 inhibited the formation of K48-linked polyubiquitin chains and stabilized proteasome substrates. It was proposed that Rad23 was a negative regulator of proteasomal degradation. However, the function of shuttling factors is likely to strongly depend on their concentration since excessive amounts of Rad23 will decrease the probability that Rad23 loaded with ubiquitylated cargo will bind to the proteasome. In this respect it is noteworthy that each of these experiments was performed with either the addition of excess Rad23 to *in vitro* systems or by overexpression of Rad23 *in vivo* (Chen et al., 2001; Ortolan et al., 2000; Raasi and Pickart, 2003). A subsequent study addressed this issue and indeed revealed that Rad23 can recruit polyubiquitylated proteins to the proteasome and facilitate their degradation (Verma et al., 2004).

Recently the chaperone Cdc48 has been considered for an emerging role in the delivery of substrates to the proteasome. In a proposed escort pathway for polyubiquitylated proteins, the substrates are guided to the proteasome in a hand-over mechanism (Figure 6). Cdc48 as the central unit in this pathway serves as a scaffolding platform that binds via its ubiquitin-binding co-factors polyubiquitylated proteins and recruits multiple enzymes including E3 and E4 and DUBs, before they are delivered to the proteasome by the shuttle factors Rad23 or Dsk2 (Richly et al., 2005). As discussed above, a possible role for the unfoldase activity of Cdc48 has been proposed in the context of substrate delivery. As such, initial unfolding of substrates before they encounter the proteasome has been found as a likely scenario and would combine Cdc48's functions in recruiting proteins and its unfoldase activity in targeting for proteasomal degradation (Beskow et al., 2009; Finley, 2009).

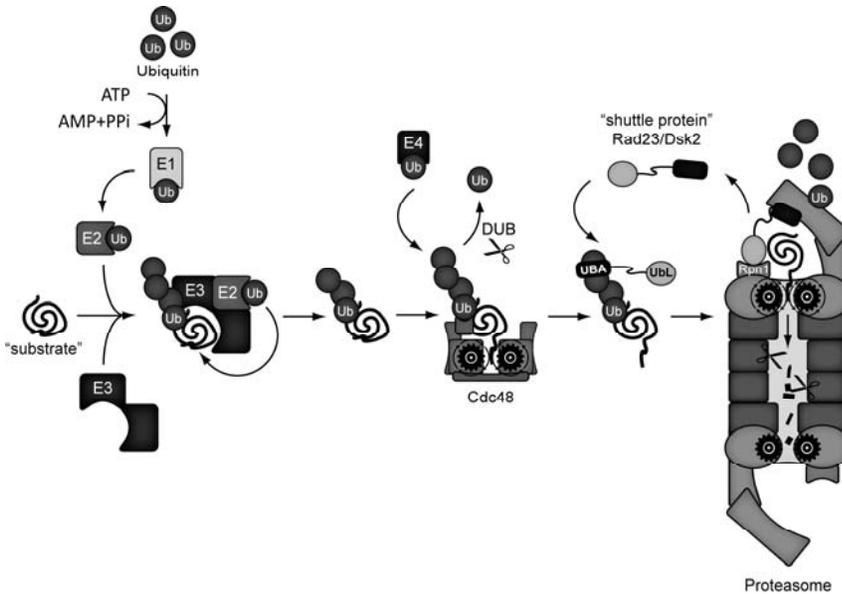


Figure 6 The role of Cdc48 and shuttle factors in ubiquitin-dependent proteasomal degradation. The substrate is ubiquitylated through a cascade of E1, E2, and E3 enzymes. The polyubiquitylated protein binds directly to the proteasome or, alternatively, is recognized by the Cdc48^{Ufd1/Npl4} complex. Shuttle factors bind to the polyubiquitin signal and deliver the substrate for degradation. Their UbL domains bind to the Rpn1 subunit of the 19S regulatory particle. Rad23 and Dsk2 resist proteasomal degradation and are released from the proteasome.

