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Ubiquitin-free routes into the proteasome

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Abstract. The majority of proteasome substrates identified to date are marked for degradation by polyubiquitinylation. Exceptions to this principle, however, are well documented and can help us understand the process proteasomes use to recognize their substrates. Examples include ornithine decarboxylase, p21/Cip1, TCRα, IκBα, c-Jun, calmodulin and thymidylate synthase. Degradation of these proteins can be completely ubiquitin-independent or coexist with ubiquitin-dependent pathways. Uncoupling degradation from ubiquitin modification may reflect the evolutionary conservation of mechanisms optimized for highly specialized regulatory functions.

Key words. Proteasome; proteolysis; ubiquitin-independent; ornithine decarboxylase; antizyme; IκBα; c-Jun.

Introduction

Proteasomes usually demand a polyubiquitin tag as the price of admission, but a select group of labile proteins has found a way to elude this inspection. Some of the evaders have been identified, but only systematic investigation can uncover the full scope of this trickery. The ubiquitin-proteasome paradigm of destruction has been affirmed from beginning (ubiquitin activation) to end (proteasomal engulfment and degradation of substrate), and explains much about how cells dispose of unwanted proteins. However, because this prevailing paradigm provides a ready method for searching out and identifying novel substrates, reliance on this approach might cause us to underestimate the global frequency of proteasomal substrates that do not use ubiquitin tagging. Unbiased screening methods may therefore be needed to determine the true prevalence of alternate rites (and rights) of passage. Beyond the question of their prevalence, ubiquitin-independent cases can be used to test and extend our understanding of degradation mechanisms. The prevalence of proteasome substrates that do not require ubiquitin has been considered previously [1–3]. We will discuss here the following questions: What substrates have been identified that do not depend on ubiquitin and how are they processed in its absence? What characteristics of proteasomal degradation differ for ubiquitin-dependent and independent substrates? To what extent are they similar? What general conclusions can emerge from such a comparison?

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The route to immolation

A brief description of the relevant aspects of proteasome architecture will delineate the challenges encountered by a substrate on its pathway to destruction. Compartmentalized proteases are found in all three domains of life: archea, prokaryotes and eukaryotes [4]. In eukaryotic cells, the 26S proteasome consists of two dissociable assemblies, a cylinder-shaped 20S core plus 19S regulatory complexes that cap one or both ends. The 19S assemblies mediate functions associated with substrate recognition and insertion. The catalytic sites that convert protein substrates to peptides lie in the interior of the 20S cylinder. The cylinder consists of a stack of four rings, each ring with seven proteins. The overall configuration is $\alpha7$: $\beta7$: $\beta7$: $\alpha7$, with distal α rings flanking an internal pair of β rings. The α rings surround a central axial pore, which is occluded by the amino terminal extensions of some of the α subunits; together these form a tight mesh [5]. Entry to the catalytic chamber requires that this meshwork be disentangled by association with the regulatory complex [6]. The dimensions of the open pore dictate that a protein threading the pore must be in an unfolded configuration (recent data suggest, however, that more than one polypeptide chain may pass at once [7], supporting mod-

els that allow endoproteolytic cleavage [8, 9]). Once initiated, entry usually continues to completion, reducing the entire protein to peptides. Folding and unfolding activity is intrinsic to the 19S assembly and is promoted by ATP hydrolysis [10]. The 19S assembly is further subdivided into two subassemblies [11]: the 'base', proximal to the α rings, and the distal 'lid'. The base contains six distinct ATPase subunits that form a ring and are associated with two additional non-ATPase proteins. The base, juxtaposed to the α7 ring, is well positioned to initiate threading of unfolded substrates into the α heptamer pore, possibly through the axis of its own hetero-hexameric ATPase ring. Two of the ATPase proteins have been associated with particular functions: the Rpt2 ATPase participates in opening the α ring pore [6], and the Rpt5 ATPase is involved in recognizing polyubiquitylated proteins [12]. Hinged to the base is a second subassembly termed the lid; base and lid together form the 19S regulator assembly. The lid subassembly contains 10–12 distinct proteins, each present in equal number, whose roles are more obscure. Lid and base may be dissociated in vitro by high salt concentrations or by certain mutations of lid constituents. The lid is essential for proteolysis of ubiquitylated substrates – the 20S proteasome plus base can degrade a protein that has been artificially denatured, but does not degrade a polyubiquitylated substrate. A specific protein within the lid is required for cleaving ubiquitin chains from the proteasome substrate [13, 14], a process that must take place at or near the time of substrate insertion into the 20S cylinder. In addition to the proteasomal proteins, which are present at fixed stoichiometric ratios, a significant number of additional proteins, most of unknown function, are found associated with the 26S proteasome or 19S regulatory assembly when these are isolated under gentle conditions [15].

