Abstract

Many biological processes rely on targeted protein degradation, the dysregulation of which contributes to the pathogenesis of various diseases. Ubiquitin plays a well-established role in this process, in which the covalent attachment of polyubiquitin chains to protein substrates culminates in their degradation via the proteasome. The three-dimensional structural topology of ubiquitin is highly conserved as a domain found in a variety of proteins of diverse biological function. Some of these so-called “ubiquitin family proteins” have recently been shown to bind components of the 26S proteasome via their ubiquitin-like domains, thus implicating proteasome activity in pathways other than protein degradation. In this chapter, we provide a structural perspective of how the ubiquitin family of proteins interacts with the proteasome.
© 2004 Elsevier B.V. All rights reserved.

Keywords: Protein degradation; Ubiquitin; Ubiquitin-like domain; Proteasome

1. Introduction

Ubiquitin-mediated protein degradation is one of the major mechanisms for controlled proteolysis, which is crucial for many cellular events, including the control of life span of regulatory proteins, the removal of improperly folded proteins that would otherwise aggregate, and the production of immunocompetent peptides [1–6]. The role of ubiquitin in degradation is its most extensively studied function: polyubiquitin chains are covalently attached to proteins and serve as a signal for their recognition and degradation by the 26S proteasome [2]. Ubiquitin has also been found to be involved in protein trafficking [7–11], DNA repair [12–14], signaling pathways [15], and transcription [16,17].

A highly conserved ubiquitin structural fold is present in numerous eukaryotic proteins and has ancestral ties to the prokaryotic protein ThiS [18]. Ubiquitin family proteins have been defined by their structural homology with ubiquitin and are divided into two families: type I ubiquitin-like proteins and type II ubiquitin-like domain proteins. Like ubiquitin, these proteins are involved in a variety of different biological pathways. Some ubiquitin-like domains have been found to bind proteasomal subunits, although the functional significance of these interactions remains the subject of much speculation. In this chapter, we illustrate how the interactions between ubiquitin family members and proteasomal subunits may be characterized by NMR spectroscopy, and we discuss the implications of such interactions for proteasome activity.

2. The ubiquitin family of proteins

2.1. A prominent role for ubiquitin in targeted protein degradation

The best-characterized role of ubiquitin is in targeted protein degradation by the 26S proteasome, which consists...
of two 19S regulatory particles and a 20S core. Proteins are posttranslationally modified by ubiquitin via an enzymatic cascade, which begins with the formation of a thioester bond between a ubiquitin-activating enzyme (E1) and the C-terminal glycine of ubiquitin. This activated ubiquitin is subsequently transferred to a ubiquitin-conjugating enzyme (E2) [19–21]. An E3 ubiquitin ligase then catalyzes the formation of an isopeptide bond between ubiquitin and lysine residues of a specific target protein, either by directly accepting the ubiquitin and transferring it to the substrate, or by bringing the substrate into proximity with the E2–ubiquitin complex. This process is repeated, with more ubiquitin moieties being covalently conjugated to those already in the nascent polyubiquitin chain. These polyubiquitin chains are recognized by the 19S regulatory complex of the 26S proteasome and the proteins to which they are conjugated are degraded [22].

2.2. Type I ubiquitin-like proteins

The first class of ubiquitin family proteins, the type I ubiquitin-like proteins, are similar to ubiquitin: they can either exist freely or be covalently attached to other proteins via an enzymatic cascade (Fig. 1A). Like ubiquitin, they are expressed as fusion products, allowing several moieties to be expressed during each transcription/translation cycle. The fusion proteins are subsequently separated into functional molecules by isopeptidase cleavage at the C-terminus, where there is usually a diglycine motif [23–26].

