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THE UBIQUITIN-PROTEASOME SYSTEM DURING PROTEOTOXIC STRESS

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Front cover: Ub-R-YFP MelJuSo cells treated with proteasome inhibitor
(Illustration by V. Menéndez Benito)

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"C'est le temps que tu as perdu pour ta rose

qui fait ta rose si importante"

Saint Exupéry (Le Petit Prince)

A mis padres

ABSTRACT

The dual function of the ubiquitin-proteasome system in protein quality control and as a master regulator of vital cellular processes places the system in a delicate position. This might be particularly relevant under patho-physiological conditions such as endoplasmic reticulum stress, which provoke the accumulation of aberrant proteins. This scenario, also referred to as proteotoxic stress, is associated with numerous devastating disorders, including a large number of neurodegenerative diseases like Alzheimer's disease, Parkinson's disease and polyglutamine diseases. The question is whether the ubiquitin-proteasome system is able to promptly and adequately respond to these challenging conditions without compromising its other functions.

One of the difficulties approaching these questions is the lack of systems for monitoring the functionality of the ubiquitin-proteasome activity *in vivo.* We have generated fluorescent reporter substrates for monitoring the functionality of the ubiquitin-proteasome system in cell lines. In addition, we have developed a transgenic mouse model constitutively expressing one of these reporters. Finally, we have designed and characterized a novel fluorescent activity probe that permits specific labeling of proteasomes *in vitro* and *in vivo*.

We have subsequently used these models to gain insight into the mechanisms contributing to the long term accumulation of deleterious proteins during proteotoxic stress. These studies revealed that proteotoxic stress conditions compromises the functionality of the ubiquitin-proteasome system. In these circumstances, ubiquitin-proteasomal degradation is still taking place but it is suboptimal. Detailed analysis of the dynamics of ubiquitylation in living cells suggests that ubiquitin is a rate limiting factor during stress conditions. Importantly, we found that whereas the ubiquitin-proteasome was able to remove the majority of the accumulated substrates once the cells have recovered from the stress condition, the cells were unable to clear accumulated aggregation-prone substrates. This observation might explain the preferential accumulation of such substrates in conformational diseases.

LIST OF PUBLICATIONS

This thesis is based on the following papers and manuscripts that will be referred to in the text by their Roman numerals:

I Lindsten K*, **Menéndez-Benito V***, Masucci MG, Dantuma NP. (2003) A transgenic mouse model of the ubiquitin/proteasome system. *Nature Biotechnol.* 21 (8):897-902

II **Menéndez-Benito V**, Verhoef LG, Masucci MG, Dantuma NP. (2005) Endoplasmic reticulum stress compromises the ubiquitin-proteasome system. *Hum. Mol. Genet.* 14 (19):2787-99

III Verdoes M*, Florea BI*, **Menéndez-Benito V**, Witte MD, van der Linden WA, van den Nieuwendijk AM, Hofmann T, Berkers CR, van Leeuwen FWB, Groothuis TA, Leeuwenburgh MA, Ovaa H, Neefjes JJ, Filippov DV, van der Marel GA, Dantuma NP, Overkleeft HS. A fluorescent broad spectrum proteasome inhibitor for labeling proteasomes *in vitro* and *in vivo*. *Submitted*

IV **Menéndez-Benito V***, Salomons FA*, Dantuma NP. A transient depletion of free ubiquitin after proteotoxic stress contributes to the accumulation of proteasomal substrates. *Manuscript in preparation.*

* These authors contributed equally to the work

RELATED PUBLICATION

Menéndez-Benito V, Heessen S and Dantuma NP. (2005) Monitoring of ubiquitin-dependent proteolysis with green fluorescent protein substrates. *Methods Enzymol.* 339:490-511

ABBREVIATIONS

APC	Anaphase promoting complex
CMV	Cytomegalovirus
DUB	Deubiquitylating enzyme
E1	Ubiquitin activating enzyme
E2	Ubiquitin conjugating enzyme
E3	Ubiquitin ligating enzyme
E4	Chain elongation factor
ER	Endoplasmic reticulum
ERAD	ER-associated degradation
FACS	Fluorescence-activated cell sorter
FP	Fluorescent protein
GAr	Glycine Alanine repeat
GFP	Green fluorescent protein
JAMM	JAB1/MPN/Mov34
MHC	Major histocompatibility complex
ODC	Ornithin decarboxylase
OUT	Otubain protease
PAN	Proteasome activating nucleotidase
PGPH	Post-glutamyl peptide hydrolase
RUB1	Related to ubiquitin 1
SCF	Skp1/Cul1/F-box
SCA	Spinocerebellar ataxia
SUMO	Small ubiquitin-related modifier
UBA	Ubiquitin associated domain
UBB	Ubiquitin B
UBC	Ubiquitin-conjugating E2
UBD	Ubiquitin binding domain
UBL	Ubiquitin-like modifier
UbL	Ubiquitin-like domain
UCH	Ubiquitin C-terminal hydrolase
UEV	Ubiquitin-conjugating enzyme variant
UPR	Unfolded protein response
USP	Ubiquitin-specific proteases
YFP	Yellow fluorescent protein

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1. GENERAL INTRODUCTION

A major breakthrough in cell biology has been the discovery that protein turnover is a regulated and dynamic process orchestrated by the ubiquitin-proteasome system. The elegant work of Rose, Hershko and Ciechanover during the late 70s, lead to the discovery of the ubiquitin-mediated protein degradation, which was awarded with the Nobel Prize in Chemistry in the year 2004. Since the discovery of regulated protein degradation, the progress in the understanding of the mechanisms involved in ubiquitin-dependent proteasomal degradation has been spectacular. Nowadays, the ubiquitin-proteasome system is known as a versatile mechanism that plays a vital role in virtually every cellular process (124).

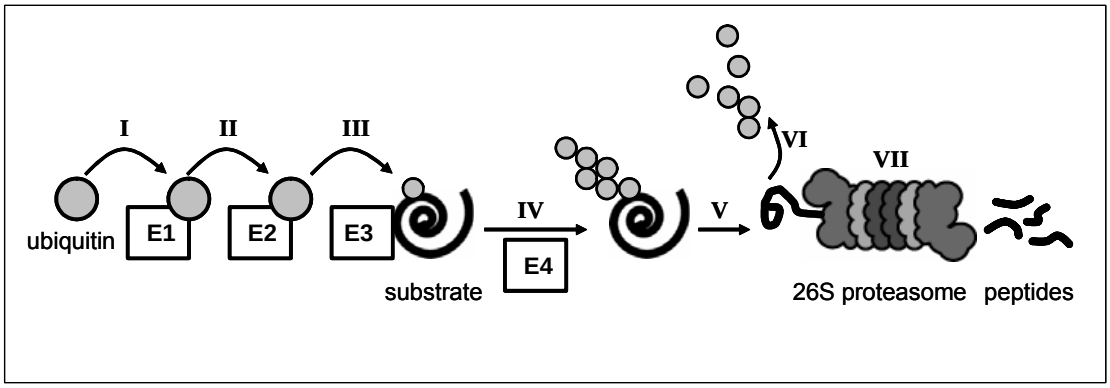

Figure 1. *Schematic representation of the ubiquitin-proteasome system.* (**I**) Ubiquitin is activated by the E1. (**II**) Activated ubiquitin is then transferred to E2. (**III**) The protein substrate is specifically recognized by E3 and ubiquitin is transferred from E2 to the substrate. (**IV**) Successive conjugation rounds result in the formation of a poly-ubiquitin chain. In some cases an E4 is involved in elongation of the poly-ubiquitin chain. (**V**) The poly-ubiquitin chain is recognized as a degradation signal. (**VI**) Binding of the poly-ubiquitylated substrate to the proteasome results in deubiquitylation, unfolding and translocation through the proteasome. (**VII**) The substrate is degraded into small peptides.

The ubiquitin-proteasome system consists of ubiquitin, a multi-enzymatic system comprised of ubiquitin-activating (E1), ubiquitin-conjugating (E2) and ubiquitin-ligating enzymes (E3) and the proteasome; a self compartmentalised multisubunit protease. Degradation of substrates occurs in a regulated and sequential order (79, 104). In the presence of a degradation signal ubiquitin is conjugated to the protein substrate via a three-step

mechanism catalysed by E1, E2 and E3. Successive conjugation rounds result in the formation of a poly-ubiquitin chain. Finally, the poly-ubiquitylated substrate binds to the proteasome and is subsequently deubiquitylated, unfolded, translocated into the proteolytic chamber of the proteasome and degraded into small peptides (Fig. 1).

Degradation of proteins by the ubiquitin-proteasome system is of vital importance for nearly all cellular processes. First, the exact temporal and spatial inactivation of many key proteins that regulate processes such as cell cycle progression, apoptosis, transcription and development, are controlled by ubiquitin-proteasomal degradation. Second, the ubiquitin-proteasome system is implicated in the immune response, since it generates the majority of the peptides presented by the major histocompatibility (MHC) class I (83). Finally, the ubiquitin-proteasome system is responsible for the clearance of the aberrant proteins in the cellular environment (Fig. 2). In addition, there are many non-proteolytic functions associated with the ubiquitin-proteasome system, such as DNA repair, membrane transport, chromatin-remodeling, transcription and signaling pathways (62, 76, 107, 145).

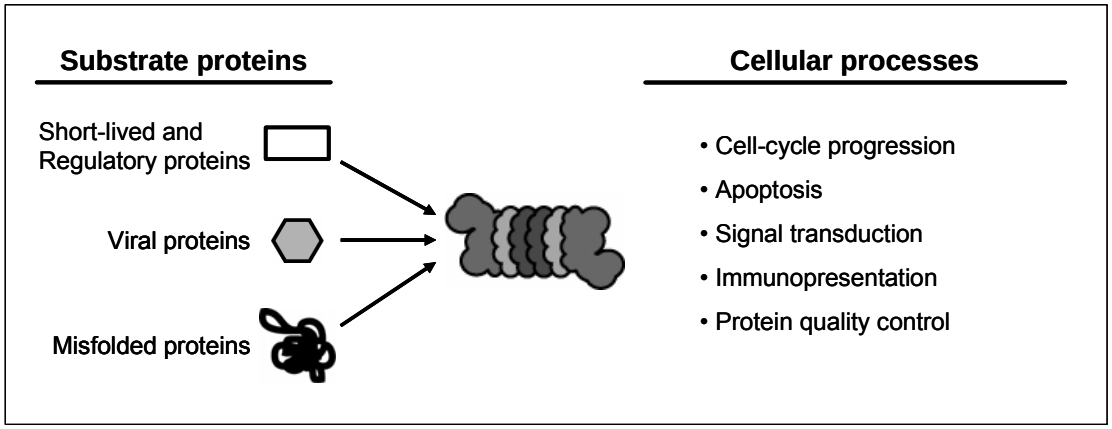

Figure 2. *Functions of the ubiquitin-proteasome system in protein degradation.* The ubiquitin-proteasome system controls the degradation of short-lived and regulatory proteins such as cell-cycle regulators, transcriptor factors and tumor suppressors, which are involved in a variety of basic cellular process. Additionally, the ubiquitin-proteasome system plays an important role in the clearance of misfolded and damaged proteins. Viral proteins are also degraded by the ubiquitin-proteasome system and the resulting peptide fragments will be use for MHC class I presentation.

The dual function of the ubiquitin-proteasome system as a sentinel of protein quality control and as a master regulator of vital cellular processes places the system in a delicate position. This situation might be particularly relevant under conditions such as heat shock, hypoxia, oxidative stress or endoplasmic reticulum (ER) stress that cause a rapid accumulation of misfolded and damaged proteins, a condition known as proteotoxic stress. The question is whether the ubiquitin-proteasome system is able to promptly and adequately respond to these challenging conditions without compromising its house-keeping functions. The work described in this thesis approaches this question with the development of chemical, cellular and animal models for monitoring the functional status of the ubiquitin-proteasome system. These models have been subsequently used to study the response of different proteasomal degradation pathways to proteotoxic stress conditions. The findings presented in this thesis shed light on some of the factors controlling the dynamics and functionality of the ubiquitin-proteasome system in normal and pathological conditions.

2. TARGETING PROTEINS FOR DEGRADATION

2.1. Ubiquitin

Ubiquitin is a small protein consisting of 76 amino acids and is expressed in all eukaryotic cells. It is encoded by a multigene family with a very unusual organization in that it consists of several monomeric and multimeric ubiquitin genes (202, 275). The monomeric genes code for ubiquitin moieties fused to ribosomal proteins. The multimeric genes encode ubiquitin precursors, containing repeats of ubiquitin immediately adjacent to each other. Both forms are post-translationally processed to ubiquitin monomers by ubiquitin C-terminal hydrolases (UCH). The amount of monomeric ubiquitin loci and the number of ubiquitin tandem repeats in the multimeric ubiquitin locus varies among different species. However, the amino acid sequence of ubiquitin is extremely conserved among species. For instance, yeast and human ubiquitin differ only in three amino acid residues.

Ubiquitin was the first protein discovered to be covalently attached to other proteins by an isopeptide bond (84). In general, this isopeptide bond is formed between the C-terminal glycine of ubiquitin (Gly76) and the ε-NH_2 group of an internal lysine residue of the targeted protein. In addition, some proteins are substrates for a linear ubiquitin conjugation to the α-NH_2 group of the N-terminal residue (37). The current knowledge is that protein modification by ubiquitin can be mediated either by attachment of a single ubiquitin (mono-ubiquitylation) or a poly-ubiquitin chain (poly-ubiquitylation), in which is ubiquitin is conjugated to the preceding ubiquitin by the formation of an isopeptide bond.

Poly-ubiquitination was shown to serve as a signal that targets proteins for degradation (39, 40, 105, 280). In addition, a number of non-proteolytic functions of ubiquitin have been discovered during the last decades, such as endocytosis, signal transduction, chromatin remodelling and DNA repair [for review, see (237, 269)]. Many of these functions are controlled by mono-ubiquitylation and by the formation of particular ubiquitin-chains which differ in length or in structure [for review, see (107, 211)].

In the past few years an increasing number of proteins sharing structural similarities with ubiquitin have been identified. Among these are the

ubiquitin-like modifiers (UBLs), such as SUMO (small ubiquitin-related modifier), RUB1 (related to ubiquitin 1) and Apg12, which have the capacity to be enzymatically conjugated to substrates. Modification by ubiquitin-like proteins is important for diverse biological processes such as DNA repair, autophagy and signal transduction [for review, see (110, 240)].