Degradation and polyubiquitylation uncoupled

The standard model requires that polyubiquitylation and degradation not merely be coincident but that the first cause the second. For some proteins these processes coincide, but can be decoupled experimentally, implying that degradation does not require the modification. These include p21/Cip1, the TCRα subunit of the T cell receptor, IκBα, c-Jun and calmodulin.

The Cdk inhibitor p21/ WAf1/Cip1 is a labile protein that is degraded by the proteasome and is ubiquitylated in vivo. Nonetheless, a variety of experimental manipulations designed to impair ubiquitin modification of p21 did not stabilize the protein [16]. First, expression of a mutant form of ubiquitin without lysines (incorporation of which terminates ubiquitin chain extension) did not alter p21 turnover, but did stabilize a positive control protein that depends on ubiquitin conjugation for its turnover. Second, all six lysines in p21 were mutated to arginines, depriving p21 of ubiquitylation targets. No ubiquitin chains were detected in association with the lysine-less p21, but were readily seen with the wild-type protein. Despite this, the lysine-less mutant and wild-type p21 had similar turnover. This was true using a variety of conditions of expression and measures of turnover, including pulse chase analysis and accumulation in response to proteasome-specific inhibitors. Lastly, an attempt was made to rule out an amino-terminal free amine group as a potential target of ubiquitin chain addition, as has been described for the MyoD protein [17]. N-terminal fusion of epitopes which stabilize MyoD and other proteins known to be degraded through N-terminal ubiquitylation failed to stabilize either wild-type or lysine-less p21. This last experiment cannot fully exclude ubiquitin modification at a free N-terminal amine, nor exotic chemistry that postulates as yet undocumented sites for ubiquitin addition. (Late note: very recent evidence [18] shows that p21 can be polyubiquitylated at the free amine of its N-terminal methionine, and that this suffices for its degradation.) However, these experiments demonstrated that experimental maneuvers that strongly impaired ubiquitin modification had no effect on turnover. The case for ubiquitin-independent proteasomal degradation of p21 could be further strengthened by in vivo evidence in animal cells using conditional defects in the ubiquitin system and by in vitro experiments with purified constituents. Nonetheless, p21 hoists a warning flag – coincidence of ubiquitylation with proteasome-mediated turnover does not establish causality.

For several other proteins, ubiquitin chain modification is associated with turnover, but the two can be experimentally dissociated. The T-cell-antigen receptor chain is a protein complex that assembles in the endoplasmic reticulum (ER) and is ultimately displayed on the cell surface. The TCRα subunit, if expressed without the other proteins of the complex, cannot assemble and is retro-transported from ER back to cytoplasm. There it is ubiquitylated and degraded by the proteasome. A lysine-less TCRα was created, and its degradation properties compared to those of wild-type TCRα. Both were labile, and both accumulated in cells treated with a proteasome inhibitor [19]. Co-expression with TCRα of a catalytically inactive form of the mammalian E2 Ubc7 led to accumulation of TCRα and impaired its turnover [20], implying that Ubc7 is involved in this form of regulation. However, expression of the mutant Ubc7 caused similar accumulation of the lysine-less TCRα protein. Impaired E1 function and expression of chain-terminating ubiquitin mutants also impaired turnover of both the wild-type and lysine-less TCRα [21]. Evidently, a functional ubiquitation system promotes TCRα turnover, but that protein may not be its direct target. Again, an in vitro system reconstituting ER extraction, ubiquitylation and proteasome degradation events would help exclude N-terminal modification as a rescue

hypothesis. A similar lysine mutagenesis strategy was used to test the requirement for ubiquitin in the proteasomal degradation of IκBα, a protein that binds to and regulates NFκB function. Replacing all lysines with arginines in a region sufficient for unstimulated degradation had no effect on proteasome-dependent turnover [22].