SUMO-1 (Small Ubiquitin-related MODifier) is a much studied type I ubiquitin family protein. It is conserved from yeast to mammals and its sequence is 18% identical to that of ubiquitin. The list of substrates for SUMO-1 is growing steadily and the effects of SUMO-1 conjugation are diverse. Sumoylation may affect the intracellular localization of the proteins to which they are conjugated. For example, the sumoylation of RanGAP1 targets it to the nuclear pore complex [27] and the attachment of SUMO-1 to the PML protein (identified as a fusion protein in promyelocytic leukemia patients) is crucial for its localization to nuclear bodies [28]. The conjugation of SUMO-1 to transcription factors may regulate their activity, as has been shown for c-Jun, p53, and c-Myb [29–31]. Sumoylation may also protect proteins from degradation, as it does for Ls-Bax [32]. There is evidence for cross-talk between the ubiquitination and sumoylation machinery [14], the mechanism and functional significance of which are still unclear. Interestingly, increased levels of sumoylated proteins have been found to be associated with polyglutamine diseases such as Huntington’s disease [33].

---

![Fig. 1. Schematic diagram illustrating the structural domains within several type I (A) and type II (B) ubiquitin family members. Proteins included are NEDD8; SUMO1, small ubiquitin-related modifier-1; FUBI, failure of ureteric bud invasion protein; UCRP, ubiquitin cross-reactive protein precursor; hHR23a, human homolog of Rad23-a; hHR23b, human homolog of Rad23-b; hPLIC-1, human proteasome ligase interaction component-1; hPLIC-2, human proteasome ligase interaction component-2; ubiquitin-3; A1Up, ataxin-1 ubiquitin-like interacting protein; NUB1, NEDD8 ultimate buster-1; BAG1, BCL-2 binding atanogene-1 protein; Parkin; ElonginB; HERP, homocysteine-responsive endoplasmic reticulum-resident ubiquitin-like domain member 1 protein; OASL, 59-kDa 2-5'-oligoadenylate synthetase like protein; Gdx, ubiquitin-like protein from chromosome Xq28; U7I3, ubiquitin conjugating enzyme 7 interacting protein 3; S3A1, splicing factor 3 subunit 1; BAT3, HLA-B-associated transcript 3; Uch14, ubiquitin carboxyl-terminal hydrolase 14. Ubiquitin-like and ubiquitin-associated domains are represented as UBL and UBA, respectively. Other identified functional domains within these proteins are also included as BAG, bcl-2-associated atanogene domain; COL, collagen-like domain; IBR, in between ring finger domains; PAP, polyA-related domain; ProR, proline-rich region; SYNTH, 2-5'-oligoadenylate synthetase N-terminal region; SWAP, suppressor-of-white-apricot splicing regulator domain; UCH-1, ubiquitin carboxyl-terminal hydrolase family 1; UCH-2, ubiquitin carboxyl-terminal hydrolase family 2; XPC, XPC-binding domain; ZnF, zinc finger domain.](image-url)
Nedd8, also known as RUB (Related to Ubiquitin) in yeast, is a type I ubiquitin family member that functions in targeted protein degradation. The Nedd8 conjugation pathway is conserved and essential in many eukaryotes, including Schizosaccharomyces cerevisiae, Arabidopsis, and humans, but notably not in Saccharomyces cerevisiae (S. cerevisiae) [34]. The only known substrates for Nedd8 are cullins, which are a key component of the SCF ubiquitin ligase complexes. Neddylation of the cullins is important in the regulation of SCF function. For example, Nedd8 conjugation to Cul-1 activates ubiquitin-mediated proteolysis of p27 and of IκBα [35–39]. In Arabidopsis, either deletion or overexpression of Nedd8 conjugation pathway components impairs SCF function. Therefore, regulation of neddylation is crucial for proper SCF function [40,41].

SUMO and Nedd8 are two of the best characterized type I ubiquitin family members. Despite their similarity to ubiquitin, they play entirely different roles in the cell. Indeed, the posttranslational modification of proteins by type I ubiquitin family members has been shown to have a wide variety of biological consequences, including regulation of subcellular localization, regulation of protein/protein interactions, and protection from degradation.

2.3. Type II ubiquitin-like domain proteins

Type II ubiquitin-like domain proteins constitute the second group of ubiquitin family members. They are defined by the presence of ubiquitin-like domains within the context of a larger protein. These ubiquitin-like domains lack the C-terminal diglycine motif and are not cleaved or ligated to other proteins (Fig. 1B).