2.7 RECOGNITION AND PROCESSING OF THE SUBSTRATE AT THE PROTEASOME

Polyubiquitylated proteins bind to the proteasome via the specific ubiquitin receptors. The docking onto the proteasome is followed by deubiquitylation and unfolding of the substrate, two strictly coupled events (Verma et al., 2002; Yao and Cohen, 2002). The mechanism of translocation of the substrate into the catalytic core is not yet clarified. A possible scenario suggests that unfolding is driven by translocation (Kenniston et al., 2003; Lee et al., 2001; Pickart and Cohen, 2004; Prakash et al., 2004). According to this model the proteasome would need an interaction site to apply mechanical force on the substrate, while the counteracting resistance for unravelling is provided by the narrow entry into the core particle. In line with this model it has been found that the proteasome needs an unstructured or loosely folded polypeptide of a certain length for initiation of degradation (Takeuchi et al., 2007; Verhoef et al., 2009). Fully folded proteins might not reach deep enough into the centre of the ATPase ring in order to get

processed to allow efficient degradation of the substrate (Prakash et al., 2004). Consistent with these results is the observation that the proteasome preferentially degrades those proteins out of complexes that carry an unstructured initiation site while leaving the other binding partners intact (Prakash et al., 2009) and it has been proposed that this mechanism is responsible for the stability of some proteins that can interact with the proteasome without facing destruction like shuttle factors delivering proteins for their degradation without facing destruction themselves (Schrader et al., 2009).

3 AIMS

The work presented in this thesis focused on understanding the requirements and constraints for efficient proteasomal degradation and specifically aimed to investigate the mode of action by which the UBA2 domain stabilizes the shuttle factor Rad23.

These were the specific aims for the papers included in this thesis:

- Characterization of the minimal length requirement for efficient ubiquitin-dependent proteasomal degradation (**Paper I**).
- Analysis of the role of the ubiquitin-dependent chaperone Cdc48 in the degradation of soluble substrates (**Paper II**).
- Elucidation of the molecular mechanism responsible for the protective effect of C-terminal UBA domains (**Paper III and IV**).

4 METHODOLOGY

This chapter describes the general principles behind the techniques that have been used in the laboratory work for this thesis. Additional information about the protocols that were used in different experiments can be found in the *Material and Methods* sections of the respective papers.

4.1 GREEN FLUORESCENT PROTEIN-BASED PROTEASOME SUBSTRATES

The Dantuma group has developed a reporter system for ubiquitin-dependent proteasomal degradation based on the green fluorescent protein (GFP) (Figure 7) (Dantuma et al., 2000; Neefjes and Dantuma, 2004).

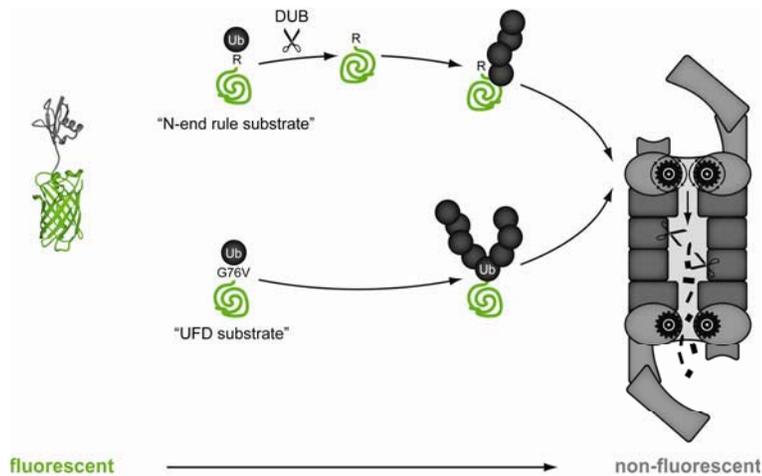


Figure 7 GFP-based proteasome substrates. Upper panel: The N-end rule substrate Ub-R-GFP is processed by DUBs. This generates a GFP protein with an N-terminal arginine that is recognized by a specific E3 ligase to assemble a polyubiquitin chain on the protein. Lower panel: The UFD substrate Ub^{G76V}-GFP is engineered by the replacement of the C-terminal glycine residue of the ubiquitin with valine (G76V). This turns the N-terminal moiety into a non-cleavable ubiquitin that functions as a degradation signal.

By converting GFP into substrates of the ubiquitin/proteasome system, proteasomal degradation can be readily monitored in cells. The fluorescent protein is targeted for proteasomal degradation by introduction of a constitutive degradation signal. The Ub-arginine-GFP (Ub-R-GFP) and Ub^{G76V}-GFP reporters (Dantuma et al.,

2000), which I used in this study, are constitutively degraded by the N-end rule (Varshavsky, 1996) or the UFD pathway (Johnson et al., 1995), respectively.