In vitro analysis using purified proteasomes was used to investigate the requirement for ubiquitin in the turnover of c-Jun, a labile transcription factor. c-Jun is polyubiquitylated in vivo, and that modification is functionally important for stability: phosphorylation by MAP kinase impaired modification and stabilized the protein [23]. Nonetheless, recombinant c-Jun was degraded efficiently by purified 26S proteasomes without ubiquitin [24]. Calmodulin provides still another example of a protein for which ubiquitylation can be dissociated from degradation by the proteasome. Prolonged incubation of calmodulin causes changes in the conformation of its Ca^{++} binding region, the result of formation of isoaspartyl residues. Both native and 'aged' calmodulin were found to be proteasome substrates, but the extent of proteolysis in HeLa cell extracts was not correlated with the extent of ubiquitylation [25]. In a purified ubiquitin-free system, the addition of Ca^{++} strongly inhibited turnover of the native protein, but had little effect on 'aged' calmodulin turnover. In this model of age-dependent protein damage, isoaspartyl residue formation leads to conformational alterations and inability to respond to a ligand that can induce a non-degradable conformation in the undamaged native protein.

Degradation without polyubiquitylation

The cases considered thus far are ones in which ubiquitin and turnover both make an appearance, and the question is how to untangle whether or under what circumstances the first causes the second. Ornithine decarboxylase (ODC) is different. Regulation of this enzyme depends primarily on turnover by the proteasome, a process whose rate is controlled by end products of the metabolic pathway initiated by ODC (reviewed in [26]). Yet ODC modification by ubiquitin does not occur, and can therefore play no part in this process. In a baroque series of events, ODC turnover is instead regulated by the protein antizyme. Polyamines are the downstream product of ODC. They are essential and ubiquitous in living cells, but become toxic if present at excessive levels. Polyamine accumulation stimulates antizyme production by a unique mechanism. The antizyme messenger RNA (mRNA) contains two overlapping reading frames that must be aligned by +1 translational frameshifting to produce a functional protein. Polyamines strongly stimulate this frameshifting, linking antizyme production to polyamine levels [27]. Antizyme acts in several ways to limit polyamine accumulation. It restricts polyamine uptake by cells through a poorly understood mechanism. Its primary action, however, is to bind to ODC, forming thereby an enzymatically inactive heterodimer. In addition to acting as a stoichiometric inhibitor, antizyme promotes the degradation of ODC by improving its affinity for the proteasome about eightfold [28]. One antizyme molecule can direct the destruction of many ODC molecules, for ODC is engulfed and consumed while antizyme (usually) slips away to strike again.

How does the proteasome recognize ODC? It does so by molecular mimicry. ODC/AZ and a model ubiquitylated protein cross-compete for degradation; even a naked polyubiquitin chain can competitively inhibit ODC turnover [28]. The ODC C terminus, its recognition element, must therefore engage the same binding site of the proteasome that recognizes ubiquitin conjugates. ODC is seemingly independent of ubiquitin, but in fact adapts ubiquitin-like camouflage. Adaptative mimicry is usually a way to confuse predators, but ODC uses it instead as a way to invite destruction.

Thymidylate synthase, required for the biosynthesis of deoxyTMP, was very recently found to be degraded by proteasomes without ubiquitin conjugation [29]. Like ODC, thymidylate synthase has an unstructured terminal region not required for enzymatic activity, in this case at the N terminus. Truncation of the initial six amino acids was shown to stabilize the protein. The biochemical role of this N-terminal region in proteasome interaction remains to be determined.

Substrates created or enhanced by experimental artifice

A recurrent theme that emerges from these studies is the importance of conformational flexibility in substrate recognition or processing by the proteasome. Even proteins that are recognized through a ubiquitin chain require unfolding for downstream processing. Those which are recognized without such a modification are globally loosely folded, e.g. p21/Cip1 [30], or contain a disordered domain which is necessary for interaction with the proteasome, e.g. ODC [31, 32]. The 19S proteasome regulator interacts with misfolded proteins with non-native conformations [33], a capacity that probably reflects this mode of substrate recognition. Chemical or physical denaturation has been used to create or enhance proteasome substrates. These findings augment our view of what is required for recognition and processing by the proteasome. Chemical denaturation of ovalbumin [1], ovomucoid [14] and lysozyme [34] enhances their proteolysis by the proteasome. Oxidation can even convert proteins into substrates that are utilized by the 20S proteasome in preference to the 26S proteasome (reviewed in [35]).

All roads converge, but where?