The S. cerevisiae protein Rad23 and its two human homologs, hHR23a and hHR23b, are type II ubiquitin-like domain proteins, with a ubiquitin-like domain at their N-terminus and two ubiquitin-associated domains. Their best-characterized function is in DNA repair, particularly in nucleotide excision repair [42] and in base excision repair [43]. In S. cerevisiae, Rad23 has also been implicated in spindle pole body duplication and cell cycle progression [44–46]. Overexpression of Rad23 suppresses the toxicity caused by overexpression of N-end rule pathway components [47], and both the yeast and human homologs have been shown to interact with proteasomal subunits as well as with ubiquitin itself [12,48–51]. These data suggest an additional mechanistic role for Rad23/hHR23 homologs in the ubiquitin–proteasome pathway.

Similar observations have been noted for the S. cerevisiae protein Dsk2 and its human homologs hPLIC-1, hPLIC-2, and ubiquitin. They are also type II ubiquitin-like domain proteins with a ubiquitin-like domain at the N-terminus and a ubiquitin-associated domain at the C-terminus. Like Rad23/hHR23, the three human homologs have been shown to interact with the proteasome as well as with ubiquitin [48,51–53]. They have also been shown to bind the E3 ubiquitin ligase E6AP [52,54]. These interactions suggest that the PLIC proteins are functionally linked to the ubiquitin–proteasome pathway, which is further supported by reports that they regulate the stability of the GABA_A receptor and of presenilin [55,56]. Regulation of the GABA_A receptor is important for inhibitory neurotransmission and mutations in presenilin are associated with early-onset Alzheimer’s disease, thereby implicating the PLIC proteins in proper nervous system function. In addition to its role in ubiquitin-mediated protein degradation, Dsk2 has been implicated in spindle pole body duplication in S. cerevisiae [44].

The contribution of another type II ubiquitin-like protein, parkin, to the pathogenesis of neurodegenerative disease has been better defined than that of the PLIC proteins. Mutations in parkin are linked to autosomal recessive inherited juvenile parkinsonism. Parkinson’s disease is characterized by the loss of dopaminergic neurons in the substantia nigra and by the presence of Lewy bodies, which are cytoplasmic inclusions that contain ubiquitin. Defects in the proteasome are linked to Parkinson’s disease, and inhibition of the proteasome can cause the neuronal apoptosis and inclusion formation that are the hallmarks of this disease [57,58]. In fact, parkin is itself an E3 ubiquitin ligase, whose substrates include the Pael receptor, CDCrel-1, O-glycosylated alpha-synuclein, parkin-associated endothelin-like cell receptor, and synphilin. Accumulation of certain substrates, such as the Pael receptor and synuclein, is also associated with neurodegeneration [59].

There are many other type II ubiquitin family members that cannot be discussed here due to space constraints. They play essential roles in diverse functional pathways, including DNA repair [42], apoptosis [60–62], protein folding [63–66], and signaling [67]. As described above, however, there is increasing evidence pointing to a role for some type II ubiquitin family proteins in the ubiquitin–proteasome pathway. The mechanism by which they do so is presently the subject of intense study in several laboratories.

2.4. Interactions between type II ubiquitin-like domain proteins and the proteasome

Various type II ubiquitin family members have been found to interact directly with the proteasome. Besides Rad23/hHR23 and Dsk2/PLIC, the list of such proteins includes the chaperone cofactor BAG-1 and the Nedd8-interacting protein NUB1 [68]. All of these, except for BAG-1, have been shown to bind the S5a regulatory subunit of the proteasome. S5a is found free in the cell and is also part of the 19S regulatory particle, where it is located between the lid and base subcomplex. HHR23a and hPLIC-2 have been shown to interact with S5a via their ubiquitin-like domains [68].
Recent reports have identified other proteasomal subunits that can also bind ubiquitin or ubiquitin-like domains. The AAA ATPase S6' (also known as Rpt5 in yeast) is found in the base of the proteasome, within the 19S regulatory particle, and has been shown to interact with polyubiquitin chains only when it is within the proteasomal complex [69]. As an ATPase, S6’ might provide the link between substrate recognition by polyubiquitin chain binding and the subsequent unfolding of the substrate that must occur in preparation for its translocation into the proteolytic chamber of the 20S proteasome core [69].