4.2 PROTEASOME INHIBITION

The proteasome degrades substrates into small peptides by the activity of three catalytic sites. As mentioned above, the proteasome has three different peptidase activities. To demonstrate that a protein is a substrate of the proteasome, the steady-state levels of the protein are compared in the presence or absence of proteasome inhibitor. The synthetic proteasome inhibitor used in these studies is the commonly used peptide-aldehyde MG132. Proteasome inhibitors are powerful tools for studying questions related to the ubiquitin/proteasome system. However, their use in yeast is hindered by the impermeability of the cell wall/membrane (Lee and Goldberg, 1996). To transiently permeabilize the cell wall/membrane, a chemical method can be used that does not require genetic manipulations of the yeast strain. Using medium with 0.1 % L-proline as sole nitrogen source and a small amount of sodium dodecylsulfate (SDS) (0.003%) the cell wall/membrane can transiently become permeable for small chemical compounds, like proteasome inhibitors (Liu et al., 2007).

4.3 PROTEIN TURNOVER ANALYSIS

A frequently used method to measure protein half-lives in yeast is referred to as promoter shut-off experiments. Target genes expressed under the galactose-inducible GAL1 promoter can be turned off rapidly after switching cells to glucose containing medium. As an option, protein synthesis can be inhibited at the same time by cycloheximide, which abrogates overall protein synthesis. The turnover rate of a protein can be determined by following its decay after shutting off the promoter. Aliquots are taken at several time points and total protein extracts are prepared for SDS-polyacrylamide gel electrophoresis (PAGE) and Western blot analysis.

4.4 FLOW CYTOMETRIC ANALYSIS

Flow cytometry is a powerful tool to count cells in a stream of fluid. A laser beam is directed onto the stream and multiple electronic detectors are aimed at the point where the stream passes through the beam. Each cell passing through the beam scatters the ray and fluorescent proteins expressed will be excited. The combination of scattered and emitted fluorescent light is picked up by the detectors and can be analyzed. The flow cytometric analysis in these studies was performed on a FACScalibur (Beckton&Dickinson) and the data were analyzed with CellQuest software.

4.5 WESTERN BLOT ANALYSIS

The western blot is an analytical technique used to detect specific proteins in a given sample of total cell lysates. It uses SDS-PAGE gel electrophoresis to separate proteins before they are transferred to a membrane (typically nitrocellulose or PVDF). After the transfer, the membranes are blocked and probed using antibodies specific to the protein of interest. The protein bands can be visualized by using chemiluminescence. For this purpose, the membrane is incubated with peroxidase-conjugated serum specifically binding to the first applied antibody.

5 RESULTS AND DISCUSSION

5.1 PAPER I

Minimal length requirement for proteasomal degradation of ubiquitin-dependent substrates

The first study included in the thesis aimed to investigate the unusual stability of the naturally occurring UFD substrate UBB+1, which is a protein found in neurodegenerative disorders. Interestingly, UBB+1 resembles a UFD substrate, consisting of an ubiquitin molecule with an additional 19 aa stretch at its C-terminus. The UFD pathway has been commonly used to target proteins of interest for ubiquitin-dependent proteasomal degradation (Johnson et al., 1992).

We found that UBB+1 and canonical UFD substrates with extensions below a critical length were inefficiently degraded by the proteasome in HeLa and neuroblastoma cells, a phenomenon that was found to be conserved in budding yeast. Extending the C-terminal polypeptide turned both UBB+1 and the designed UFD substrates into short-lived proteins. Our data suggested that the proteasome requires a short unfolded polypeptide stretch of at least 20 to 25 aa in order to efficiently process the substrate. These results are consistent with another study that was carried out with a ubiquitin-independent ornithine decarboxylase (ODC) fusion substrate. It was observed that ODC fusions need a loosely structured region of 20 to 30 aa for efficient degradation (Takeuchi et al., 2007), a length requirement that is comparable to the minimal length for UFD substrates.

We also observed that neuroblastoma cells expressing the UFD substrates that do not fulfill the length requirement had a general inhibitory effect on ubiquitin-dependent proteasomal degradation. This could be monitored with co-expression of the commonly used UFD reporter substrate Ub^{G76V}-GFP (Dantuma et al., 2000) that accumulated in the presence of short UFD substrates. This effect was not detectable in cells expressing the unstable ^{myc}Ub-fusions with extensions longer than 25 amino acids. It has been shown before that accumulation of UBB+1 has an inhibitory effect on ubiquitin-dependent proteolysis (Lindsten et al., 2002). This is of particular interest since dysfunction of the ubiquitin/proteasome system may play a role in the pathophysiology of neurodegenerative diseases (Ciechanover and Brundin, 2003; Sherman and Goldberg, 2001). Our data suggest that the limited length might be

responsible for the unusual stability of UBB+1. While the exact mechanism responsible for its inhibitory activity on the ubiquitin/proteasome system remains unclear, this atypical UFD substrate has given new insights into the prerequisites for efficient proteasomal degradation that are conserved in eukaryotes.