In general, substrates of the proteasome invoke shared capacities that include conditionality, association, denaturation, pore opening, signal clearance, entry, translocation and proteolysis. Physiologic substrates are often conditionally degraded, having half-lives that are longer or shorter, depending on changing or transient functional requirements of the cell. They must associate with the proteasome, thereby achieving a high local concentration. They must be unfolded, an active process for those that arrive in a folded state. The axial pore of the α heptamer ring must be cleared to open an entry path. The degradation tag, if it is to be preserved for further use (or to prevent it from sterically obstructing substrate entry), must be cleared and recycled. The unfolded substrate polypeptide must be inserted into the pore and subsequently made to complete the processive translocation process, resulting in the complete engulfment of the polypeptide chain and its proteolysis to peptides. These elements may be considered as steps that take place approximately sequentially as listed. In comparing the specific properties of ubiquitin and ubiquitin-independent substrates, these steps may be thought of as a checklist of similarities or differences. Because the overall process for the two classes of substrates must converge at some point in this sequence of events, differences seem more likely to emerge at earlier steps than at later steps.

The validity of this principle, the greater possibility of differences at earlier steps, is apparent from consideration of the first of these steps, conditionality. ODC offers the best-understood case of ubiquitin-free proteasomal degradation and its conditional control. Degradation of ODC is accelerated by polyamines, which act by augmenting antizyme. Antizyme is for ODC the functional equivalent of polyubiquitin, but the molecular particulars are radically different for the two marking systems. At what stage in the sequence of events do the mechanisms of antizyme-mediated and ubiquitin-mediated degradation converge? Our data [28] indicate convergence at the very next definable step, proteasomal association. ODC:AZ and polyubiquitin share a site of proteasomal recognition or, more accurately, refuse to share, as their binding is competitive. This finding together with the postulate of convergence implies that ODC and ubiquitylated substrates are processed by similar mechanisms at all steps after proteasomal association. However, such a conclusion follows only if degradation consists of a linear series of events, each one dependent on the preceding event and triggering the next. This is clearly too simple. (Things should be made as simple as possible – but no simpler. – *A. Einstein*). For example, tag clearance differs in the two cases: polyubiquitin must be removed by cleaving covalent bonds, an action dependent on an intrinsic proteasome enzymatic activity, while antizyme need only dissociate from ODC and drift away.

Ubiquitylation is versatile, specific and effective – why use something like antizyme?

Advocates of open standards computer software provide source code to all comers, thereby encouraging widespread access, flexibility and jointly shared development by a community of programmers and users [36]. As happened with e-mail communication, an open standards approach can result in a broadly adapted means of communication. The open standards approach competes in the marketplace with proprietary systems. These may offer special capacities to purchasers and subscribers, but, because they withhold coding detail from the public, can develop further only in the hands of their creator and may resist evolutionary change. A virtue and defect of proprietary systems is their limited interoperability with other communications systems. This is bad if you want to send e-mail to your friends, but good if you want to avoid sharing secrets with outsiders or avert unintended crosstalk. The ubiquitin system has been extensively exploited and extended by opportunistic evolutionary developers. Antizyme in this metaphor exemplifies a closely held proprietary system for control of polyamines, which can resist global perturbation of the ubiquitin system and does dependable work for its regular customers. Personal computers using Macintosh operating systems cannot be made victim to viruses directed against Windows-based PCs, and proprietary communications methods are immune to junk e-mail. Similarly, biology cannot easily make a mess of antizyme.

Antizyme is the central component of a specialized regulatory system devoted to polyamines. As a specialist, it need do only a few things well, and therefore does not require design complexity. Antizymes not only exert control, but also sense polyamine levels through their unique translational frameshifting mechanism. Because polyamines bind to RNA with high affinity, a sensing mechanism dependent on mRNA frameshifting may elegantly solve a bioengineering problem that cannot be handled by mere proteins, such as those involved in ubiquitylation.

In eukaryotes ranging from fission yeast to man, antizymes are present and do similar work. They are an ancient solution that has been retained deep into evolutionary time. Stephen Jay Gould has emphasized the ‘roles of contingency and chance in the history of life’ [37]; adaptations may be conserved not because they are uniquely optimal evolutionary solutions but merely because they arose in the past and continue to work. Polyamine regulation may have resisted updating to a more ‘modern’ system because antizyme adequately solves multiple design problems, including sensing and control of multiple functionally related effectors.

Proteins that find their way into the proteasome without showing a ubiquitin ticket have been stigmatized as a

quasi-criminal class [2]. As these become more familiar, we may learn to recognize their actions as a clever form of mimicry which falls within accepted behavioral norms.

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