Studies in yeast recently revealed that two additional components of the base subcomplex, Rpn1 and Rpn2, bind ubiquitin-like domains. These two proteins are non-ATPase subunits whose human homologs are S2 and S1, respectively. One report demonstrated that Rpn1 alone is capable of direct interaction with the ubiquitin-like domains of Rad23 and Dsk2 [50]. Based on data from cross-linking experiments, another research group proposed that these two ubiquitin-like domain proteins each bind an Rpn1/Rpn2 protein complex within the proteasome [70].

3. Structural comparisons of ubiquitin family proteins

3.1. Ubiquitin has a highly conserved structure

The structure of ubiquitin was revealed by X-ray crystallography [71,72]. The ubiquitin fold forms quickly and cooperatively [73] around a hydrophobic core and is highly stable. It consists of five β-strands, an α-helix of 3.5 turns and a 3_10-helical turn (Fig. 2A) [71,72]. The first and last β-strands (β1 and β5) are oriented parallel to each other. All other neighboring β-strands are anti-parallel.

A stereoview of the ubiquitin structure is presented in Fig. 3, where certain residues have been highlighted in red. These residues correspond to those that are conserved in over 90% of ubiquitin family members. They are also highlighted in the alignment shown in Fig. 4, which compares type I ubiquitin-like proteins, ubiquitin-like domains from type II ubiquitin family proteins, and ubiquitin. As seen in Fig. 3, these conserved residues are directed towards the protein interior and are mostly hydrophobic. A conserved salt bridge between ubiquitin residues K27 and D52 (colored blue in Fig. 3) connects the long α-helix with the loop following β4. The conserved residues and salt bridge are important for the stabilization of the ubiquitin fold.

3.2. Ubiquitin family members have a similar protein fold but different electrostatic surface potentials

The structures of several ubiquitin family members have already been elucidated and are represented in Fig. 2. The solution structures of SUMO-1 and of the ubiquitin-like domain of hPLIC-2 have been solved by NMR spectroscopy [74,75]. The structure of the hPLIC-2 ubiquitin-like domain was used to build a model of this domain in hHR23a, by creating an alignment based on NMR data of the regular secondary structural elements as well as hydrogen bonding patterns between the β-strands [75].

As displayed in Fig. 2, SUMO-1 and the ubiquitin-like domain from hPLIC-2 have similar structural topology to ubiquitin. A detailed inspection, however, reveals that the orientation of β3 and β5 relative to α1 in the hPLIC-2 ubiquitin-like domain is more similar to that of ubiquitin than to that of SUMO-1. A quantitative analysis of the backbone architecture of these three proteins supports this observation, as the root mean square deviation to the ubiquitin-like domain of hPLIC-2 for backbone atoms of residues situated in helices or β-strands is 2.0 Å for ubiquitin and 2.8 Å for SUMO-1.

Although the ubiquitin-like domains of hPLIC-2, ubiquitin, and SUMO-1 all have similar folds, their electrostatic surface potentials differ significantly. Fig. 5 displays the electrostatic potential mapped onto a surface diagram for the ubiquitin-like domains of hHR23a (Fig. 5A) and hPLIC-2 (Fig. 5B), ubiquitin (Fig. 5C) and SUMO-1 (Fig. 5D). The
orientation displayed on the left in this figure is rotated by 180° relative to that on the right. Basic, acidic, and hydrophobic regions are indicated in blue, red, and white, respectively. As seen in the orientation on the left, SUMO-1 is dramatically more acidic than both ubiquitin and type II ubiquitin-like domains. The face in question includes E67 and E89 in SUMO-1 and, as discussed below, is implicated in binding S5a.