5.2 PAPER II

Cdc48-mediated unfolding as a critical determinant for the need of polyubiquitin chains in proteasomal degradation

Our finding that degradation of substrates requires a minimal length for being efficiently processed by the proteasome (**Paper I**) encouraged us to investigate further the role of unstructured polypeptide sequences in degradation. We took advantage again of the commonly used fluorescent UFD substrate Ub^{G76V}-GFP (Dantuma et al., 2000) that is targeted for degradation through the well-described UFD pathway. Polyubiquitylated proteins are escorted to the proteasome, involving the Cdc48^{Ufd1/Npl4} chaperone complex, the elongation factor Ufd2, and the shuttle factor Rad23 (Richly et al., 2005).

Accordingly, we found that the degradation of the canonical UFD substrate Ub^{G76V}-GFP was strictly depending on Cdc48. Extending the substrate with a short unstructured polypeptide at its C-terminus alleviated the need for Cdc48 in proteasomal degradation. This observation was consistent with previous results for the mammalian homolog p97 (Beskow et al., 2009). Remarkably, the unstructured polypeptide had to fulfill the same length requirements as observed in **Paper I**.

Next, we observed that the presence of an unstructured initiation site not only allowed the substrate to be degraded independent of Cdc48, but also at the same time abrogated the need for polyubiquitylation. Substrates having no or short extensions were stabilized in the absence of UFD-mediated polyubiquitylation, whereas the fast degradation of Ub^{G76V}-GFP with a 20 aa extension was unaffected. This interesting observation encouraged us to investigate the role of the non-cleavable N-terminal ubiquitin moiety in UFD substrates in targeting for degradation. We found that the Cdc48-independent substrate was still targeted for proteasomal degradation by the N-terminal ubiquitin albeit independent of polyubiquitylation.

The novel finding that monoubiquitin efficiently targets proteins for degradation raises the question of how this substrate is recognized by the proteasome. In this respect, it is interesting that the intrinsic proteasomal ubiquitin receptor Rpn13 binds monoubiquitin with high affinity (Husnjak et al., 2008). It remains to be clarified whether this is of functional significance for the degradation of Cdc48-independent substrates. However, it has been shown that processing of precursor proteins by the proteasome can be driven by monoubiquitylation (Kravtsova-Ivantsiv et al., 2009; Rape et al., 2001).

We turned to an alternative proteasome interaction motif and took advantage of the UbL domains of the shuttle factors Rad23 and Dsk2. These two proteins interact with the Rpn1 subunit of the proteasome independent of polyubiquitylation. We found that the UbL-GFP fusion protein was a stable protein but introduction of an unstructured initiation site resulted in rapid degradation by the proteasome. Our data that the proteasome alone is not able to unfold certain substrates prompted us to speculate that a primary role of the UFD pathway might be to assure that tightly folded substrates lacking initiation sites are first delivered to the Cdc48^{Ufd1/Npl4} complex before they are handed over to the proteasome for their degradation (Figure 8).

Unstructured polypeptides also appear to play major roles in degradation initiation in prokaryotic targeting systems, where they are integrated in the degradation tags. The ClpAP and ClpXP proteases require a short SsrA peptide tag conjugated to C-termini of substrates in order to degrade the protein (Gottesman et al., 1998), and, in addition, the prokaryotic ubiquitin-like protein (Pup) in *Mycobacterium tuberculosis*, carries an N-terminal structural element that allows the prokaryotic proteasome to initiate degradation (Striebel et al., 2010). We show that the generation of an unstructured initiation site by the ubiquitin-selective chaperone complex Cdc48^{Ufd1/Npl4} is the critical event in the degradation of UFD substrates that determines the need for polyubiquitin chains in this process. We conclude that the strict requirement for polyubiquitylation in the degradation of proteasome substrates does not necessarily have to be attributed to polyubiquitin-dependent binding at the proteasome but may instead reflect the involvement of polyubiquitin-specific proteins acting upstream of the proteasome. Interesting in respect to the stability of the shuttle factors Rad23/Dsk2 is our observation that proteasome reporter substrates based on the UbL domains of these proteins failed to be degraded, unless they carry an unstructured initiation site (**Paper IV**).