2.2. Ubiquitylation

The formation of an isopeptide bond between ubiquitin and its target protein is the result of a multi-step process that requires the coordinated action of at least three different type of enzymes: ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2) and ubiquitin-ligating enzyme (E3) [for review, see (79, 104)].

In the first step, ubiquitin is activated by the formation of a high energy thiol-ester linkage between the C-terminal carboxyl group of ubiquitin and the thiol group of a cysteine residue within the catalytic site of E1. This first reaction requires the hydrolysis of an ATP molecule. The activated ubiquitin is then transferred to a cysteine residue of one of several E2s. In the third reaction, the concerted action of E2 and a member of the E3 family results in the ubiquitylation of the substrate protein that is specifically bound to E3. For most substrates, the first ubiquitin moiety is attached to the ε-NH_2 group of an internal lysine, but some proteins are substrates for a linear ubiquitin conjugation to the α-NH_2 group of the N-terminal residue (37).

Once the first ubiquitin is attached to the substrate it can become the acceptor of a new ubiquitylation cycle and, through successive rounds of ubiquitylation, the poly-ubiquitin chain is formed. Since ubiquitin has seven lysine residues (positions 6, 11, 27, 29, 33, 48 and 63) that are potential ubiquitylation sites, several linkages could occur that would result in chains with different structures and functions (206). Most commonly, poly-ubiquitin chains are linked through Lys48 and are the canonical signal for proteolysis. Although less common, Lys29 chains have been shown to be form *in vivo* and to target a particular subset of protein substrates for degradation (126, 167). Another naturally occurring poly-ubiquitin chain is linked through Lys63. This chain has been shown to be involved in a number of processes, including endocytosis

(71), DNA repair (113) and translation (249), but it does not appear to target proteins for degradation. Poly-ubiquitin chains linked through Lys6 have been associated with the E3 activity of BRCA1, a breast and ovarian cancer-specific tumor suppressor (185, 196, 284). The BRCA1-dependent localization of Lys6 poly-ubiquitin chains to DNA repair foci suggest a possible function in DNA repair (185). Other poly-ubiquitin chains remain poorly characterized and their biological functions remain unknown.

2.3. Ubiquitylating enzymes

Research over the last decade has revealed that ubiquitylation is mediated by an immense number of E2/E3 pairs that are susceptible to sophisticated ways of regulation. In contrast with the multiplicity of E2s and E3s, there is only one E1 in yeast (98) and deletion of this gene is lethal (177). In mammals E1 exist as two isoforms, resulting from alternative translation initiation sites (43, 93). The E1a isoform is phophorylated and it has been proposed that this modification regulates the cell cycle dependent E1a nuclear localization (44). Studies on E1 carried on during the early years of the ubiquitin-field were particularly informative. In fact, the identification of the mouse cell line ts85 as a E1 temperature sensitive mutant together with the observation that ts85 cells tend to arrest in the G2 phase of the cell cycle was the first clue that ubiquitylation has a role in cell cycle progression (65).

Eleven different E2s have been identified in yeasts (Ubc1-8, 10, 11 and 13) and more than 30 in higher eukaryotes. These proteins share a catalytic core domain of about 150 amino acids that contains a conserved cysteine residue that binds ubiquitin. Individual E2s are able to associate with different E3s, and a single E3 ligase may associate with more than one E2. This diversity of interaction may increase the range of substrates that can be recognized by the ubiquitin-signalling cascade. Moreover, it also suggests that there might be mechanisms regulating the formation of specific E2/E3 pairs in the cell.

E3s are responsible for the specificity in substrate recognition and therefore constitute a very diverse group of enzymes in terms of size, functional domains and number. E3s belong to two main subfamilies: HECT (homologous

to the E6-AP1 C-terminus)-domain E3s and RING (really interesting new gene)-finger domain E3s. The mechanism for E2/E3 mediated ubiquitylation varies for the different E3s. For HECT-domain E3s, ubiquitin is transferred from E2 to a cysteine residue on the E3 and then to the E3-bounded substrate. On the other hand, the RING finger-domain E3s directly catalyze the transfer of ubiquitin from E2 to the protein substrate.

HECT-E3s are characterized by a 150 amino-acid C-terminal domain. This domain contains a conserved cysteine residue that is the acceptor of ubiquitin (233). The N-terminal domains mediate substrate recognition and vary among the different HECT E3s. The RING finger domain is a small domain of about 50 amino acids defined by a conserved Zn^{+2}-chelating His/Cys-rich domain (20) that, when present in an E3, mediates the E3/E2 binding (68). Frequently, RING-finger proteins are part of multisubunit complexes. For example, in the family of Skp1/Cul1/F-box (SCF) proteins, a scaffold protein Cullin connects the RING-finger Skp1 with one of the multiple F-box protein, which is responsible for specific substrate recognition. Moreover, F-boxes only recognize substrates that have been previously phosphorylated [for review, see (30, 205, 213)]. Another multisubunit RING-finger E3 is the anaphase-promoting complex (APC) [for review, see (33)]. APC is composed of a stable APC core, formed from at least eleven proteins, and one of three variable activators. The binding of the APC activator is regulated during the cell cycle and confers substrate specificity. Moreover, the phosphorylation status of APC also regulates its activity. The coordinated action of SCF and APC sequentially degrading cell cycle regulators ensures cell cycle progression (281).

A family of proteins involved in the formation of poly-ubiquitin chains are characterized by a modified version of the RING finger-motif that lacks the hallmark Zn^{+2}-chelating residues, named U-box domain (97). The best characterized U-box proteins are the yeast protein “ubiquitin fusion degradation protein 2” (Ufd2) and the “C-terminus of the Hsc70 interacting protein” (CHIP). The U-box proteins Ufd2, CHIP and other proteins that do not contain U-box domain have been characterized by their role in ubiquitin-chain elongation and have been termed E4 (140, 147). It is still a matter of debate whether the E4s proteins have intrinsic E3 activity or not.

It should be emphasized that the activity and specificity of these enzymes is susceptible to regulation by post-translational modification, by interaction with activating/repressing partners and by subcellular localization. All these characteristics are essential for an accurate spatial and temporal targeting of specific protein substrates during the life of each cell.

2.4. Deubiquitylation

Enzymatic reversibility is a significant quality of the ubiquitylation reaction. The heterogeneous group of deubiquitylating enzymes (DUBs) have important roles in the ubiquitin-proteasome system. First, DUBs maintain the free ubiquitin pool in the cell by processing the inactive ubiquitin precursors (poly-ubiquitin and ubiquitin fused to ribosomal proteins), rescuing ubiquitin from intracellular nucleophiles and recycling ubiquitin from poly-ubiquitylated substrates committed for degradation. Second, the removal of the poly-ubiquitin chain once a substrate is bound to the proteasome facilitates degradation (see chapter 3.2). This process has also the beneficial effect of protecting the proteasome from the inhibitory effect of unanchored poly-ubiquitin chains. On the other hand, DUBs can inhibit ubiquitin-mediated degradation by prematurely disassembling poly-ubiquitin chains. Finally, DUBs can also modulate non-proteolytic functions of ubiquitin, such as membrane protein trafficking and signal transduction [for review, see (4)].

DUBs catalyze the cleavage of ubiquitin-linked molecules (ubiquitin-ubiquitin and ubiquitin-substrate) after the last residue of ubiquitin (Gly76). The majority of the known DUBs are cysteine proteases for which a variety of catalytic domains have been described [for review, see (195)]. The first DUBs to be identified were the ubiquitin C-terminal hydrolases (UCHs). *In vitro*, UCHs have preference for small ubiquitin-adducts (154). Consequently, it has been proposed that UCHs rescue ubiquitin that is incorrectly conjugated to intracellular nucleophiles, such as glutathione and polyamines. Additionally, UCHs might be involved in the processing of ubiquitin precursors. Interestingly, the isopeptidase UCH37 cleaves ubiquitin chains in a sequential manner, starting from the most distal ubiquitin (152) and has been shown to be associated with the proteasome (114, 152) (see chapter 3.2).

The largest family of DUBs is comprised of ubiquitin-specific proteases (Ubp or USP). These enzymes have modular domains, which include the conserved catalytic domain and different ubiquitin-binding and protein-protein interacting domains, and these domains might be involved in the specificity in the recognition of a particular type of ubiquitin modification or a specific substrate. Some Ubp enzymes have important roles recycling ubiquitin: Ubp6/USP14 associates with the proteasome and participates in the cleavage of ubiquitin isopeptide bonds and the yeast Ubp14 disassembles unanchored ubiquitin chains (see chapter 3.2). Other Ubp enzymes have been implicated in the regulation of specific proteins. For instance, HAUSP is essential for the stabilization of the tumor suppressor p53 (160).

The number of families containing potential DUBs is constantly increasing with the continuous discovery of new proteins with deubiquitylating activity. These enzymes play key regulatory roles in a variety of cellular processes. Despite the importance of DUBs, the mechanisms concerning substrate-specificity and their mode of regulation are still not understood.

2.5. Ubiquitin-binding proteins

The discovery of proteins that specifically recognize mono- and poly-ubiquitin has contributed to the understanding of how protein-modification can be use as a signalling event. To date, nine different ubiquitin-binding domains (UBDs) have been identified [for review, see (108)]. Some examples of UBDs are the ubiquitin-associated (UBA) domain, the ubiquitin-interacting motif (UIM) and CUE (coupling of ubiquitin conjugation to ER degradation) domain. UBDs interact with the same hydrophobic patch of ubiquitin that is also used by the ubiquitylating-enzymes E1, E2 and E3 (90, 117). Nevertheless, each UBD has a particular three-dimensional structure, which, in some cases, could be the basis for a different affinity for mono-ubiquitylated versus poly-ubiquitylated substrates or even for a particular type of ubiquitin chain.

UBDs are present in many different proteins involved in a great range of cellular processes. Several components of the ubiquitylation machinery have been shown to have UBDs. For example, structural studies

indicated that the UEV (ubiquitin-conjugating enzyme variant) domain in the E2 Ubc13 is responsible for the specificity of synthesis of Lys63 poly-ubiquitin chains (178, 263). UBDs are also frequently found in DUBs, but their role remains unclear. One possibility is that, in analogy to the Ubc13 UEV domain, the affinity for a particular ubiquitin chain type is determined by the identity of the UBDs. Another possibility is that UBD might mediate inter- or intra-molecular interactions that modulate the activity of the DUB. A different group of UBD containing proteins are involved in the recognition of poly-ubiquitylated substrates, as for example, the proteasome subunits Rpn10/S5a and Rpt5/S6' (see Chapter 3.2). In addition, some UBD containing proteins might shuttle poly-ubiquitylated substrate to the proteasome [for review, see (59)]. Moreover, UBDs are also important mediators of non-proteolytic functions of ubiquitin.

The existence of many different UBD containing proteins that specifically recognize ubiquitin-tags reflexes the complexity and versatility of ubiquitin as a post-translational modifier. It is anticipated that a more detailed study of this new stratum of the ubiquitin-signalling will shed some light in the understanding of how ubiquitin can control different cellular processes.

3. PROTEASOMAL DEGRADATION

3.1. The proteasome

Although proteolysis is a process of vital importance for cellular survival, uncontrolled proteolysis could have catastrophic consequences. The three kingdoms of life have resolved this paradox by sequestering active proteolytic sites into gated multi-subunit proteolytic chambers [for review, see (11, 210)]. The eukaryotic chambered protease is called the proteasome and is composed by two complexes: the 20S core particle and the 19S regulatory particles. The 19S regulatory particles can bind at one end of the 20S core (19S-20S) or at both ends (19S-20S-19S), forming the so-called 26S proteasome [for review, see (79, 210)]. In some cases, the 20S core particle is bound to other regulatory particles, such as PA28 (see Chapter 3.4).

The 20S core particle is a hollow cylinder composed by four stacked rings, as shown by X-ray crystallography (86). Each of the outer rings is composed by seven different α-subunits and each of the inner rings is composed by seven different β-subunits, forming the symmetrical structure: (α_{1-7})-(β_{1-7})-(α_{1-7})-(β_{1-7}) (Fig. 3). In each β-ring there are three β-subunits with threonine-protease activity. The active sites of these proteases are facing an inner cavity within the β-rings, termed proteolytic chamber, which can be accessed by a narrow channel formed by the α-subunits. The N-termini of the α-subunits form a network that obstructs the entrance, suggesting that the proteolytic chamber is gated (85, 141). Docking of the regulatory particles might control the opening and closing of this channel.