4. Definition of the S5a-binding surfaces on ubiquitin family proteins

4.1. Using NMR spectroscopy to map protein interaction surfaces

Use of NMR spectroscopy allows structural insight into the different binding properties of ubiquitin and of the
Fig. 5. Comparison of the electrostatic surface potential of ubiquitin family proteins. The electrostatic potential is mapped onto the surface diagrams of the ubiquitin-like domains of hHR23a (A) and hPLIC-2 (B), ubiquitin (C), and SUMO-1 (D). The orientation of the figures on the left is identical to that presented in Fig. 2 whereas that on the right is rotated by 180°. The parameters used to generate each of these surface potentials are −42 to −21 and 42 to 21 kT. This figure was generated with GRASP [97].
ubiquitin-like domains with S5a, and enables the S5a-binding surface of the ubiquitin-fold to be characterized. It is a powerful tool for the mapping of protein interaction surfaces. Two-dimensional $[^{1}H,^{15}N]$-HSQC spectra acquired on $^{15}N$-labeled proteins display resonance cross-peaks for all amide nitrogen and proton atoms and therefore allow all residues (excluding proline) to be simultaneously observed. The chemical shift at which atoms appear in NMR spectra is sensitive to their local chemical environment. Atoms at interaction surfaces thus tend to experience the greatest changes in their environment upon ligand binding, assuming that there are no structural changes resulting from the interaction. The chemical shift perturbations that these atoms undergo can be monitored by $[^{1}H,^{15}N]$-HSQC spectra and can thus be used to map binding surfaces [75,76].

We have used the $[^{1}H,^{15}N]$-HSQC spectra of $^{15}N$-labeled ubiquitin-like domains of hPLIC-2 or hHR23a alone and with equimolar ratios of S5a to study their interaction surfaces with S5a (Fig. 6). Comparison of the $[^{1}H,^{15}N]$-HSQC spectra acquired either alone or in complex with S5a reveals the disappearance of signal that originates from resonance broadening, which is in turn due to the increased rotational correlation time of the complex and chemical exchange from switching between the free and bound states. The increased rotational correlation time is caused by the increased molecular weight of the complex [77]. For example, in the case of the hPLIC-2 ubiquitin-like domain, the molecular weight is increased from 14 kDa in the free form to 55 kDa when complexed with S5a.

In the $[^{1}H,^{15}N]$-HSQC spectrum acquired on hPLIC-2 ubiquitin-like domain in complex with S5a, some strong
crosspeaks are observable. These originate from the N-terminal and C-terminal regions of our hPLIC-2 construct and include the first 30 residues of hPLIC-2 protein (Fig. 1) and 22 residues following the ubiquitin-like domain that were added to facilitate purification. All of these residues are randomly coiled, whether the hPLIC-2 construct was alone or complexed with S5a.

Although residues in the ubiquitin-like domains are severely broadened, a detailed comparison of the \([1H,15N]\)-HSQC spectra of the ubiquitin-like domains of hPLIC-2 and of hHR23a reveals that some resonances experienced chemical shift changes upon addition of S5a. At equimolar concentration with S5a, all of the crosspeaks could be observed in 1D traces along the nitrogen and proton dimensions. By comparing the 1D traces in both dimensions of \([1H,15N]\)-HSQC spectra acquired in the absence or presence of an equimolar concentration of S5a, we were able to measure the change in the amide nitrogen and proton chemical shift.

The chemical shift changes were then plotted as shown in Fig. 7 according to Eq. (1),

\[
\sqrt{(0.2\delta_N^2 + \delta_H^2)}
\]

where \(\delta_N\) and \(\delta_H\) represent the changes in nitrogen and proton chemical shifts (in ppm), respectively, upon S5a addition. These data are much easier to interpret in the context of the three-dimensional structure and were therefore mapped onto the surface diagrams of the respective proteins as illustrated in Fig. 8. In this figure, residues experiencing greatest perturbation in their amide nitrogen and proton chemical shifts, indicating the highest

---

Fig. 7. NMR chemical shift perturbation data for the ubiquitin-like domains of hPLIC-2 ubiquitin-like domain and hHR23a. Data for the ubiquitin-like domains of hPLIC-2 (A) and hHR23a (B) are shown. These data are displayed for each residue according to Eq. (1), \(\sqrt{(0.2\delta_N^2 + \delta_H^2)}\), where \(\delta_N\) and \(\delta_H\) represent the change in nitrogen and proton chemical shifts (in ppm) upon S5a addition, respectively. Residues that were excluded from this analysis include P40, P49, N51, and F57 of the hPLIC-2 ubiquitin-like domain and P21, P42, and P60 of the hHR23a ubiquitin-like domain.
involvement in the interaction, are colored dark blue. The residues in white are those that do not experience changes in the resonance frequencies of their amide nitrogen and proton atoms, and which are thus not at the S5a contact surface. Residues, including proline, whose chemical shift perturbation could not be measured, are indicated in red.