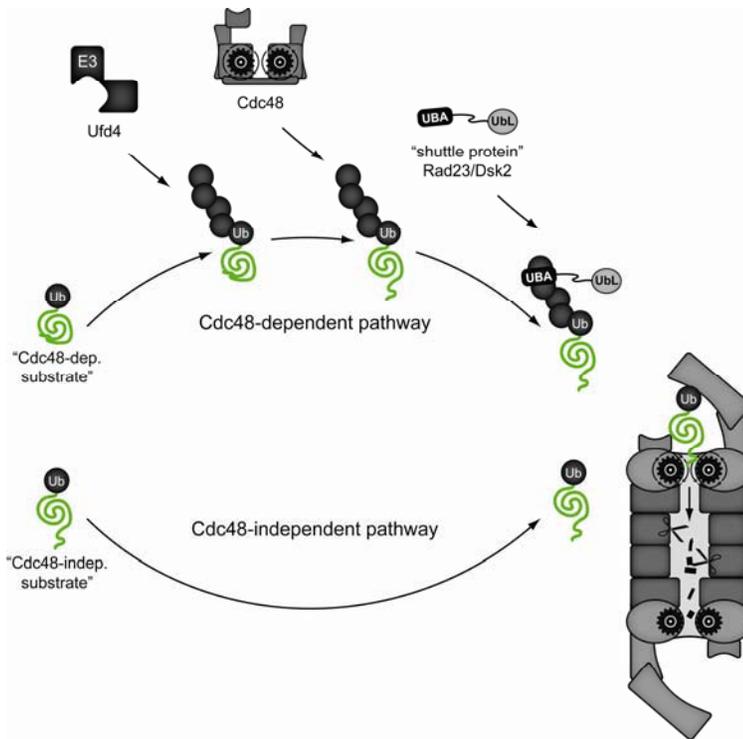


Figure 8 Model for Cdc48-dependent and Cdc48-independent degradation. The canonical UFD substrate Ub^{G76V}-GFP is degraded in a Cdc48-dependent fashion, following the escort pathway (Richly et al., 2005). First, the protein is ubiquitylated by the Ufd4 HECT E3 ligase. Subsequently, the modified substrate is bound by the Cdc48^{Ufd1/Npl4} complex to generate an initiation site. Finally, the shuttle factor (e.g. Rad23) binds the polyubiquitin chain of the substrate and delivers it to the proteasome for degradation. An unstructured initiation site bypasses not only the Cdc48 requirement, but also renders the degradation independent of polyubiquitylation and Rad23. The protein is targeted by its N-terminal uncleavable ubiquitin moiety to the proteasome.

5.3 PAPER III

Mutant p62/SQSTM1 UBA domains linked to Paget's disease of bone differ in their abilities to function as stabilization signals

Cellular stabilization signals have added a new layer of complexity to the regulation of the ubiquitin/proteasome system. The UBA2 domain functions as an intrinsic stabilization signal that prevents degradation of the proteasome-interacting protein Rad23. More recently, it has been reported that the UIM domain of the transcription factor Met4 protects its host protein by preventing the formation of K48-linked polyubiquitin chains that are sufficiently long to lead to proteasomal degradation (Flick

et al., 2006). While the mode of action by which the UBA2 domain protects Rad23 from degradation remains unknown, it is likely to deviate from the model proposed for Met4, since Rad23 has to interact with the proteasome in order to fulfill its task in shuttling substrates to the proteasome (Elsasser and Finley, 2005). Although it seemed plausible that the molecular mechanism for UBA domain-mediated protection against proteasomal degradation would also implicate ubiquitin binding. This hypothesis was in particular attractive since the UBA and UIM domains only share their ability to bind ubiquitin chains (Dikic et al., 2009). This brings up the question of how the protective effect of the UBA domain is accomplished. In this study we wanted to gain insight into the protective mechanism mediated by UBA domains. For this purpose, we have taken advantage of four naturally occurring mutant UBA domains of the protein p62 and used them to study the role of ubiquitin binding and structural integrity in UBA-mediated protection. Interestingly, multiple mutations clustered within or adjacent to p62's UBA domain have been linked to sporadic and familial cases of Paget's disease of bone (PDB) (Laurin et al., 2002). The etiology of PDB is poorly understood but it is characterized by a focal increase in bone turnover causing fractures and bone deformations (Ralston et al., 2008).