The 19S regulatory particle can be divided in two sub-complexes: the base and the lid (80). The base is connected to the α-subunits of the 20S core particle by a ring formed by six ATPases subunits: Rpt1/S7, Rpt2/S4, Rpt3/S6, Rpt4/S10b, Rpt5/S6' and Rpt6/S8 (Fig. 3). These ATPases belong to the AAA (ATPase associated with a variety of cellular activities) family, which often form ring-like oligomers that function as molecular chaperones (204). Each of the base ATPases is encoded by a different gene. Moreover, similar mutations in different ATPases result in diverse phenotypes, indicating that each ATPase subunit is functionally unique (229). Two additional components

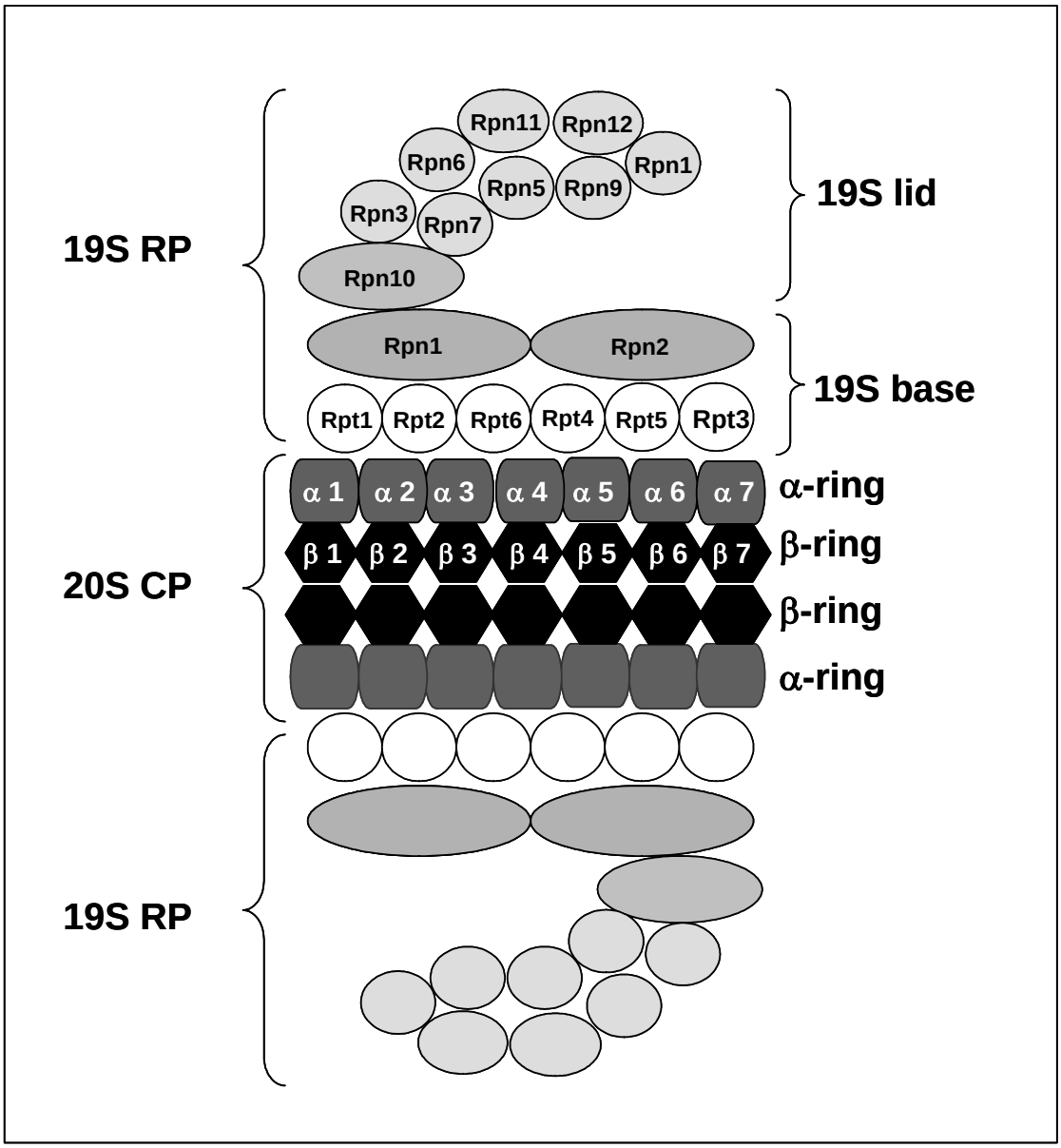

Figure 3. *The subunit composition of the 26S proteasome*. The proteasome consist of the 20S core particle (CP) and the 19S regulatory particle (RP). The 20S CP is build up by two α-rings and two β-rings, each ring containing seven different subunits. The 19S RP is composed of two subcomplexes: the base and the lid. The different subunits are indicated using the nomenclature of *Saccharomyces cerevisiae* 26S proteasome. Proteins that associate transiently with the proteasome are not indicated. Note that the positions of the individual subunits in this figure do not necessary reflect the quaternary structure of the proteasome subcomplexes. Rpn, regulatory particle non-ATPase; Rpt, regulatory particle ATPase. Adapted from Pickart and Cohen (210).

of the base are the non-ATPase subunit Rpn1/S2 and Rpn2/S1 (Fig. 3). These proteins are the biggest subunits of the 19S regulatory particle and have been proposed to serve as a platform for proteins that transiently interact with the proteasome (see chapter 3.2). The lid of the 19S is situated on top of the base and contains at least eight different subunits: Rpn3/S3, Rpn5, Rpn6/S9, Rpn7/S10a, Rpn8/S12, Rpn9/S11, Rpn11/S13 and Rpn12/S14 (Fig. 3). The Rpn10 subunit interacts with subunits from the base and from the lid (70). Furthermore, RPN10 deletion provokes the dissociation of the 19S regulatory particle into two subcomplexes, indicating that the Rpn10 subunit contributes to stabilize the interaction between the base and the lid of the 19S regulatory particle (80). Additional proteins might contribute to the stabilization of the lid-base association.

Besides these constitutive proteasome subunits, there are several proteins that interact transiently with the proteasome, including E2s, E3s, DUBs, shuttling factors containing UBDs and molecular chaperones (64, 157, 272). Some of these proteins interact with the proteasome through a ubiquitin-like domain (UbL), characterized by a striking similarity with the three-dimensional structure of ubiquitin. Thus, the proteasome is a dynamic structure forming transient interactions with different factors that are necessary for temporal and spatial regulated proteolysis.

3.2. The roles of the 19S regulatory particle in degradation

Docking of the 19S regulatory protein to the 20S proteolytic core is a requirement for ubiquitin-dependent proteasomal degradation. Yeast that lacks the Rpn10 subunit has inefficient degradation of poly-ubiquitylated substrates, indicating that the 19S lid plays essential roles in ubiquitin-dependent proteolysis (80). Likewise, the 19S base has been shown to be implicated in opening the gate of the 20S core particle (141), in unfolding the protein substrates (24, 218) and in the translocation of substrates into the proteolytic chamber (141). Although the specific sequence of events leading to substrate degradation remains elusive, some particular functions of different components

of the 19S in proteasomal degradation have been discovered and are described below.

One of the major tasks of the 19S regulatory particle is the recognition of substrates that should be degraded. Although some substrates have been reported to be degraded independently of ubiquitylation (242, 294), or to be targeted by N-terminal ubiquitylation (18, 25, 37), the bulk of the proteasomal substrates are identified by the presence of a poly-ubiquitin chain. Two 19S subunits that bind poly-ubiquitin chains have been identified: Rpn10/S5a and Rpt5/S6'. The 19S lid subunit Rpn10/S5a was the first protein found to function as a ubiquitin-binding protein (112, 292). Notably, whereas the yeast Rpn10 subunit is predominantly dissociated from the proteasome (262, 278), the mammalian S5a is not found in a free state (103), indicating that the yeast Rpn10 might have additional functions. The 19S base subunit Rpt5/S6' has recently been shown to interact with poly-ubiquitin chains in an ATP dependent manner (150). It is likely that other proteasome subunits might participate in substrate recognition. Regarding the affinity for poly-ubiquitin chains, studies with model substrates have shown that the 19S has preference for binding chains composed of four or more ubiquitins (254). In addition to 19S components Rpn10/S5a and Rpt5/S6', proteins that associate transiently with the proteasome have been proposed to participate in substrate recognition. Most of these proteins, like Rad23, Dsk2 and Ddi1, belong to the UbL-UBA family, characterized by the presence of one UbL domain and one or several UBA domains (see Chapter 2.5). UbL-UBA proteins are able to bind poly-ubiquitin chains through the UBA domain (279) and proteasomes through the UbL domain (232). These proteins have been proposed to work as shuttling factors that bring poly-ubiquitinated proteins to the proteasome (96). Nevertheless it should be noticed that, in certain cases, interaction with an UbL-UBA containing protein can also result in protection of the poly-ubiquitin chain and thereby preventing the degradation of the poly-ubiquitylated protein (219). This suggests that the function of UbL-UBA containing proteins might be determined by the binding substrate or by additional interacting proteins.

Another important function preceding protein degradation is the removal of the poly-ubiquitin chain from the protein substrate. The Rpn11/S13 subunit of the 19S lid has been shown to be involved in substrate deubiquitylation (271, 287). Rpn11/S13 cleaves the isopeptide bond between

the substrate and the first ubiquitin in the chain releasing the intact poly-ubiquitin chain (287). Additionally, two DUBs can transiently associate with the proteasome: UCH37 (114, 152) and Ubp6/USP14 (21, 157). Nevertheless, these proteins are not essential for proteolysis, indicating that either they are substrate-specific or that there functions are redundant. UCH37 cleaves the poly-ubiquitin chain sequentially, starting from the distal ubiquitin, which lead to the proposal that UCH37 might work as a molecular clock (152).

Native proteins have to be unfolded to be able to pass through the narrow 20S central channel. The 19S ATPase subunits have been proposed to participate in the unfolding of substrates based on their analogy to the ATPase components from bacterial and archaebacterial ATP-dependent proteases. Bacterial and archaebacterial chambered proteases have AAA-ATPase homo-oligomeric rings and do not have a lid. These ATPases unfold native proteins in a signal-dependent manner in a process that requires ATP-binding for association with the substrate and ATP hydrolysis for unfolding (26, 131, 192). Experiments with archaebacterial 20S proteasomes and the PAN (proteasome-activating nucleotidase) regulatory complex, a homolog of the eukaryotic 19S ATPases, have demonstrated that ATPase activity is also required for translocation of the substrates to the 20S catalytic core (13). Although it has not been investigated in detail in eukaryotic proteasomes, it is possible that substrate recognition, unfolding, opening of the proteasomal gate and translocation into the proteolytic chamber are coordinated by the ATPase subunits of the 19S base. An interesting question is whether these processes are coupled or not.

3.3. Degradation in the 20S core particle

Once a substrate has been successfully deubiuquitylated, unfolded and translocated into the proteolytic chamber of the 20S core particle, it cannot escape from destruction. The 20S core particle contains six proteolytic active sites, three on each β-ring (Fig. 4). These three subunits belong to the group of N-terminal nucleophile hydrolases, which use a nucleophilic group from the side chain of the N-terminal amino-acid to break peptide bones. A special characteristic of the proteasome active sites is that, unlike any other protease,

they use an N-terminal threonine as nucleophilic group (243). The three β-catalytic subunits are synthesized as inactive precursors and once incorporated into the 20S core particle they are activated by intramolecular proteolysis that result in the exposure of the N-terminal threonine residues (35, 55, 236).

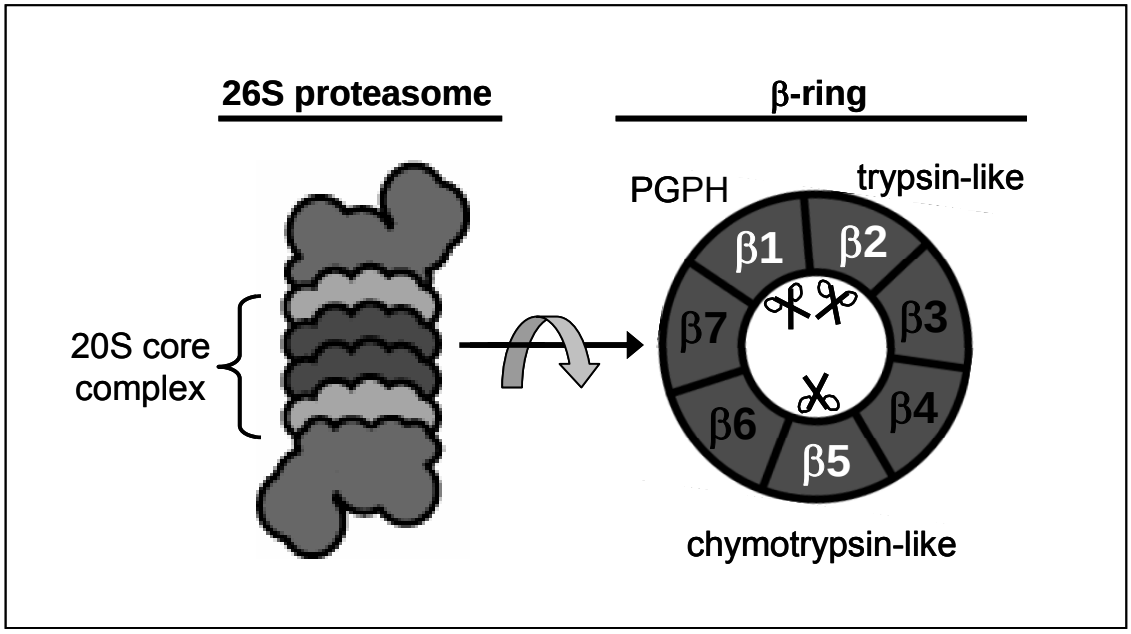

Figure 4. *The proteolytic subunits of the proteasome.* The proteolytic active sites are located in the β1, β2 and β5 subunits of the 20S core particle, facing the proteolytic chamber of the proteasome. These activities are referred to as post-glutamyl peptide hydrolyzing (PGPH), trypsin-like and chymotrypsin-like, respectively.

Each of the three catalytic subunits in a β-ring have particular specificities (54, 198). The β5 or chymotrypsin-like subunit, cleaves preferentially after hydrophobic residues; the β2 or trypsin-like subunit, cleaves after basic residues and the β1 or post-glutamyl peptide hydrolase (PGPH) subunit, cleaves after acidic subunits. Moreover, the β1 subunit and, to a minor extend, the β5 subunit are also able to cleave after branched amino-acid residues (Leu, Ile, Val) (28, 29, 54, 176). Importantly, the activity of the 20S catalytic core is not a mere addition of the three protease activities. First, the individual β51 β2 and β5 subunits are only functional when incorporated into the 20S core particle. Second, it has been shown that the binding of substrates to a non-identified non-catalytic β-subunit participates in proteolysis by regulating the activity of the β-catalytic sites (135, 190, 235). Overall, the 20S

core particle is able to cleave all types of peptide bonds to achieve processive proteolysis. Although proteasomes usually degrade proteins completely into small peptides varying between 3 and 23 amino acids (133), in a few cases they work as endoproteases and yield two protein products of different biological activity (168). This proteasomal role is essential for the function and regulation of certain transcription factors [for review, see (221)].

3.4. Other proteasomal complexes

In mammals, the proteasome plays a pivotal role for the immune defence by generating peptides for MHC class I presentation. During infection, the release of the cytokine interferon-γ (IFN-γ) optimizes antigen presentation by upregulating MHC class I and TAP (transporter associated with antigen processing). In addition, IFN-γ induces the expression of three inducible β catalytic subunits (iβ), named iβ1/LMP2, iβ2/MECL1 and iβ5/LMP7. These subunits replace the constitutive subunits β1, β2 and β5 in *de novo* synthesized 20S core particles, forming a complex known as immunoproteasome that have different cleaving site preferences and faster cleavage rate [for review, see (137, 138)].