The S5a contact surface revealed by this technique is localized to an analogous surface on the hHR23a and hPLIC-2 ubiquitin-like domains, and predominantly involves residues located in the third, fourth, and fifth β-strands (Fig. 8). Reference to Fig. 5 reveals that the residues at the S5a contact surface of each of these ubiquitin-like domains are hydrophobic with peripheral basic residues.
Furthermore, titration experiments indicate that the ubiquitin-like domains of hPLIC-2 and hHR23a each bind S5a with a 1:1 stoichiometry [75].

We used the above technique to study the interaction between mono-ubiquitin and S5a. Competition experiments between mono-ubiquitin and polyubiquitin chains suggest that polyubiquitin chains preferentially bind the 26S proteasome [22]. From our NMR studies, we found that mono-ubiquitin binds S5a and that the contact surface on ubiquitin is analogous to those of the ubiquitin-like domains of the type II ubiquitin family proteins, hHR23a and hPLIC-2 [75] (Fig. 8C). This surface includes three residues (L8, I44, and V70) that had been previously identified by mutagenesis studies as important for proteasome-binding [22,78,79]. Contact with I44 and V70 would be structurally difficult without contact with R42. Accordingly, we found that the positively charged residues R42 and Q49 are also within the S5a contact surface.

4.2. Comparison of S5a-binding surfaces on ubiquitin family proteins

We were also able to compare the relative S5a-binding affinity of ubiquitin to the ubiquitin-like domains of hPLIC-2 and hHR23a. This analysis was done by comparing the spectral changes of each of these proteins at identical molar ratios with S5a. Ubiquitin can bind S5a at two different segments, only one of which can bind the ubiquitin-like domains of hPLIC-2 and hHR23a [80–82]. Therefore, the ubiquitin-like domains should be saturated with S5a when they are at equimolar concentration whereas ubiquitin binding is expected to be complete when ubiquitin is in twofold excess over S5a.

We indeed observed complete binding at equimolar concentration with S5a for the ubiquitin-like domains, which indicates that the complexes formed by these proteins are specific. In contrast, saturation of ubiquitin required a threefold molar excess of S5a, indicating that ubiquitin complexes weakly with S5a and dissociates easily [75]. Examination of the electrostatic surface potential of ubiquitin (Fig. 5C) indicates that the weaker binding between ubiquitin and S5a is probably due to the charged and polar residues on the relevant surface of ubiquitin, including R42, Q49 and the nearby D52.

To test whether there are other binding sites for the proteasome on the hPLIC-2 ubiquitin-like domain, we performed site-directed mutagenesis of a few residues in the hPLIC-2 ubiquitin-like domain that had been identified as important for S5a-binding, namely, H99A, I75A and A77S [75]. The resulting mutant hPLIC-2 ubiquitin-like domain could bind neither S5a nor the 26S proteasome. Therefore, the surface of the hPLIC-2 ubiquitin-like domain that is involved in binding S5a is also required for its interaction with the proteasome. It is possible that the ability of the hPLIC-2 ubiquitin-like domain to bind S5a is crucial for its interaction with the proteasome. Alternatively, if other proteasomal subunits bind this ubiquitin-like domain, they do so at the same surface as S5a.

4.3. Using structure-based homology of protein surfaces to predict proteasome subunit interaction

Subtle differences in electrostatic charge or hydrophobicity may underlie significant alterations in function, as exemplified by the decreased binding affinity of ubiquitin for S5a that arises from the presence of the charged and polar residues on its S5a-binding surface.

The type I ubiquitin-like protein SUMO-1 has a very different electrostatic surface from ubiquitin as well as from the ubiquitin-like domains of either hPLIC-2 or hHR23a (Fig. 5). The surface in SUMO-1 that is analogous to the S5a-binding surfaces of the hPLIC-2 and hHR23a ubiquitin-like domains and of ubiquitin is substantially more negatively charged. Therefore, we expected that SUMO-1 would not bind S5a. This prediction was confirmed by titration experiments on 15N-labeled SUMO-1 with unlabeled S5a, where no perturbations of the SUMO-1 spectra were observed in the presence of S5a [75]. Although SUMO-1 shares a fold very similar to that of the other proteins in this study, it has evolved very different surface properties, presumably to perform different functions not directly involving proteolysis or binding to the proteasome.