First, we introduced the wild-type and mutant UBA domains into the GFP-based N-end rule reporter substrate Ub-R-GFP and analyzed their effect on the substrate's half-life. We observed that the UBA domain of p62 shares with the UBA2 domain of Rad23 its ability to inhibit proteasomal degradation of reporter substrates. Introduction of the wild-type p62 UBA domain into Ub-R-GFP caused a significant increase in protein steady-state levels and a prolonged half-life. This opened the possibility to exploit a number of natural occurring UBA mutants of p62 that varied in their ability to bind ubiquitin (Cavey et al., 2005). We found that both, wild-type and mutant UBA domains, can function as *cis*-acting stabilization signals to protect the reporter substrate from proteasomal degradation. Most surprising was the observation that the UBA^{G425R} mutant domain of p62, a fully folded UBA domain but severely impaired in its ubiquitin binding activity, is still able to delay proteasomal degradation of the reporter substrate. We observed a correlation between the folding state and the protective effect of the mutant UBA domains tested. The different mutations in the UBA domain of p62 led to a variation of structural stabilities of the domains and we observed that fully folded domains like the wild-type UBA domain and the mutant domains UBA^{P392L} and UBA^{G425R} were able to prolong the half-life, whereas mutant

domains with reduced thermal stability, like UBA^{G411S} and UBA^{M404T}, were not able to protect or even further destabilized the reporter substrate.

This approach suggested that structural integrity is likely important for UBA-mediated inhibition of proteasomal degradation. Notably, the data do not exclude a contributing role for ubiquitin binding since all ubiquitin binding-deficient domains were less efficient in decelerating degradation than the wild-type UBA domain. Our data show that the thermal stability of the domains correlates with the protective effect indicating that structural integrity may play an important role in UBA-mediated protection of proteins.

5.4 PAPER IV

UBA-mediated inhibition of protein unfolding prevents proteasomal degradation

In **Paper IV**, we studied how C-terminal UBA domains protect shuttle factors from proteasomal degradation.

We found that the UBA-mediated protection from proteasomal degradation is a shared feature of the ubiquitin receptors Rad23 and Dsk2. Both proteins share the ability of shuttling substrates to the proteasome and need to physically interact with the proteasome (Verma et al., 2004).

Next, we compared side-by-side the protective effects of the C-terminal UBA domains of Rad23 and Dsk2 and the UIM domain of Met4 in the context of the GFP-based N-end rule reporter substrate. In contrast to the UBA domains, the UIM of Met4 was not able to protect Ub-R-GFP from proteasomal degradation, which suggested that divergent molecular mechanisms are responsible for protection of their native proteins. This finding was in agreement with the fact that features of Rad23 or Dsk2 are hard to reconcile with a Met4-type of mechanism, since interaction with the proteasome plays a central role in shuttling substrates for proteasomal degradation.

Testing for the critical determinants of UBA-mediated protection, we found that the C-terminal position of the protective UBA domain is critical for the stabilizing effect. Our observation that efficient proteasomal degradation of UbL-based fluorescent reporters is strictly dependent on the presence of an unstructured initiation site (**Paper II**) and the finding that the structural integrity of the UBA domain important for the stabilizing effect (**Paper III**), encouraged us to investigate how these domains could

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interfere with the initiation of degradation. We found that C-terminal extensions were able to abrogate the protective effect in reporter substrates and Rad23 (Figure 9). This interference with stabilization was dependent on the length of the extension, which corresponded to the minimal length requirements for unstructured initiation sites (**Paper I**) (Takeuchi et al., 2007). Our observations suggest that these proteins are shielded from the destructive activities of the proteasome by elements that prevent the initiation of degradation subsequent to the actual binding to the proteasome. This mechanism is compatible with the observed transient interactions of these shuttle factors with the proteasome and fundamentally different from the mechanism proposed for Met4.