IFN-γ induction also results in the upregulation of PA28α and PA28β, which form a heptameric ring known as 11S regulatory particle, PA28α/β or REGα/β. Similarly to the 19S cap, the PA28α/β particle can assemble with the 20S core particle and is believed to open the α-subunit gate, as observed in X-ray crystallographic studies of a complex formed between the yeast 20S core particle and the PA28 homolog from the protozoan *T. brucei* (274). On the other hand, PA28α and PA28β do not have ATPase activity or ubiquitin-binding sites and it is likely that only unfolded proteins and small peptides could enter the 11S docked 20S catalytic core (172). Although PA28α/β has been shown to change the spectrum of products generated by the proteasome (32) and to be important for MHC class I antigen presentation (57, 172, 217), the molecular mechanism is still unclear and appears to be dependent on the processed peptide (189, 246) [for review, see (83, 222)]. PA28α and PA28β are constitutively expressed and are assembled into

different proteasome complexes (11S-20S-11S, 11S-20S and hybrids 19S-20S-11S) (252), but the biological roles of the 11S complex remain unclear.

Furthermore, the 20S core particle can associate with, at least, two other regulatory proteins: PA28γ and PA200. PA28γ, a protein that is related to PA28α and PA28β but that is not inducible by IFN-γ, forms a homo-heptameric complex that assembles with the 20S. In contrast with the 11S, which reside primarily in the cytoplasm, PA28γ is mainly found in the nuclei (277). The biological roles of PA28γ are currently under investigation. PA28γ-knockout mice are viable with the only abnormality being a slight decrease in growth rate (188). Moreover, primary fibroblasts from PA28γ-knockout mice are defective in cell cycle progression (188). The steroid receptor co-activator SRC-3 has been recently identified as the first PA28γ-20S biological substrate (162). Importantly, SRC-3 activity is involved in mammary-gland development and it has a potent activity in promoting transformation and breast tumor formation (163), suggesting a potential tumor suppressor role for PA28γ. Further studies are necessary to explore the biological functions of PA28γ-20S mediated proteasomal degradation. The most recently discovered 20S core particle regulator is a large (200KDa) nuclear protein known as PA200. Electron-microscopic studies have shown that PA200 is able to interact with the α-subunits of the 20S opening the channel. It is likely that, similar to PA28, PA200 facilitates the entrance of substrates or the exit of products from the 20S core particle. A possible role of PA200-20S in DNA repair has been suggested, but further studies are necessary to clarify the biological function of PA200 (259).

4. FEATURES DETERMINING PROTEIN HALF-LIFE

4.1. Degradation signals

Normally, the lifespan of a protein is determined by the presence of degradation signals that target the protein for proteasomal destruction. Some short-lived proteins have constitutive degradation signals that result in a high and constant degradation rate throughout the life of the cell. Most of the regulatory proteins are characterized by the presence of conditional degradation signals resulting in a spatially or temporally controlled degradation. There are at least three ways to regulate conditional degradation: i) by modifications in the substrate, such as conformational changes, formation of inter- or intra-molecular interaction, phosphorylation, hydroxylation or glycosylation; ii) by confining signal-recognition to a particular cellular compartment and iii) by regulating the availability or the activity of the molecule that recognizes the signal, normally an E3.

Some of the degradation signals that have been identified are: the N-end rule, PEST sequences, the destruction box, the KEN box, the ubiquitin fusion degradation signal (UFD), hydrophobic patches and the ornithine decarboxylase (ODC) domain. The N-end rule was the first identified degradation signal, and consist of a N-terminal destabilizing amino-acid in the vicinity of an internal Lys, which is the site for poly-ubiquitylation (9, 10, 267). The N-end rule has a hierarchic structure, characterized by primary, secondary and tertiary destabilizing amino acids. The primary N-terminal destabilizing amino acids are bulky hydrophobic (Phe, Leu, Trp, Tyr, Ile) and basic (Arg, Lys, His) residues, which are directly recognized by the Ubr1/E3α (yeast/mammals) E3 (267). The secondary destabilizing amino acids are Glu and Asp, which can become destabilizing upon the linkage of an Arg in the α-NH_2 in a reaction catalysed by the ATE1-encoded isoforms of Arg-tRNA-protein transferase (149). Finally, the tertiary destabilizing Gln and Asn can be converted into the secondary destabilizing Glu and Asp by de-amination followed by arginylation. Moreover, it has been recently shown that in mammals, N-terminal Cys can also be arginylated once it has been oxidised by nitric oxide (118). Several studies have shown that the N-end rule is involved in several biological

processes as peptide import and chromosomal segregation in yeast (220, 257), apoptosis in *Drosophila melanogaster* (56) and cardiovascular development in mice (149). Recently, the first physiological substrates of the mammalian N-end rule have been identified (156). Interestingly, these substrates are proteins involved in cardiovascular development and are targeted for degradation by N-terminal Cys in a nitric oxide-dependent manner (118).

PEST signals were identified through comparative analyses of the amino-acid sequences of several short lived proteins (228). These analyses lead to the observation that short lived proteins frequently contain a region characterized by the presence of an amino-acid stretch with high content of Pro, Glu, Ser and Thr (PEST), flanked by a region with basic amino acids (Arg, Lys or His). Based on this common feature, an algorithm to calculate the probability for a given motif to act as a degradation signal was developed. PEST signals are required for degradation of several proteins, such as the tumor suppressor p53, the inhibitor of NFκB, IκBα and many cyclins [for review, see (223)]. In many cases, phosphorylation within the PEST signal is required for degradation (36, 143, 153). Although PEST signals are unstructured domains, the fact that phosphorylation is required for degradation suggest a sophisticated recognition system.

The destruction box and the KEN box were identified as degradation signals present in mitotic cyclins (81, 209). In addition to cyclins, destruction boxes are found in the budding yeast Ps1 (285) and the fission yeast Cut2 (41), which control sister-chromatids cohesion during anaphase. Degradation of all these proteins is mediated by the cell cycle-dependent APC (see Chapter 2.3).

The ubiquitin fusion degradation (UFD) signal is characterized by the presence of an N-terminal ubiquitin moiety that cannot be cleaved by UCHs or other proteases. In these circumstances, ubiquitin is recognized as a degradation signal leading to ubiquitylation within the ubiquitin moiety and subsequently degradation of the UFD containing protein (125, 126). Although the enzymatic system required for degradation of UFD containing proteins have been fully characterized in yeast (126), the physiological relevance of this pathway remains unclear. So far, the only UFD substrate identified is the aberrant form of ubiquitin UBB^{+1} (167), which is originated by erroneous translation of the transcript derived from the ubiquitin B (UBB) gene. UBB^{+1}

levels are elevated in certain neurodegenerative diseases (260, 261). Interestingly, recent findings indicate that certain proteins are degraded upon the conjugation of ubiquitin to the α-NH_2 group of the N-terminal amino-acid [for review, see (37)]. Some examples include the myogenic transcriptional switch protein MyoD (25), the cell cycle regulator p21(18) and several viral proteins (8, 60, 119, 224). Even though it is not known what triggers the conjugation of this first linear ubiquitin, it is tempting to speculate that N-terminal ubiquitylated substrates are recognized as UFD substrates.

The degradation signal Deg-1 was found in the yeast transcriptional regulator Mat2α. The ER-associated E2s Ubc6/Ubc7 (see Chapter 5.2.2) have been shown to be involved in ubiquitylation of Mat2α (111, 127). A particular feature is that Deg-1 is a hydrophobic domain that mediates the binding in the hetero-dimer Mat1α/Mat2α. If this interaction is disrupted, the hydrophobic Deg-1 domain is exposed and subsequently Mat2α is degraded. Interestingly, yeast screens resulted in the identification of artificial degradation signals that required Ubc6/Ubc7 for degradation and that were characterized for the presence of hydrophobic patches, such as the CL1 artificial degradation signal (77, 78). Thus, it could be hypothesized that exposure of hydrophobic domains is a degradation signal present in orphan subunits, misfolded and damaged proteins.

Although most proteins are targeted to the proteasome by poly-ubiquitylation, several proteins have been reported to be degraded by the proteasome in a ubiquitin-independent manner [for review, see (115)]. The best characterized ubiquitin-independent proteasomal substrate is ODC, a key enzyme in the synthesis of polyamides. Polyamide production is regulated by a negative feedback mechanism: polyamides stimulate the synthesis of antizyme, which in terms inactivates ODC by disrupting the functional ODC-homodimers and by accelerating the proteasomal degradation of ODC (161). Using reconstituted systems it has been shown that ODC is degraded by the 26S proteasome in ATP-dependent ubiquitin-independent manner and that antizyme accelerates this degradation process (187). The degradation signal resides in a C-terminal 37 amino-acid stretch that contains a PEST sequence and it is transferable to other proteins (116, 294). The intriguing question is how the proteasome recognizes ODC and other ubiquitin-independent substrates.

Although *in vitro* experiments have shown that the 19S regulatory particle can interact with hydrophobic patches of denatured proteins and thereby bypass the ubiquitylation step, it is not known whether this mechanism can occur *in vivo* (250).

4.2. Stabilization signals

The hypothesis that certain domains can delay or inhibit proteasomal degradation was based on the discovery of a stabilizing domain in the viral protein EBNA-1 (Epstein-Barr virus nuclear antigen). This domain is a long repetitive sequence consisting of glycine and alanine that protects EBNA-1 from proteasomal degradation (48, 158, 159, 244). The Epstein-Barr virus exploits this stabilization signal to escape from immuno-surveillance and extent the half life of this crucial viral protein.

It has been postulated that stabilization signals might be a widespread mechanism present in cellular proteins as well (50). A recent study has shown that the Rad23 is protected from ubiquitin-dependent proteasomal degradation by a C-terminal UBA domain (101). Stabilization signals have two potential functions. First, these signals might enable a protein to exert a function while being ubiquitylated or while interacting with the proteasome. For instance, in the context of the UbL-UBA proteins, a stabilizing domain would allow these proteins to escort ubiquitylated substrates to the proteasome without being degraded themselves in the process. Second, the presence of such signals could be a general phenomenon that controls protein stability and that would offer new platforms for regulation. Nevertheless, the existence of stabilization signals as a general mechanism is still a matter of speculation.

5. THE UBIQUITIN-PROTEASOME SYSTEM IN PROTEIN QUALITY CONTROL

5.1. Protein quality control

Protecting the integrity of the cellular proteome is essential to guarantee an adequate cellular function. Folding of nascent polypeptides is a difficult process because it has to be accomplished in a very dense macromolecular environment. Incorrect folding could be hazardous, since accumulation of non-native polypeptides might lead to the formation of aggregates. *De novo* protein folding occurs in a protective environment formed by an elaborated system constituted of chaperones, chaperonins and co-factors [for review, see (95)]. Nonetheless, over one quarter of all newly synthesized proteins are degraded directly after synthesis, perhaps indicating failure reaching the correct native conformation (225, 238). A particularly intricate process in protein folding is the proper assembly of subunits from multimeric complexes. Proteasomal degradation of unincorporated subunits prevents the exposure of hydrophobic domains and consequent formation of aggregates and guarantees that the constituents of multimeric complex are present in stochiometric amounts.

Furthermore, even if a protein has been successfully folded it can be damaged afterwards. In fact, the cellular milieu is a rather hostile environment where different agents, such as enzymes, reactive metabolites and radicals, can produce irreversible damage to the proteins. This circumstance can be aggravated by subtle changes in the cellular environment, like temperature or pH fluctuations. The quality control mechanisms protect the cellular proteome by three procedures: proof-reading, refolding and degradation [for review, see (82)]. The chaperone machinery associates with damaged proteins based on its ability to recognize non-native structures, prevents aggregation and promotes refolding. Additionally, damaged proteins are degraded by the ubiquitin-proteasome system. Recently, it has been shown that chaperones and cofactors can link client substrates with the ubiquitylation machinery (42, 170), suggesting that there is a direct crosstalk between the folding and degradation pathways (175).

Protein quality control pathways are necessary in compartments where protein synthesis and maturation takes place: the cytosol (95), the ER (58, 91, 248), the mitochondria (5) and the nucleus (72).

5.2. Protein quality control in the ER

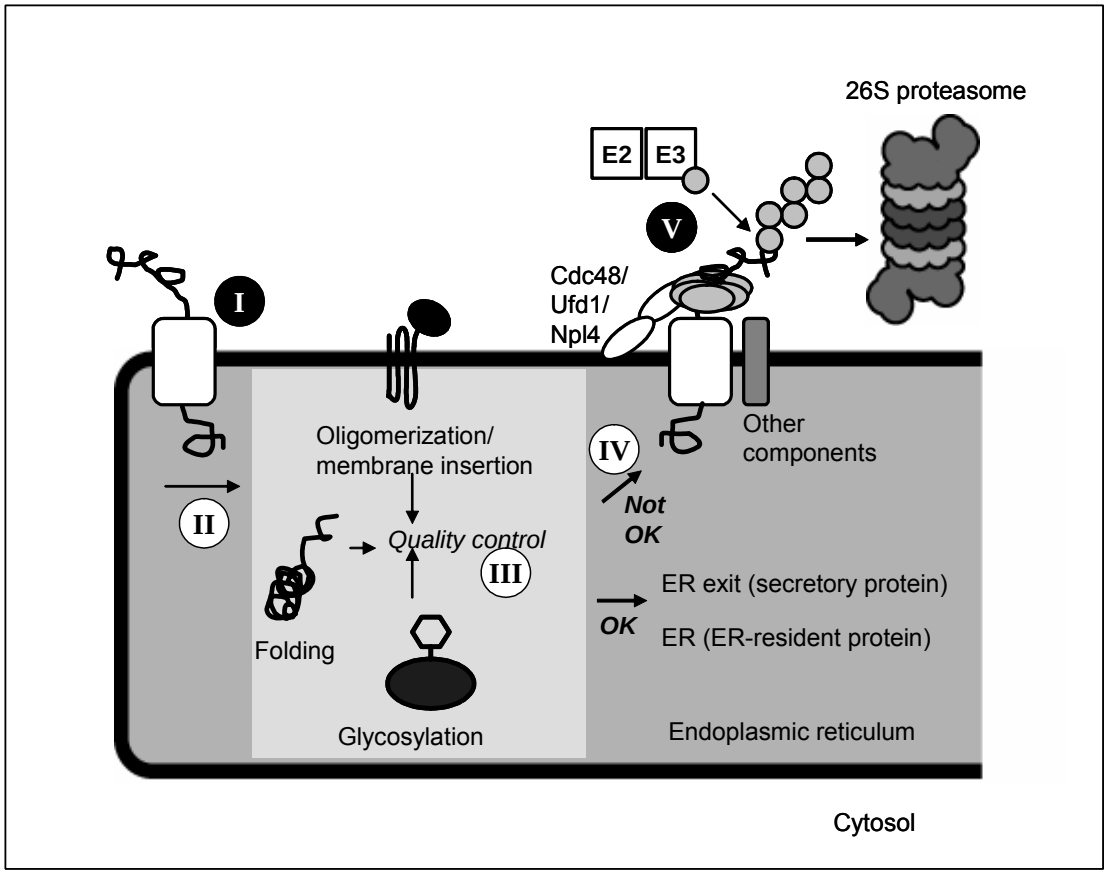

Figure 5. *Protein quality control mechanisms in the endoplasmic reticulum (ER) and ER-associated degradation (ERAD).* (**I**) Proteins are translocated into the ER in an unfolded state through the Sec61p complex. (**II**) Folding of nascent proteins starts during translocation and is assisted by molecular chaperones residing in the ER lumen. In the ER, translocated proteins undergo post-translational modifications, such as N-linked glycosylation and oligomerization. (**III**) Dedicated protein quality control mechanisms monitor the integrity of the proteins in the ER. If the protein has been properly folded and matured it will reach its final destination (**IV**) Aberrant and misfolded proteins will be escorted to a putative channel that facilitates their export from the ER (see text for details). (**V**) Lysine residues at the cytoplasm are ubiquitylated by specific E2/E3s and dislocation is completed with the help of Cdc48/Ufd1/Npl4 .The poly-ubiquitylated protein is subsequently degraded in the cytosol by the proteasome.