We have extended our structural analysis of type II ubiquitin family proteins to the chaperone cofactor BAG-1, which has also been reported to bind the 26S proteasome [83]. BAG-1 binds the E3 ubiquitin ligase CHIP, and the BAG-1/CHIP complex accepts protein substrates from the 70-kDa heat shock molecular chaperones Hsc/Hsp70 for ubiquitin-mediated protein degradation [84]. We generated a model structure of the BAG-1 ubiquitin-like domain from the experimentally determined structure of the ubiquitin-like domain of hPLIC-2, based on the alignment shown in Fig. 4. The secondary structural diagrams of both ubiquitin-like domains are thus the same. The BAG-1 ubiquitin-like domain surface potential at the β-sheet face is similar to that in the ubiquitin-like domains of both hPLIC-2 and hHR23a (Fig. 9), suggesting that this surface of BAG-1 will also be used to contact the S5a subunit of the proteasome.

5. Functional implications of the interactions between type II ubiquitin family proteins and the proteasome

5.1. The role of S5a in ubiquitin-mediated protein degradation

S5a binds polyubiquitin chains [85,86] as well as a subset of type II ubiquitin family members, including hHR23, PLIC, and NUB1. HHR23 and PLIC additionally bind ubiquitin [53,87] and NUB1 binds NEDD8 [87]. It has therefore been proposed that these type II ubiquitin family
proteins could serve as linkers between ubiquitin/NEDD8-conjugated proteins and S5a. This hypothesis is not relevant to *S. cerevisiae*, where the yeast homolog Rpn10 lacks the second ubiquitin-binding site that is required for interaction with type II ubiquitin family members [50]. Instead, the Rpn1 and Pre2 proteasomal subunits have been found to recognize Dsk2 and Rad23 in yeast [50,53]. In fact, deletion of the Rpn10 from *S. cerevisiae* does not result in any significant defect in ubiquitin-mediated proteolysis, suggesting that it is not essential for recognition of polyubiquitinated substrates [88]. Other proteasomal subunits may also bind polyubiquitin chains, and indeed, the S6' ATPase in the proteasomal base has recently been identified as a ubiquitin receptor within the proteasome [69]. The ability of the proteasome to bind polyubiquitin chains, however, is decreased in the absence of Rpn10/S5a [50], indicating that this subunit may be important for some ubiquitin-binding function of the proteasome.

Most of the Rpn10/S5a within a cell is free of the proteasome, and only a small proportion is found associated with the proteasome at steady-state levels. Interestingly, free S5a, but not proteasome-associated S5a, has been shown to bind polyubiquitin strongly [69]. Also, deletion of both the RAD23 and RPN10 genes in *S. cerevisiae* results in a synthetic phenotype, with pleiotropic defects that include increased levels of polyubiquitinated proteins [89]. This accumulation is also observed in a mutant strain with a double deletion of RAD23 and DSK2, as well as in a triple deletion mutant of RAD23, DSK2 and RPN10 [90]. Therefore, Rad23, Dsk2 and Rpn10 may have yet undefined but overlapping functions in ubiquitin-mediated protein degradation.

Given the biological necessity of targeted protein degradation, it would not be surprising if there were redundancy in polyubiquitin chain recognition by the proteasome. It is also clear from the studies mentioned above that S5a plays an important role in this process. Nonetheless, S5a may not be the major polyubiquitin chain receptor within the proteasome and its interaction with polyubiquitin may serve other purposes. Furthermore, the ability of free S5a to bind polyubiquitin indicates that it may play an additional role in ubiquitin-mediated degradation, albeit one that is still not mechanistically defined.

5.2. The role of Rad23/hHR23 proteins in the ubiquitin–proteasome pathway

Various type II ubiquitin family proteins are believed to play a role in the ubiquitin–proteasome pathway. They include parkin, BAG-1, NUB1, Rad23/hHR23, and Dsk2/PLIC. Such connections have caused new scientific questions to emerge as they implicate proteasome activity in novel functional pathways. This discussion will focus on Rad23/hHR23, since it is one of the more extensively studied of these proteins.