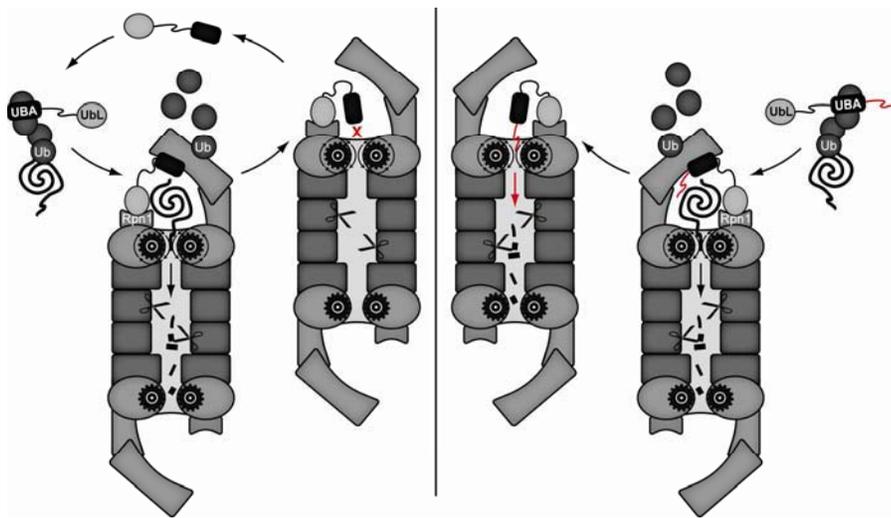


Figure 9 Model for UBA-mediated protection. The schematic drawing shows a shuttle factor (Rad23/Dsk2) that delivers an ubiquitylated substrate to the 26S proteasome. It binds the proteasome via the Rpn1 subunit to deliver the substrate bound to its UBA domain. The substrate is deubiquitylated and transferred into the catalytic core by the help of the AAA-ATPase ring in the base of the regulatory particle. Wild-type Rad23/Dsk2 contains C-terminal UBA domains that resist to initiation of protein degradation. As a consequence the shuttle factor is not degraded and released from the proteasome (left panel). In case Rad23/Dsk2 exposes an unstructured initiation site C-terminally of its protective UBA domain, the shuttle factor itself becomes subject of the unfolding machinery of the proteasome and is degraded (right panel).

It has previously been shown that proteasomes, when interacting with protein complexes, display a strong preference for those proteins that contain unstructured initiation sites. This may play a significant role in selecting proteins for their

degradation at the proteasome (Prakash et al., 2009). Our findings are fully consistent with this model and suggest that the presence or absence of unstructured initiation sites are used as a means to distinguish between substrates designated for degradation and proteasome-interacting proteins to be spared.

Our results suggest that resistance to initiation of protein degradation is the primary factor responsible for the protective effect of UBA domains. This may explain why some UBDs fail to protect from proteasomal degradation (Heessen et al., 2005), whereas mutant UBA domains with severely impaired ubiquitin binding abilities are still able to delay degradation (**Paper III**). It is intriguing that the UIM and UBA domains, two small domains that have little in common except their ubiquitin binding ability, target two independent events that are both critically required for efficient proteasomal degradation. It remains to be identified how widespread the usage of ubiquitin binding domains for protecting proteins from proteasomal degradation is.

6 GENERAL DISCUSSION AND PERSPECTIVES

“To degrade or not to degrade?” is a simple question the proteasome is confronted with continuously. Having a closer look, this simple issue turns out to be more complex than initially anticipated. It is well established that polyubiquitin chains are the canonical signals for targeting substrates for proteasomal degradation. However, it has also been shown that directly tethering proteins to the proteasome by other means can be sufficient to facilitate their destruction (Janse et al., 2004). How are proteasomes then able to distinguish between the substrates deemed for degradation and proteasome-interacting proteins? Or more simply put, why are certain proteins that encounter the proteasome efficiently and rapidly degraded whereas others are spared and released?

Most striking in this respect are the shuttle factors that deliver substrates for degradation and thus transiently interact with and function in close proximity to the proteasome. Our data suggest that these proteins are shielded from the destructive activities of the proteasome by elements that prevent the initiation of degradation (**Paper IV**). This finding is in agreement with the proposed selective nature of protein degradation at the proteasome and gives the first example from nature that the presence or absence of initiation sites is used as a means to distinguish between substrates designated for degradation and proteasome-interacting proteins to be spared. Before a protein can reach the catalytic sites, a critical step is the unfolding of the protein, since the proteolytic chamber can only be reached through a narrow channel (Groll et al., 2000). ATPases in the base of the regulatory particle have been shown to be responsible for this unfolding step (Navon and Goldberg, 2001). How is this accomplished? A possible scenario suggests that unfolding is driven by translocation through the narrow channel (Kenniston et al., 2003; Lee et al., 2001; Pickart and Cohen, 2004; Prakash et al., 2004). In this case, the proteasome would need an interaction site to apply mechanical force on the substrate, while the counteracting resistance for unravelling is provided by the residual protein stuck at the narrow entry of proteasome. To this end, a possible interaction site should reach deep enough into the centre of the ATPase ring in order to get processing started. Recent results are indeed fully consistent with this model. The proteasome needs an unstructured or loosely folded polypeptide of a certain length (**Paper I**) as an initiation site for degradation to allow efficient degradation of the substrate (Prakash et al., 2004). Our data shows that the structural integrity rather than ubiquitin binding is important for

UBA-mediated stabilization (**Paper III**) providing further evidence for a lack of initiation as the protective mechanism. This model gives a possible explanation of why extending the shuttle factor Rad23 with an unstructured polypeptide of a certain length turns it into a short lived protein (**Paper IV**). Supportive evidence comes from the observation that of protein complexes interacting with the proteasome, the proteasome preferentially degrades those proteins that carry an unstructured initiation site while leaving the other subunits intact (Prakash et al., 2009). This may also explain why some proteins can functionally operate in close proximity to the proteolytic complex without becoming degraded (Schrader et al., 2009).