Quality control mechanisms are especially necessary in the ER. First, the ER is dedicated to the production of proteins for the secretory pathway. Many of

these proteins harbour hydrophobic transmembrane domains, which have to be inserted in membranes correctly. Furthermore, ER-client proteins undergo modifications, such as N-terminal glycosylation, disulphide bond formation and assembly of multimeric complexes. To accomplish these functions, the ER is equipped with a variety of proteins, such as chaperones, lectins, glycan-processing enzymes and oxido-reductases, which assist in the folding and maturation of secretory proteins. Finally, the ER is also responsible of controlling that those proteins that fail the maturation process are not exported down in the secretory pathway.

The ER has a sophisticate proof-reading system. Proteins that are detected as anomalous are directed by the ER-associated degradation (ERAD) pathway for destruction by the cytosolic ubiquitin-proteasome system (Fig. 5). Importantly, the ERAD pathway also plays a role in the regulated degradation of short-lived ER-resident proteins, such as HMG-CoA reductase, a rate-limiting enzyme in cholesterol biosynthesis (92). Because of the physical separation between the recognition machinery and the degradation machinery, the elimination of ER-resident proteins involves the translocation of the substrate to the cytoplasm in a process that involves poly-ubiquitylation, mobilization by the Cdc48/Ufd1/Npl4 ATPase complex and finally degradation by the proteasome [for review, see (182)].

5.2.1. Substrate recognition

Studies with model ERAD substrates in yeast have revealed that the inspection for hydrophobic and misfolded domains is not limited to the luminal domains of the proteins, and, in fact, it appears that the first checking control scans the cytosolic domains (268). If the protein passes this quality control checkpoint, then the inspection continues in the lumen of the ER. Given the conformational diversity of ER-proteins, it is not surprising that there are also multiple pathways for recognition of misfolded proteins that will look for hydrophobic patches, unpaired cysteines and immature glycans. One of the best characterized recognition mechanism is the calnexin/calreticulum system for glycoproteins [for review, see (102)]. The glycoprotein is trapped in the calnexin/calreticulum cycle until the native conformation is achieved, but if the

process takes too long time the oligosaccharide branch is cleaved by ER-α-1,2-mannosidase-I. If this occurs, the glycoprotein is recognized by the lectin EDEM and the substrate is targeted for ERAD (184, 199). Other mechanisms screening for aberrant proteins involve a number of chaperons, such as BiP, that interacts with hydrophobic domains (215). Additionally, the oxido-reductases protein disulfide isomerase (PDI) (256) and Eps1p (265) also target misfolded proteins for degradation.

5.2.2. Substrate retro-translocation, poly-ubiquitylation and delivery to the proteasome

The mechanism underlying the translocation of ERAD substrates from the ER to the cytosol is not completely understood. One of the most debated subjects is the identity of the channel for retro-translocation. The Sec61 complex, which mediates the translocation of nascent polypeptides into the ER, was originally proposed to also function in retro-translocation (12, 51, 75, 207, 212, 214, 247, 273, 276, 296). Recent studies have indicated that the translocation of certain ERAD substrates could be mediated by the yeast Der-1p (139) and the mammalian homologue Derlin-1 (164, 290) assisted by Derlin-2 and Derlin-3 (200). Nevertheless, it appears that a Derlin-1 based channel would only be required for the translocation of certain proteins and thus, it is expected that in the following years additional translocation channels will be discovered.

One of the requisites for translocation is poly-ubiquitylation of the ERAD-substrate at the cytosolic face of the ER membrane. Studies on yeast have been crucial for the identification of some of the ubiquitylation pathways governing ERAD. Many of the proteins involved in ubiquitylation of ERAD substrates are ER-transmembrane proteins and cytosolic proteins anchored to the ER by cofactors.

Several studies have shown that the Cdc48/Ufd1/Npl4 complex is necessary for translocation of misfolded ERAD substrates (123, 288). Cdc48 (and the mammalian homologue p97 or VCP) is a AAA-ATPase and forms a hexameric ring with a central channel, reminiscent of the bacterial chaperone ClpP (52, 295). Each Cdc48 subunit contains two ATPase domains and an N-

terminal domain that can interact with different cofactors that assist Cdc48 in its multiple functions. Interestingly, Cdc48/Ufd1/Npl4 is able to bind both poly-ubiquitylated and non-ubiquitylated substrates (183, 289). Although it is clear that Cdc48/Ufd1/Npl4 is necessary for the translocation of ERAD substrates, the mechanisms are still poorly understood. Some recent studies shed some light on this issue. For instance, it has been shown that in mammalian cells Cdc48/Ufd1/Nlp4 is bound to the ER membrane by association with Derlin-1 and with the membrane protein VIMP (290). Other studies have shown that in yeast the ER membrane protein Ubx2 associates directly with Cdc48/Ufd1/Npl4, with an E3 (interactions with Doa10 and Hrd1 have been shown) and simultaneously binds through UBA domains to poly-ubiquitylated substrates (194, 239). Additionally, it has been demonstrated that the Cdc48/Ufd1/Npl4, in coordination with the UbL-UBA proteins Rad23, Dsk2 or Ufd2, is involved in the delivery of poly-ubiquitylated proteins to the proteasome (59, 227). Since Rad23, Dsk2 and Ufd2 have been shown to be also important for degradation of ERAD substrates (179, 251), it is tempting to speculate that a similar flow through ubiquitin-binding proteins brings the ERAD substrates to the proteasome.

5.3. Protein quality control during stress

Several physiological and pathological conditions, such as high temperatures, inflammation, hypoxia or ischemia can cause fluctuations in the intracellular environment that result in protein damage. Many proteins involved in protein-quality control are typically upregulated in response to these conditions. For instance, the heat shock response leads to the transcriptional upregulation of many molecular chaperones that protect the cellular proteome from stress [for review, see (174)]. Besides the general activation of chaperones under stress conditions, ubiquitin has been known to be a heat shock protein for many years (66, 67, 69). Notably, the stress inducible genes encode ubiquitin precursors, containing repeats of ubiquitin immediately adjacent to each other. This elegant approach to efficiently produce ubiquitin suggests that large amounts of ubiquitin are required for cell survival under environmental stress.

Likewise, a variety of insults could lead to protein misfolding in the ER. These include nutrient depravation, changes in Ca^{+2} concentration and alterations in redox balance. Disruption of ER homeostasis results in the activation of a complex signalling cascade named the unfolded protein response (UPR) [for review, see (129, 282)]. The UPR involves at least three mechanisms to cope with misfolded ER-substrates: a general attenuation of translation, an upregulation of structural components of the ER and ER-resident chaperones, and an upregulation of certain components of ERAD (58, 182). If the condition cannot be reversed, the prolonged UPR will eventually lead to apoptosis [for review, see(23)].

Besides these acute insults, patho-physiological conditions can also affect the ER environment in a relatively subtle and chronic manner. In some cases, the exquisite protein quality control machinery and the robustness of the UPR allow the cells to successfully adapt to these demanding situations. An illustrative example are professional secretory cells, such as plasma cells, pancreatic β cells, hepatocytes and osteoblasts, which assisted by a chronic UPR, are able to synthesize, fold and secrete enormous amounts of proteins [for review, see (282)]. Nevertheless, in some pathological conditions the UPR does not manage to restore homeostasis in the ER, either because the UPR signalling is defective or because the overproduction of a mutant protein overwhelms the UPR capacity. The etiology of many diseases, such as neurodegenerative diseases, cystic fibrosis and muscle degenerative disorders is significantly related to impaired ER homeostasis and activation of the UPR.

5.4. ER stress and the unfolded protein response

The ER chaperon BiP is the master regulator of the UPR. In normal conditions, BiP is bound to the transducer proteins IRE1, PERK and ATF6. When the concentration of misfolded proteins in the ER increases, BiP releases from the transducer proteins and binds to the misfolded proteins instead, resulting in the activation of the signal transducers (17). The immediate response is a general inhibition of protein biosynthesis. This response is mediated by activation of the ER-resident serine/threonine kinase PERK that, upon

dissociation of BiP, phosphorylates the translational initiator factor eIF2α, repressing its activity (94, 234).

The second line of defence is an alteration of transcriptional programs through IRE1 and ATF6. IRE1α and IRE1β are ER-membrane bound endo-ribosomes that mediate mRNA splicing of the X-box-binding protein (XBP1) mRNA (27). The splicing of XBP1 results in a translational frame shift in the XBP1 transcript that now encodes an active transcription factor (291). ATF6 is an ER membrane-bound transcription factor that is activated by intramembrane proteolysis upon ER stress (99). The transcriptional pathways activated by the UPR result in the upregulation of structural components of the ER and ER-resident chaperones such as BiP and GRP94, in an attempt to facilitate normal folding of the proteins that are accumulated in the ER. The second effect is the upregulation of several components of the ERAD pathway, including several E2s and E3s (255), and Derlin-1 and Edem (200, 291). It is noteworthy that even though the three transduction factors PERK, IRE1α/β and ATF6 are present in all cell types, the activation of the UPR could be different depending of the circumstances. For instance, during ER stress concomitant to B-cell differentiation IRE1α is activated whereas PERK is not (73, 293).

If the ER stress condition cannot be reversed, the prolonged UPR will trigger signalling pathways leading to apoptosis. Although the mechanism of ER stress-induced apoptosis remains unclear, several mediators have been identified: IRE1, caspase-12 and PERK/CHOP. IRE1 activates c-Jun-N-terminal inhibitory kinase which leads to activation of the JNK protein kinase and mitochondria/Apaf1-dependent caspase activation (258). The murine caspase-12 is an ER-associated effector of the apoptotic caspase-cascade (191). Activation of the PERK during ER stress induces the translation of the transcription factor ATF4, which subsequently activates the transcription of the transcription factor CHOP (173). Abrogation of CHOP expression inhibits ER stress induced cell death (297).

6. THE UBIQUITIN-PROTEASOME SYSTEM IN CONFORMATIONAL DISORDERS

The protein quality control mechanisms are powerful means to avoid damage and misfolding of proteins. Nevertheless, in certain patho-physiological conditions the dedication of this machinery is not sufficient and accumulation of anomalous proteins occurs. Aberrant proteins can have deleterious effects in all cellular processes and is particularly dangerous in post-mitotic cells of long-lived organisms, where the toxicity can be sustained over extended periods of time. Proteotoxic stress is correlated with a large number of human disorders known as conformational diseases (31) (Table 1). To this group belong cystic fibrosis, retinitis pigmentosa, α1-antitrypsin deficiency and a large number of neurodegenerative diseases, including polyglutamine diseases, Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis and prion encephalopathies. This heterogeneous group of diseases are characterized by the accumulation of a particular protein that acquires an abnormal, aggregation-prone conformation that is toxic for the cells. Understanding how these toxic proteins escape from the protein quality control mechanisms would be important for the development of new therapies.

6.1. Protein aggregation during conformational diseases

The presence of insoluble protein aggregates in the affected tissues and cells is a communality of conformational diseases. Examples of these aggregates are intracellular tangles and extracellular plaques in Alzheimer's disease, Lewy bodies in Parkinson's disease, cytoplasmic bodies in certain muscle myopathies and Mallory bodies in several liver disorders. Although the protein aggregates found in conformational diseases vary in protein composition, localization and shape, they always contain various components of the ubiquitin-proteasome system and chaperones, suggesting an attempt of the protein quality control mechanisms to reverse the situation (3, 45).

Table 1. *Examples of conformational diseases*

Disease	Main aggregate component	Characteristic aggregates
Alzheimer's disease	Amyloid β-peptide (Aβ40- Aβ42) Hyperphosphorylated Tau	Extracellular plaques Intracellular oligomers Intracellular neurofibrillary tangles
Parkinson's disease	α-Synuclein	Intracellular Lewy bodies
Frontotemporal dementia with parkinsonism	Tau	Intracellular neurofibrillary tangles
Polyglutamine diseases : Huntington's disease Spinocerebellar ataxias (SCA1-3, 7)	Long polyglutamine stretches: Mutant Huntingtin Mutant Ataxin1-3, 7	Cytoplasmic and nuclear inclusions
Creutzfeld-Jakob disease (CJD) /spongiform encephalopathies	Prion protein (PrP^{SC})	Extracellular plaques Intracellular oligomers Extracellular oligomers
Amyotrophic lateral sclerosis (ALS)	Mutant Superoxide dismutase (SOD)	Intracellular inclusions
Z α1-antitrypsin deficiency	Z-α1-antitrypsin	Intracellular inclusions (hepatocytes)

At first, it was assumed that the formation of aggregates was a stochastic process driven by the exposure of hydrophobic domains in misfolded proteins. However, studies overexpressing the cystic fibrosis transmembrane conductance regulator (CFTR), an inefficiently folded integral membrane protein, showed that the excess of CFTR is actively delivered to inclusion bodies by dynein retrograde transport to the microtubule organizing centre (MTOC) (100, 128, 186). Based on these observation, it has been hypothesized that the formation of such intracellular inclusions, termed aggresomes, is an active physiological response to the excess of misfolded proteins (253). Whether the aggregates are a cause or a consequence of conformational diseases is a very controversial issue. Some studies indicate that aggregate formation inhibits ubiquitin-dependent proteasomal degradation (14, 15, 120, 208), whereas others indicate that protein aggregation is a protective mechanism (6, 231).