Rad23/hHR23 has a well-characterized function in nucleotide excision repair but much data currently support an additional role for it in the ubiquitin–proteasome pathway. However, this functional role of Rad23/hHR23 remains highly controversial. Early evidence suggested that Rad23/hHR23 interaction with the proteasome was essential for nucleotide excision repair as deletion of the ubiquitin-like domain, which is required for proteasome binding, causes increased UV-sensitivity in cell lines [12]. Later work provided evidence that the 19S regulatory particle of the proteasome interacts with Rad23 to play a nonproteolytic role in nucleotide excision repair [91]. This hypothesis is based in part on the observation that defects in the 19S particle cause defective nucleotide excision repair, whereas those in the 20S core, where proteolysis occurs, do not [91].

Other data indicate that Rad23/hHR23 may function in ubiquitin-mediated protein degradation independent of nucleotide excision repair. In fact, there is evidence that it can both inhibit and promote ubiquitin-mediated protein degradation. The inhibitory role is noticeable in studies where the protein expression level is high. More specifi-
cally, high levels of Rad23 have been shown to inhibit the formation of polyubiquitin chains in vitro and to stabilize proteolytic substrates in vivo [49,92]. This inhibition was found to be dependent on the presence of the ubiquitin-associated domains of Rad23/hHR23 and their ability to interact with ubiquitin [49]. It has therefore been suggested that Rad23 is important for the translocation of ubiquitinated proteins to the proteasome [49]. Notably, the yeast homolog Rpn10 also appears to play a role in this process. In the presence of high levels of Rad23, the interaction between ubiquitinated proteins and the proteasome is detectable and Rpn10-dependent [93]. It is possible that this role of Rad233 has implications for nucleotide excision repair as its interaction with ubiquitinated proteins increases after DNA damage [93].

In addition to its inhibitory role described above, Rad23/hHR23 has also been shown to promote the degradation of certain proteins. These two seemingly contradictory roles might be substrate-dependent. This hypothesis is exemplified by comparing the effects of Rad23/hHR23 overexpression on p53 and Rad4. Such overexpression results in decreased steady-state levels and transcriptional activity of p53 [94]. In contrast, Rad23 overexpression inhibits the ubiquitination of the nucleotide excision repair factor Rad4, which forms a complex with Rad23 to recognize damaged DNA [95]. This inhibition is associated with an increased rate of DNA repair, suggesting that regulation of Rad4 levels by Rad23 is important for proper nucleotide excision repair function [95].

5.3. A model of how hHR23 may function in the recruitment of ubiquitinated proteins to the proteasome

Proper Rad23/hHR23 function requires both the N-terminal ubiquitin-like domain and the ubiquitin-associated domains [93]. As described above, the ubiquitin-like domain mediates binding to S5a [68] whereas the ubiquitin-associated domains interact with polyubiquitin chains [49,51,87]. Therefore, Rad23/hHR23 can and does facilitate the formation of ternary complexes with the proteasome and ubiquitinated substrates [93]. Also, deletion experiments in yeast have shown that Rad23/hHR23 and Rpn10/S5a play overlapping roles in ubiquitin-mediated protein degradation [89,90].

Data from the genetic and biochemical experiments described above, together with biophysical data from NMR spectroscopy experiments, suggest a model of how hHR23a and S5a function together to recruit ubiquitinated proteins to the proteasome. S5a has higher affinity for the ubiquitin-like domain of hHR23a than for ubiquitin, which readily binds the C-terminal ubiquitin-associated domain of hHR23a [98]. As illustrated in Fig. 10, in the ternary complex, the polyubiquitin chain bound to the ubiquitin-associated domain of hHR23a could be in close proximity to the ubiquitin-binding segments of S5a, which interacts with the ubiquitin-like domain of hHR23a. Polyubiquitin chains could thus be transferred from hHR23a to S5a, enabling them to bind S5a with little entropic penalty. This mechanism may represent one of multiple strategies for the regulation of ubiquitin-mediated protein degradation.

Acknowledgements

We are grateful to Dr. Patton Fast and University of Minnesota Supercomputing Institute for providing resources necessary for making figures in this review.

References