However, at the same time, the preference of proteasomes for substrates with unstructured initiation sites raises the question of how the cell deals with other tightly folded proteins. It is interesting in this respect that a UbL-GFP fusion protein is not degraded without an unstructured initiation site. In addition, the degradation of Ub^{G76V}-GFP is strictly depending on Cdc48 (**Paper II**), suggesting that the proteasome alone does not seem to be able to unfold GFP. Cdc48 is a chaperone that has already been reported to be required for the degradation of certain other cytoplasmic substrates (Cao et al., 2003; Decottignies et al., 2004; Fu et al., 2003; Ghislain et al., 1996; Hartmann-Petersen et al., 2004; Verma et al., 2004). An attractive model would be that the unfoldase activity of Cdc48 provides substrates with a short unfolded or loosely folded polypeptide, a function that has been recently suggested for the mammalian homolog p97 (Beskow et al., 2009), giving Cdc48 a central role in proteasomal degradation. In **Paper II** we show that this holds true for yeast and in line with an important function in proteasomal degradation, our results reveal that the strict requirement for polyubiquitylation in the degradation of certain proteasome substrates does not necessarily have to be attributed to polyubiquitin-dependent binding at the proteasome but may instead reflect the involvement of the ubiquitin-selective chaperone complex Cdc48^{Ufd1/Npl4} acting upstream of the proteasome.

The fact that UbL-GFP is a stable protein suggests that it bypasses Cdc48^{Ufd1/Npl4} to specifically bind to the Rpn1 subunit of the proteasome. Although there are indications that Rad23 is polyubiquitylated (Elder et al., 2002; Ramsey et al., 2004; Watkins et al., 1993), the interaction is independent of ubiquitylation (Elsasser et al., 2002). Noteworthy, the C-terminal UBA domains of Rad23 and Dsk2 are able to render reporter substrates resistant to Cdc48 mediated unfolding. GFP-based N-end rule (unpublished data) and UFD reporter substrates (**Paper II**) are quickly degraded in a

Cdc48-dependent manner, while the UBA domains stabilized these short-lived reporter substrates (**Paper IV**). How these proteins are able to resist both, Cdc48-mediated processing and proteasomal unfolding, remains unclear. It is tempting to speculate that the UBA domains could mask or limit polyubiquitylation of these artificial substrates, similar to what has been proposed for Met4. This could either affect the interaction between Cdc48 and the substrate or, alternatively, interfere with E4 activity at Cdc48. Interestingly, without E4 activity, the ubiquitin chains on the substrates are too short for shuttle factor binding (Richly et al., 2005). Cdc48, together with Rad23/Dsk2, escorts UFD substrates to the proteasome. According to the model, gradual step-wise extension of the chain on the substrate safeguards that the protein is handed over to a shuttle factor for proteasome targeting. To this end, Cdc48 does not only coordinate the substrate recruitment but also the ubiquitin chain elongation catalyzed by the E4 enzyme Ufd2. An intriguing possibility is that interfering with ubiquitin chain elongation could inhibit the release of the substrate from Cdc48 and disrupt the escort pathway.

In addition, it would be interesting to know how Cdc48 could provide proteins with short unfolded polypeptide stretches. Notably, the ring-shaped structure of Cdc48 suggests a central channel, similar to the ATPase ring of the proteasome but recent structural data suggest that this channel is blocked by a zinc ion leaving only one possibility for substrates to enter and exit (DeLaBarre and Brunger, 2003). Since a central open channel would argue for full unfolding of the polypeptide, similar to the situation at the ATPase ring of the proteasome, it is tempting to speculate that a closed channel could instead favor the generation of short unfolded sequences.

In summary, we propose that the stabilizing mechanism of shuttle factors is based on *cis*-mediated unfolding inhibition. To this end, proteins have developed strategies to escape the cellular unfolding machineries by specifically located and tightly packed domains or by specialized targeting domains that can bypass the unfolding activity of chaperones. We and others have shown that efficient proteasomal degradation requires an unstructured polypeptide of a certain length. Our results suggest that this prerequisite for degradation enables the proteasome to decide which proteins to degrade and which proteins to spare.

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