6.2. The ubiquitin-proteasome system and conformational diseases

The fact that aberrant proteins are accumulated during these diseases indicates that there is a failure of the protein quality control mechanisms. Overexpression of different molecular chaperones have been shown to mitigate the proteotoxicity in many cellular and animal models (7, 46, 63, 130, 266), emphasizing the critical role of protein quality control in these diseases. The question remains why the ubiquitin-proteasome system fails to remove the damage proteins that eventually will be harmful for the cells [for review, see (38)].

Another fundamental question is whether there is a general inhibition of ubiquitin-proteasomal degradation in the course of these diseases. Several studies have shown that overexpression of some disease-related proteins, such as a mutant form of CFTR (14), huntingtin bearing a polyglutamine expansion (15, 122) and the aberrant ubiquitin UBB^{+1} (151, 167) negatively affect the performance of the ubiquitin-proteasome system. However, because of the dual function of the ubiquitin-proteasome system as a protein quality control mechanism and a as a master regulator of short-live proteins necessary for viability, a general inhibition of ubiquitin-proteasomal degradation normally causes rapid cell death. The long-term progression characteristic of all conformational diseases argues against the idea of a broad-spectrum impairment of the ubiquitin-proteasome system as the cause of the disease.

6.3. ER stress and conformational diseases

Several conformational diseases have been linked to the occurrence of ER stress and there are indications that impairment of the ER functioning may be involved in neuronal cell death [For review, see (165)].

In some cases, a disease related protein might cause the accumulation of ERAD substrates in the ER. For instance, loss of function-mutations in the ERAD E3 parkin, have been associated with autosomal-recessive juvenile Parkinson's (ARJPD) disease (136, 245). It has been shown

that functional parkin suppresses the toxicity of overexpressed Pael-receptor (121) and α-synuclein (169, 208) proteins. Nevertheless, the identity of the physiological substrates of parkin remains uncertain. Although it is clear that the E3 ligase activity of parkin is required for protection against stress-induced apoptosis, the mechanism remains elusive and it is possible that a downstream effect of parkin could be even more important than the prevention of accumulation of ERAD substrates (155). A causative link between protein misfolding and ER dysfunction is clear in the case of Z-α1-antitrypsin, a genetic disease caused by accumulation of a mutant form of the α1-antitrypsin in the ER (155).

In polyglutamine disorders, it has been shown that cytosolic accumulation of the mutant polyglutamine proteins in the cytoplasm activate the UPR that in turn induces ER-stress dependent apoptosis (106, 144, 197). It is unclear how the accumulation of proteasome substrates in the cytosol could affect the integrity of the ER. One possibility is that the expanded polyglutamine proteins cause a general inhibition of the ubiquitin-proteasome system that consequently impairs ERAD thereby causing ER stress. Another explanation would be that ER stress is a late event resulting from a chain of toxicity events as a consequence of the disease. In some cases, the damage in the ER is caused by a mutant protein that interferes with the UPR. For instance mutations in the γ-secretase PS1 linked with the familial form of Alzheimer's disease have been shown to impair the different signal transductors of the UPR (146).

These observations suggest that during conformational diseases, a prompt UPR might be necessary to protect the integrity of the ER. Nevertheless, if the disease causes a chronic insult on the ER and the condition is not reversed, a prolonged UPR will contribute to toxicity and cell death. Understanding the precise role of the UPR and ERAD in the etiology of conformational diseases would be instrumental for the development of new therapies.

7. MONITORING THE UBIQUITIN-PROTEASOME SYSTEM

Aberrations in the ubiquitin-proteasome system have been proposed to be a contributing factor in many diseases, including metabolic disorders, malignant transformation, conformational disorders and immunodeficiencies. In order to understand the precise role of the ubiquitin-proteasome system in these pathologies, the development of methods to monitor the ubiquitin-proteasome system is imperative. Today, the ubiquitin-proteasome system can be studied with the help of different systems, ranging from small fluorogenic substrates for quantifying the proteolytic activities of purify proteasomes to fluorescent based-reporters for monitoring the functionality of the pathway *in vivo*.

7.1. Small fluorogenic substrates

The individual activities of purified proteasomes can be quantified with the use of synthetic peptides substrates linked to a fluorogenic group. This is assay is simple and reliable, but it has certain limitations. First, this method only provides information about proteolytic processes and does not take into account upstream events such as ubiquitylation, deubiquitylation and substrate unfolding. Second, fluorogenic substrates can not be used for the study of qualitative variations, such as changes in the subcellular distribution of components of the ubiquitin-proteasome system, which might have important consequences for the functionality of the ubiquitin-proteasome system. Finally, interpretation of the results from this assay is complicated. For instance, if the analyses show a decrease in proteasomal activity it is not possible to deduce whether the remaining proteasome activity is sufficient for efficient ubiquitin-proteasomal degradation of natural substrates. Illustrative in this respect are previous observations that protein substrates for proteasomal degradation can be efficiently degraded in circumstances that provoke a substantial reduction of proteasome activities (49).

7.2. Fluorescent protein substrates

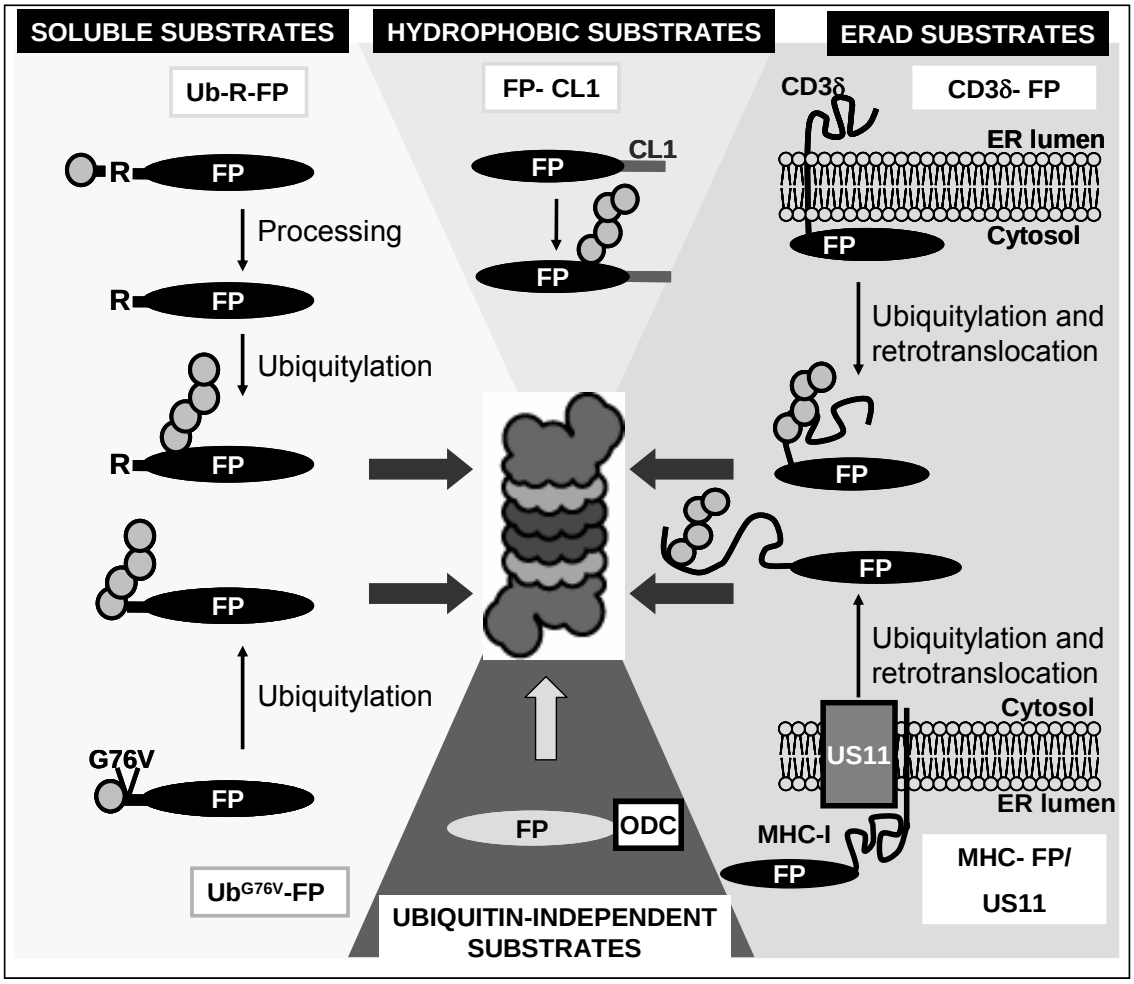

Figure 6. *Fluorescent protein (FP)-based proteasome substrates.* The following reporter substrates are represented: ubiquitin-Arg-FP (Ub-R-FP), Ub^{G76V}-FP, FP-CL1, CD3δ-FP, US11/FP-MHC class I and FP-ODC. **Ub-R-YFP** is an N-end rule substrate. The endogenous DUBs rapidly remove the N-terminal ubiquitin, which generates a FP with an N-terminal arginine residue. The N-terminal arginine is recognized by ubiquitylation enzymes resulting in the conjugation of a ubiquitin chain close to the N-terminus followed by proteasomal degradation. **Ub^{G76V}-FP** is a UFD substrate generated by replacement of the C-terminal glycine (G) of the ubiquitin moiety with a valine (V). In this case, the DUBs fail to remove the N-terminal ubiquitin. resulting in ubiquitylation of the ubiquitin moiety and subsequent proteasomal degradation. **FP-CL1** is targeted for ubiquitylation and subsequent degradation by the artificial degradation signal CL1, which is characterized by the presence of hydrophobic amino acids. **CD3δ-FP** is a substrate for ERAD, when expressed in cells lacking CD3δ-FP oligomerization partners. Co-expression of US11 and **FP-MHC class I** results in dislocation of FP-MHC class I from the ER to the cytoplasm and ubiquitylation followed by proteasomal degradation. **FP-ODC** is targeted for ubiquitin-independent proteasomal degradation by the ODC degradation signal.

Several groups have developed reporter assays that allow functional analysis of the ubiquitin-proteasome system without the limitations of fluorogenic peptides [for review, see (166, 193, 230)]. All these reporter substrates are based on the same principle: targeting a fluorescent protein (FP) for proteasomal degradation by the introduction of constitutive degradation signals (see Chapter 4.1). Some of the different fluorescent reporter systems representing different classes of proteasomal substrates that have been developed are: ubiquitin-Arg-FP (Ub-R-FP), Ub^{G76V}-FP, FP-CL1, CD3δ-FP, US11/FP-MHC class I and FP-ODC (Fig. 6).

The Ub-R-FP and Ub^{G76V}-FP reporters (49) are constitutively degraded by the N-end rule (267) and the ubiquitin fusion degradation (UFD) (126) pathways, respectively. The FP-CL1 reporter (14) is targeted for degradation by the short artificial degradation signal CL1 (77). The CD3δ-FP substrate is an ERAD substrate based on the T-cell receptor CD3δ subunit. When expressed in cells lacking the CD3δ oligomerization partners, CD3δ is translocated from the ER to the cytosol and degraded by the ubiquitin-proteasome system (286). Another ERAD substrate is based on the ability of the human cytomegalovirus (CMV) protein US11 to specifically target MHC class I molecules for dislocation from the ER and subsequently degradation by the ubiquitin-proteasomal system (164, 216). Hence, co-expression of US11 and N-terminally FP-tagged MHC class I in human cells results in a functional reporter for ERAD (132). Finally, a reporter system has been developed by fusing a FP with the mouse ornithine decarboxylase (ODC d410) degradation motif, which contains several PEST sequences (161). This degradation motive has been shown to target the ODC for ubiquitin-independent proteasomal degradation (187, 294) and it can be transferred to a FP protein resulting in proteasomal degradation of the fusion protein by the proteasome (218).

Expression of these fluorescent reporters in cell lines has been shown instrumental for the study of the ubiquitin-proteasome system in diseases (for review, see (166). Indeed, the work described in this thesis has benefited from the availability of these different reporter systems, which allowed us to study the effect of proteotoxic stress conditions in different ubiquitin-proteasomal pathways in parallel (see Chapter 10). In addition,

protein substrate reporters can be applied to the generation of animal models that will allow studying the ubiquitin-proteasome system in the context of different diseases and evaluation of new therapeutics. In this thesis, the development and characterization of the first transgenic mouse model for monitoring ubiquitin-proteasome system degradation *in vivo*, which is based in the ubiquitous expression of the UFD-FP reporter, is presented (see Chapter 10.2 and **paper II**). Another animal model for studies in the ubiquitin-proteasome system was developed by performing xenografts of UFD-luciferase reporter-expressing human cell lines in nude mice (171). The characteristics and application of these animal models are discussed in detail in Chapter 10.2 and Chapter 11.

8. PROTEASOME INHIBITORS

Since the discovery of the ubiquitin-proteasome system, the combined efforts of several laboratories have resulted in the development and discovery of synthetic and naturally ocurring inhibitors of the proteolytic activities of the proteasome. These inhibitors have been extensively used in research. Proteasome inhibitors have been instrumental for the discovery of proteins involved in ubiquitin-proteasomal degradation as well as for understanding the roles of the different proteolytic activities in many biological processes. Importantly, proteasome inhibitors have recently been introduced as a novel class of anti-cancer drugs with promising results in the treatment of multiple myeloma [for review, see (134)].

8.1. Types of proteasome inhibitors

At present, there are four major classes of proteasome inhibitors: peptide-aldehydes, peptide-boronates, peptide-vinyl sulfones and natural compounds, including epoxyketones, β-lactones and other compounds (Table 2). It is to be expected that new inhibitors might be added to this list in the future.

Table 2. *Different classes of proteasome inhibitors and their inhibitory capacity.*

Group	Proteasome inhibitors	Inhibition of β-subunits	Reversibility	Other cellular targets
Peptide-aldehydes	ALLN	β5>>>β1>β2	Reversible	Calpains and cathepsins
	MG132			
Peptide-boronates	MG262	β5>>β2>β1	Very slow on/off rates	?
	Bortozemib	β5>>β2		None found
Peptide-vinyl sulfones	NLVS	β5>>β1≅β2	Irreversible	Cathepsins
	ZLVS	β5>β2>β1		Cathepsins
	Ada-Ahx$_3$L$_3$VS	β5>β1>β2		?
	Dansyl- Ahx$_3$L$_3$VS	β5>β1≅β2		?
β-lactones	Lactacystin	β5>>>β2>β1	Irreversible	Cathepsin A TPPII
Epoxyketones	Epoxomicin	β5>>>β2	Irreversible	None found

8.1.1. Peptide-base proteasome inhibitors

Enzyme-specific inhibitors of proteases are usually composed of a peptide backbone attached to a reactive group. The peptide moiety interacts with the substrate-binding pocket of the active site, whereas the active group reacts with the catalytic amino-acid. The fact that proteasomes are the only known proteases that use an N-terminal threonine as reactive group (see Chapter 3.3) has facilitated the design of specific proteasome inhibitors.

The first proteasome inhibitors to be developed were peptide-aldehydes, such as ALLN (Ac-Leu-Leu-Nle-al) and MG132 (Z-Leu-Leu-Leu-al). Peptide-aldehydes interact preferentially with the β5 subunit, in a slow rate and reversible reaction. Two favourable properties have motivated the extensive used of peptide-aldehyde inhibitors in research: cell permeability and reversibility. Nevertheless, peptide-aldehydes are not very specific proteasome inhibitors. In fact, they are potent inhibitors of cysteine and serine proteases, such as calpains and lysosomal cathepsins. Moreover, peptide-aldehydes are instable *in vivo*.

Peptide-boronates are very potent proteasome inhibitors. For instance, the peptide-boronate MG262 is 100 times more potent than its aldehyde analogue MG132. Although in theory peptide-boronates are reversible inhibitors, the dissociation rate of the boronate-proteasome adducts is very slow and the inhibition last for hours. Moreover, peptide-boronates are very selective and metabolically stable inhibitors. These characteristics have been exploited in the search of proteasome inhibitors for therapeutic applications (see Chapter 8.3).

Peptide-vinyl sulfones (VS) are irreversible inhibitors that covalently modify the γ-hydroxyl group of the proteasomal N-terminal threonines (19, 87). This irreversible modification of the active subunits of the proteasome has been exploited for the development of active-based probes for the proteasome (see Chapter 8.2 and Chapter 10.3).

8.1.2. Peptide-derivatives and natural compounds

The *Streptomyces* metabolite lactacystin is spontaneously converted at neutral pH into clasto-lactacystin-β-lactone, which reacts with the γ-hydroxyl of the N-terminal threonine of the β5 proteasomal subunit (53, 61). There are two disadvantages associated with the use of β-lactones as proteasome inhibitors. First, β-lactones are relatively instable compounds. Secondly, β-lactones also inhibit the lysosomal peptidase cathepsin A, and the cytosolic tripeptidyl peptidase II (74).

The natural epoxyketones, epoxomicin and eponemycin, were originally found by their anti-tumor and anti-inflammatory properties. Later on, it was discovered that these properties were the consequence of a direct proteasomal inhibition (180, 181). Epoxyketones react with both the γ-hydroxyl and with the α-amino group of the N-terminal threonine (88). Serine and cysteine proteases do not have an amino group adjacent to the nucleophilic group and thus, are not susceptible to inhibition by epoxyketones, explaining the high selectivity of epoxyketones. Epoxomicin, the most potent epoxyketon, inhibits primarily the β5 proteasomal subunit.

New natural compounds have been discovered that broaden the list and properties of proteasome inhibitors. For instance, it has been shown that a compound derived from *Apiospora montagnei*, TMC-95s, is a potent and selective inhibitor of the three proteolytic activities of the proteasome (142). Interestingly, structural analyses of the interaction of this compound with the 20S core of the proteasome have revealed that it does not modify the catalytic threonine but, instead, binds non-covalently through hydrogen bonds to the three catalytic subunits (89). An interesting line of research is the systematic modification of natural compound looking for efficient and specific proteasome inhibitors (34).

8.2. The use of proteasome inhibitor as active probes

The development of proteasome inhibitors that form covalent bonds with the active groups of the catalytic subunits of the proteasome was an excellent start for the design of activity-based probes for the proteasome. These probes can

have important applications, such as monitoring the potency and selectivity for each catalytic site of known proteasome inhibitors, screening for new compounds that interfere with proteasomal activity and establishing activity profiles of different cell types in physiological and pathological conditions. Ideally, an activity-based proteasome probe should fulfil several requirements. First, it should be specific for the proteasome and it should bind the three active β- and iβ-subunits indiscriminately. Second, it should facilitate direct, accurate and sensitive detection. And, finally, it should be cell permeable and metabolically stable.

The first active probes were synthesized by adding a radio-labelled tag to peptide-vinyl sulfones. Among these, the best approach was the development of the proteasome inhibitor Ada-Y(^{125}I)Ahx$_3$ -Leu$_3$VS (132). Nevertheless, the use of the [^{25}I]-iodotyrosine renders this probe membrane impermeable and thus, limits the use of this probe to *in vitro* studies. This problem was partially solved by substituting the radio-labelled tag with the weakly fluorescent dansyl-group. Importantly, there are antibodies available that allow immuno-detection of the dansyl-group. The development of dansyl-Ahx3- Leu$_3$VS represents an additional step in the search for the ideal activity-based probe (16). Nevertheless, the low quantum yield and near UV excitation of the dansyl-group makes this compound unsatisfactory for in-gel detection and standard fluorescence microscopic techniques. In this thesis, a novel fluorescent proteasome activity probe that overcomes these problems is presented (see Chapter 10.2 and **paper II**).

8.3. The use of proteasome inhibitors in therapy

Several proteasome inhibitors have potent anti-tumor and anti-inflammatory properties (180, 181). In particular, the dipeptide-boronic acid bortezomib (also known as PS-341 and Velcade) exhibits cytotoxiciy against cancer cell lines and especially, multiple myeloma (109). These encouraging observations were pursued by clinical trials that demonstrated a remarkable anti-tumor activity, including some complete responses (226). Since 2004, the use of bortezomib has been approved as a second-line treatment for patients with multiple myeloma. The tumor-selectivity and relative low toxicity of proteasome

inhibitors are surprising given the crucial role of the ubiquitin-proteasome system in virtually all cellular processes. It has been speculated that a reason for the specific vulnerability of multiple myeloma cells towards proteasomal inhibition could be an accentuated dependency on a functional ERAD for degradation of abnormal immuno-globulins (1). Due to the important success of bortezomib, it is expected that more selective and highly potent proteasome inhibitors will be developed to treat malignant diseases.

Another interesting potential therapeutic approach would be the manipulation of the inducible catalytic subunits of the immunoproteasome, which eventually could lead to modifications in the generation of antigenic-peptides (241). Experiments with animal model have shown that proteasome inhibitors could be beneficial against unwanted immune- and inflammatory-responses such as autoimmune encephalomyelitis (2, 264), psoriases (298) and rheumatoid arthritis (203) and could be useful as immunosuppressors after transplantation (283). Moreover, a different potential strategy would be the development of compounds that interfere with other activities and components of the ubiquitin-proteasome system, such as E3s, E4s or DUBs.

9. AIMS OF THIS STUDY

The general aim of the studies presented in this thesis was to investigate the functionality of the ubiquitin-proteasome system during proteotoxic stress conditions characteristic of many human disorders.

The specific aims of this thesis were to:

I Development of a transgenic mouse model for monitoring the functionality of the ubiquitin-proteasome system *in vivo*

II Synthesize and characterize a proteasome inhibitor that would allow monitoring active subunits of the proteasome *in vitro* and *in vivo*

III Analyze the effect of proteotoxic stress conditions on the ubiquitin-proteasome system

IV Study the mechanisms underlying the long term accumulation of aberrant proteins during proteotoxic stress

10. RESULTS AND DISCUSSION

Many patho-physiological conditions are characterized by changes in the cellular environment, such as temperature fluctuations, generation of oxygen radicals or hypoxia; which provoke an increase in the amount of aberrant and misfolded proteins. The question is whether the ubiquitin-proteasome system is able to cope with these challenging situations. If the ubiquitin-proteasome system succeeds to maintain the concentration of aberrant proteins under the toxic threshold, would this effort have a detrimental effect for its house-keeping functions? One of the difficulties approaching these questions is the lack of systems to monitor the functionality of the ubiquitin-proteasome activity *in vivo.* We have addressed this need by generating fluorescent reporters to monitor the functionality of the ubiquitin-proteasome system in cell lines ((49, 230), **paper III** and **paper IV**) and in a transgenic mice (**paper I**). Furthermore, we have developed a novel fluorescent proteasome inhibitor that allows specific labeling of proteasomes *in vitro* and *in vivo* (**paper II**). We have subsequently used these models to investigate the effect of proteotoxic stress conditions in the degradation of different proteasomal substrates (**paper III** and **paper IV**) and to gain insight into the mechanisms contributing to the long term accumulation of deleterious proteins during proteotoxic stress (**paper IV**).

10.1. Generation and characterization of reporter cell lines for the ubiquitin-proteasome system

It has been previously shown that functional analysis of the ubiquitin-proteasome system can be accomplished by following the steady state levels of fluorescent reporter substrates (14, 49, 230) (see Chapter 7.2). The availability of different fluorescent reporters representing different classes of proteasomal substrates has been instrumental for the studies presented in this thesis, since it provided the possibility of studying the effect of patho-physiological conditions in several proteasomal substrates in parallel.

In these studies, we generated stable MelJuSo cell lines expressing the following fluorescent protein based ubiquitin-dependent reporters: Ub-R-YFP, Ub^{G76V}-YFP, YFP-CL1 and CD3δ-YFP, (described in

paper III). Later on, we expanded this panel with a stable MelJuSo cell line expressing the ubiquitin-independent substrate ZsGFP-ODC (described in **paper IV**).

The efficient degradation of these fluorescent reporter-substrates resulted in low fluorescence intensities. Importantly, the five reporter cell lines responded to treatment with proteasome inhibitors with a dose-dependent increase in fluorescent intensities, readily detectable by fluorescence-activated cell sorter (FACS) analysis and microscopy. Interestingly, the different nature of the reporter substrates was reflected in a different magnitude of increase in steady state levels and distinct distribution pattern in the cells. The YFP fluorescence of Ub-R-YFP and Ub^{G76V}-YFP was strongly increased in both cytosol and nucleus after proteasomal inhibition. The increase in fluorescence was weaker in YFP-CL1 MelJuSo cell lines treated with proteasome inhibition. Interestingly, accumulated YFP-CL1 was sequestered in aggresomes (see Chapter 6.1). Finally, untreated CD3δ-YFP cell line displayed a weak YFP fluorescence associated with the ER, which was clearly enhanced upon treatment with proteasome inhibitors. The ubiquitin-independent proteasomal substrate, ZsGFP-ODC, was characterized by steady state levels below detection level in normal conditions. Upon proteasomal inhibition ZsGFP-ODC accumulated evenly throughout the cells.

In summary, we have generated MelJuSo reporter cell lines that provide a versatile system to monitor four major classes of proteasomal substrate: soluble cytosolic/nuclear substrates (Ub-R-YFP and Ub^{G76V}-YFP), hydrophobic substrates (YFP-CL1), ERAD substrates (CD3δ-YFP) and ubiquitin-independent substrates (ZsGFP-ODC).

10.2. A transgenic mouse model of the ubiquitin-proteasome system

Cell lines expressing fluorescent reporters of the ubiquitin-proteasome system have been instrumental in the study of the ubiquitin-proteasome system. Nevertheless, the use of cell lines involves two important limitations. First, cell lines are not perfectly representative systems of human diseases. Second, cell

lines cannot be used for the study of tissue specific responses towards insults in the ubiquitin-proteasome system. We have approached this problem by developing a transgenic mouse model for monitoring the functionality of the ubiquitin-proteasome *in vivo*, which is described in detail in **paper I**. This transgenic mouse model is based on the constitutive expression of the UFD reporter substrate Ub^{G76V}-GFP, which was selected because of its short half-life degradation rate and low toxicity in cell culture. The Ub^{G76V}-GFP transgene was expressed from a chicken β-actin promoter with a cytomegalovirus (CMV) immediately early enhancer, which normally gives high constitutive expression in all tissues (201).

Two mouse strains, named Ub^{G76V}-GFP/1 and Ub^{G76V}-GFP/2, were generated with the reporter construct, and both strains were characterized by the presence of the transcript in all examined tissues: lung, spleen, small intestine, muscle, heart, kidney, pancreas, liver, testis and brain. As expected from the robustness of the UFD signal, degradation of the reporter substrate was so efficient that inhibition of the ubiquitin-proteasome system was required in order to detect the Ub^{G76V}-GFP protein. Indeed, primary fibroblasts, cardiomyocytes and neurons of Ub^{G76V}-GFP mice responded to treatment with different proteasome inhibitors with accumulation of the reporter substrate in a dose dependent manner.

The functionality of the reporter was also confirmed by intraperitoneal injection of the proteasome inhibitors MG132, MG262 and epoxomicin. Administration of MG262 and epoxomicin resulted in a dramatic accumulation of Ub^{G76V}-GFP in the liver, whereas MG132 did not have any detectable effect, probably due to *in vivo* oxidation of the aldehyde group (see Chapter 7.1.1). Injections with high concentration of MG262 resulted in massive reporter accumulation in liver, small intestine, pancreas and kidney and a less pronounced accumulation in spleen and lungs. Interestingly, intramuscular injection with MG262 lead to weak accumulation of Ub^{G76V}-GFP in myocytes and a particularly intense accumulation in a small population of cells that resemble satellite cells, based on their size and localization. Satellite cells are quiescent myoblasts adjacent to the muscle fiber that have a role in repair and regeneration of healthy muscle.

Finally, we investigated whether the Ub^{G76V}-GFP transgenic mouse model can reveal impairment of the ubiquitin-proteasome system as a

consequence of a pathological condition. We infected primary neurons derived from Ub^{G76V}-GFP mice with a lentivirus encoding UBB^{+1}, an aberrant ubiquitin that accumulates in the affected neurons of patients with Alzheimer's disease (260, 261) and that inhibits the ubiquitin-proteasome system in human cell lines (167). Primary infected neurons responded to UBB^{+1} with accumulation of the Ub^{G76V}-GFP reporter, demonstrating that this model can reveal ubiquitin-proteasome inhibition induced not only by classical proteasome inhibitors but also by a disease-related protein.

This transgenic mouse model provides an excellent tool to explore the status of the ubiquitin-proteasome system in different human diseases. For instance, a recent study analysing crosses between the reporter mice and a knock-in mouse model for spinocerebellar ataxia-7 (SCA-7) has shown that full blockade of the ubiquitin-proteasome system does not contribute significantly to SCA-7 related pathology (22). Nevertheless, subtle changes in the ubiquitin-proteasome system can not be excluded from this study (see also **paper III** and **paper IV**) and the understanding of the precise role of the ubiquitin-proteasome system in different diseases awaits further investigation. Finally, these mice can be used to monitor the bioavailability of proteasome inhibitors (**paper II**). Importantly, this mouse model can be instrumental to study the therapeutic potential of proteasome inhibitors and for the development of tissue and tumor specific proteasome inhibitors (see chapter 7.3).

10.3. A fluorescent activity-based probe for proteasomes

In spite of the large number of potent and selective proteasome inhibitors that have been developed during the last two decades, there was no proteasome inhibitor available that allowed monitoring proteasomal active subunits *in vivo* (see Chapter 8.2). We have developed the fluorescent proteasome inhibitor MV151, presented in **paper II**, which enables fast and sensitive labeling all three active proteasomal activities. This proteasome inhibitor was designed by linking the peptide fluorophore Bodipy-TMR and Ada-Ahx_3L_3VS, which is a potent and selective inhibitor of the three catalytic activities of the proteasome.

First, we show that MV151 is a potent proteasome inhibitor that completely blocks the three catalytic activities of the proteasome. Furthermore, proteasome labelled subunits could be easily visualized after SDS-PAGE separation followed by direct in-gel fluorescent detection. This method is easy, rapid and sensitive and allows detection of the constitutive β1, β2, β5 proteasome subunits as well as the immuno-induced counterparts. This method can be applied to characterize potency and subunit-specificity of proteasome inhibitors, as shown in detail in **paper II**.

Next, we tested the efficiency of MV151 in cell culture. Our experiments demonstrated that MV151 is cell permeable and stable. Using a HeLa cell line stably expressing the Ub^{G76V}-GFP reporter, we found that MV151 is a potent inhibitor of the ubiquitin-proteasome system. Importantly, Ub^{G76V}-GFP HeLa cells that were treated with the inactive compound MV152, in which the vinyl sulfone moiety is reduced to an un-reactive ethyl sulfone, did not accumulate the reporter. This excludes the possibility that the inhibition in the ubiquitin-proteasome was an indirect consequence attributable to toxicity of the fluorescent moiety. In addition, we could easily analyse the intracellular distribution of MV151 by monitoring its fluorescent signal. After 5 h of treatment MV151 was distributed throughout the cytoplasm and nucleus, excluding the nucleoli. Strikingly, we also observed that MV151 was accumulated in granular structures localized in the cytoplasm near the nucleus. A possible explanation for the appearance of these granular structures is that MV151 is internalized by fluid-phase endocytosis and consequently is accumulated in lysosomes. An alternative explanation is that MV151 aggregates as a consequence of proteasomal inhibition. It would be important to distinguish between these two possibilities in future investigations.

Finally, we used the Ub^{G76V}-GFP transgenic mouse model to test the bioavailability of MV151. We observed that, upon intraperitoneal administration, MV151 was primarily localized in the liver and the pancreas. Importantly, Ub^{G76V}-GFP was accumulated in those hepatocytes and pancreatic cells that contained the highest levels of MV151. It is noteworthy that high MV151 concentrations appear to be required to achieve a complete inhibition of the ubiquitin-proteasome system. This could indicate that, in normal conditions, cells are provided with excessive amounts of proteasomal activity. Illustrative in this respect, are our previous observations that, in HeLa

cell lines, substrates for proteasomal degradation can be efficiently degraded in circumstances that provoke a substantial reduction of proteasome activities (49). In the context of the MV151 injection, it would be important to rule out the remote possibility that the low Bodipy-TMR fluorescence detected in cells and tissues that are not accompanied by Ub^{G76V}-GFP reporter accumulation does not come from an inactivated or cleaved product from MV151.

In summary, we have developed a novel tool to examine activity and localization of proteasomes *in vitro* and *in vivo*. This probe can be used for many applications ranging from clinical profiling of proteasomal activity to the study of the composition, function and distribution of the proteasomes in different cell types and tissues.

10.4. The ubiquitin-proteasome system is compromised during proteotoxic stress

The hallmark of conformational diseases is the presence of proteins that undergo a conformational change from a soluble to a misfolded, aggregation-prone structure, which eventually leads to the formation of the characteristic nuclear or cytosolic protein inclusions (see Chapter 6). The reoccurring question is why these aberrant proteins are not efficiently eliminated by the ubiquitin-proteasome system. To address this question, we investigated if it was possible to saturate the ubiquitin-proteasome system by increasing the load of aberrant proteins.

Treating cells with compounds that hamper proper synthesis of ER resident proteins is known to cause a dramatic rise in ERAD substrates, leading to ER stress (see Chapter 5.4). Using the stable reporter cell lines described in Chapter 10.1, we studied the effect of ER stress on the degradation of different reporter-substrates (**paper III**). We have worked with various well-established methods to induce ER stress: tunicamycin, an inhibitor of N-glycosylation; thapsigargin, which blocks calcium entry to the ER and dithiothreitol (DTT), which reduces disulfide bridges. ER stress resulted in a delay in the degradation of the ERAD substrate CD3δ-YFP. Furthermore, there was a subtle but consistent accumulation of the soluble substrates, Ub-R-YFP and Ub^{G76V}-YFP and the misfolded substrate YFP-CL1. These results indicate

that ER stress have a general effect on different classes of proteasomal substrates.

Importantly, a similar signature accumulation was observed in Ub^{G76V}-GFP mice injected with sub-lethal doses of tunicamycin, which is known to induce transiently ER stress in the tubular epithelium of the kidney (191, 297). Detailed analysis of the kidneys of tunicamycin-treated reporter mice showed that accumulation of Ub^{G76V}-GFP was also confined to the tubular epithelium while the adjacent glomeruli were negative for the reporter substrate. Thus, induction of ER stress *in vivo* is accompanied by an increase in the levels of a soluble reporter similarly to what has been found in the reporter cell lines.

It is important to emphasize that the accumulation of cytosolic and nuclear substrates was rather modest, and represented only approximately 10-20% of the effect observed after full blockade of the ubiquitin-proteasome system with proteasome inhibitors. However, the accumulation of substrates could have important consequences. Firstly, we found that a small population of cells expressing YFP-CL1 responded to ER-stress with the formation of YFP-CL1 inclusions. This indicates that the ER stress condition is sufficient to provoke the formation of the protein deposits characteristic for conformational diseases. Secondly, when cells undergoing ER stress were additionally challenged with overexpression of UBB^{+1}, they failed to degrade this aberrant protein, which in turn caused a general impairment of the ubiquitin-proteasome system. Thus, although the efforts of the ubiquitin-dependent degradation machinery might be able to maintain the majority of the substrates within tolerable concentrations, the ubiquitin-proteasome system is nearly saturated and therefore not able to respond to any additional challenge. We conclude that a chronic ER stress compromises the activity of the ubiquitin-proteasome system.

Next, we investigated the response of the ubiquitin-proteasome system using an acute but transient stress condition (**paper IV**). For this purpose, the reporter cell lines were submitted to a 42°C heat shock for 1 hour and subsequently recovered at 37°C for several hours. Heat shock treatment induced a general accumulation of misfolded proteins throughout the cell, followed by activation of the heat shock response and the UPR. The consequences of heat shock were very similar to the effects produced by ER

stress: very high levels of CD3δ-YFP steady state levels and a modest increase for Ub-R-YFP, Ub^{G76V}-YFP and YFP-CL1.

Since heat shock was a transient insult, we were able to examine whether the accumulated reporter substrates were eventually cleared. Interestingly, whereas the soluble proteasomal substrates, Ub-R-YFP and Ub^{G76V}-YFP, were returned to basal levels, the accumulated YFP-CL1 and CD3δ-YFP were not cleared within 17 hours. Given the hydrophobic nature of the CL1 signal and the transmembrane domain from CD3δ-YFP, the behavior of these reporter substrates might be representative of aggregation-prone proteasomal substrates. There are three possible explanations that might explain the long-term accumulation of aggregation-prone proteins. First, it is possible that degradation of short-lived regulatory proteins that are essential for cell survival, such as cell cycle regulators, is prioritized over degradation of damaged and aberrant proteins. Second, since heat shock induces an overload of misfolded proteins it is likely that ubiquitin-degradation pathways specifically involved in protein quality control are saturated. For instance, the availability of specific E3 ligases that recognize hydrophobic and misfolded domains might be limiting. Third, accumulation of hydrophobic substrates might subsequently aggregate, complicating their degradation. In fact, it has been previously shown that aggregation can protect proteins from proteasomal degradation (270). The long-lasting accumulation of misfolded proteins after proteotoxic stress might explain why aggregation-prone proteins are a common characteristic of certain human diseases

10.5. Depletion of free ubiquitin contributes to proteotoxic stress

We have showed that both a chronic increase in ERAD substrates and an acute increase in aberrant proteins in the nuclei, cytoplasm and ER compromise the overall function of the ubiquitin-proteasome system. These results prompted us to investigate if a reduction of proteasome activity could account for this accumulation. We analysed whether saturation at the proteasome could account for the delayed degradation of proteasomal

substrates observed in ER stressed-cells. We found that both proteasome levels and proteasome activity were unmodified during ER stress (**paper III**). Thus, it is rather unlikely that saturation at the level of proteasomal degradation could be the cause underlying the accumulation of proteasomal substrates during proteotoxic stress.

These results suggest that the bottle neck in protein degradation is located upstream of the proteasome. Among the different components of the ubiquitin-proteasome system, the ubiquitin molecule was likely to be a limiting factor. Even though ubiquitin is one of the most abundant proteins in the cell, the majority of ubiquitin is covalently linked to other proteins and the levels of free ubiquitin are surprisingly low (47). Indeed, we found that a chronic ER stress induced a dramatic decrease in free ubiquitin levels of the cells. Similarly, heat shock was immediately followed by a transient depletion of the free ubiquitin pool and changes in the dynamic properties of ubiquitin (**paper IV**). The transient nature of heat shock-induced proteotoxic stress allowed us to follow ubiquitin during the recovery phase. We found that the heat shock was followed by upregulation of ubiquitin expression (see Chapter 5.3). Importantly, this increase in free ubiquitin levels was followed by the clearance of the soluble Ub^{G76V}-YFP and Ub-R-YFP reporters. Thus, depletion of free ubiquitin rather than proteasomal inhibition is a likely cause of the subsequent accumulation of proteasomal substrates under proteotoxic stress conditions.

11. CONCLUSIONS AND PERSPECTIVES

The studies described in this thesis have revealed that proteotoxic stress conditions lead to a compromised ubiquitin-proteasome system. In these circumstances, ubiquitin-proteasomal degradation is still taking place but it is suboptimal. In addition, our data suggests that ubiquitin, rather than the proteasome itself, is the rate limiting factor during stress conditions. Importantly, we have also showed that whereas the ubiquitin-proteasome has the means to remove the majority of the accumulated substrates after a transient stress condition, the accumulation of aggregation-prone substrates is long lasting. This observation might explain the preferential accumulation of these substrates in conformational diseases.

Based on these observations, we propose that the ubiquitin-proteasome system may progress through three phases in conformational diseases. Initially the ubiquitin-proteasome system is fully operative. The second phase is initiated by a stress condition, such as ER stress, oxidative stress, hypoxia or overexpression of a mutant protein. These conditions may compromise the ubiquitin-proteasome system causing a slow and progressive build-up of aggregate-prone substrates, which are stored in inclusions. During the third and final phase, the accumulated substrates may cause a general inhibition of the ubiquitin-proteasome system rapidly followed by cell death. Whether the cells die during the second phase or eventually face ubiquitin-proteasome inhibition may depend, among other factors, on the inherent toxicity of the accumulating proteins.

Although purely speculative, this model explains the slow progression of conformational diseases, since the second phase may start long before the onset of the disease and proceed over decades. Moreover, according to this model, several factors may determine the progression through these phases: the amount of ER-client proteins before the stress insult, the ability of the cells to upregulate the protein quality control mechanisms and the duration and intensity of the stress condition. Finally, in the majority of the conformational diseases neurons are affected. Since neurons are post-mitotic it is likely that they have gone to episodes of stress and concomitant inefficient ubiquitin-proteasome system that may have

caused the irreversible accumulation of aggregation-prone proteasome substrates in inclusions.

Further studies should be conducted to test this hypothetical model in the context of conformational diseases. Of particular interest would be to study the vulnerability of the ubiquitin-proteasome system towards proteotoxic stress conditions in different cell types. The Ub^{G76V}-GFP transgenic mouse model will be instrumental for these experiments, since it can serve as a source for different cell types with an identical genetic background expressing the Ub^{G76V}-GFP reporter. Furthermore, the reporter mouse model opens the possibility to expand our study from cell lines to organisms and investigate the status of the ubiquitin-proteasome system during proteotoxic stress in the context of conformational diseases. In fact, several laboratories are currently crossing the Ub^{G76V}-GFP transgenic mouse with different disease models. Based on our studies in cell culture, we expect that different reporter system representing distinct ubiquitin-proteasomal pathways may have different behaviour under pathological conditions. Thus, the usage of different GFP-reporter system may be necessary to gain insight into the global effect of pathological conditions on the ubiquitin-proteasome system. The recent development of a new transgenic mouse model based on the ubiquitin-proteasomal reporter substrate GFP-CL1 opens new opportunities in this respect (148). We can anticipate that the different mouse models of the ubiquitin-proteasome system can provide important information to increase our understanding of the contribution of the ubiquitin-proteasome system to human diseases.

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APPENDIX (Papers I-IV)