

A Proteasome Howdunit: The Case of the Missing Signal

Minireview

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Traditionally, it has been thought that virtually all substrates of the eukaryotic 26S proteasome must be ubiquitinated as a prelude to their destruction. Only one clear exception to this rule—ornithine decarboxylase (ODC)—was known (Coffino, 1998). However, a recent report by Sheaff et al. (2000) demonstrates that the cyclin-dependent kinase (Cdk) inhibitor p21^{Cip1} is also degraded by the proteasome in a ubiquitin-independent manner. Here, we consider whether p21^{Cip1} and ODC represent a previously underappreciated class of proteins that can be degraded by the proteasome in vivo without benefit of ubiquitination. We also discuss why, in contrast to their remarkably similar prokaryotic counterparts, eukaryotic proteasomes evolved such a strong preference for ubiquitinated substrates.

The Defendants: The 26S Proteasome and Ubiquitin

Eukaryotic cells possess several proteolytic weapons that they wield to inactivate key molecules and thereby effect regulatory switches. One such destructive weapon is the ATP-powered 2 MDa 26S proteasome, which carries out the bulk of nonlysosomal cytoplasmic proteolysis (Hershko and Ciechanover, 1998; Voges et al., 1999). The 26S proteasome is composed of two distinct sub-complexes: a 20S proteolytic cylinder flanked by 19S regulatory caps (Figure 1). The 19S particle can be further subdivided into two assemblages: the “base” and the “lid” (Glickman et al., 1998). The base contains six “AAA” ATPases and binds to the ends of the 20S cylinder

to render proteolysis ATP dependent. The lid binds the base and is thought to render proteolysis dependent upon the prior synthesis of a multiubiquitin chain on a substrate protein.

The covalent attachment of ubiquitin to acceptor lysines in a substrate (“ubiquitination”) is a multistep process (Hershko and Ciechanover, 1998) that begins with activation of ubiquitin by E1 enzyme. Activated ubiquitin is transferred from E1 to a ubiquitin-conjugating enzyme (E2), which can transfer the ubiquitin to substrate either by itself, or in cooperation with a ubiquitin ligase (E3). E3s bind directly to substrate, and confer specificity and regulation to ubiquitination. Budding yeast has one E1, eleven E2s and an unspecified number of E3s. This multitude of E2 and E3 enzymes enables the elaboration of numerous ubiquitination pathways in vivo, thus allowing for the specific and regulated turnover of a diverse array of substrates.

Once a single ubiquitin is covalently attached to a protein, additional ubiquitins can be linked to one of ubiquitin’s seven lysines, yielding a substrate-tethered multiubiquitin chain. A tetraubiquitin chain serves as the minimal targeting signal for degradation (Thrower et al., 2000). Although chain links involving K11, K29, K48, and K63 of ubiquitin have been detected in vivo, only K48-linked multiubiquitin chains have been shown to target substrates for degradation.

The Special Case of Ornithine Decarboxylase

A large body of evidence supports the scenario outlined above. Nevertheless, examples of ubiquitin-independent proteolysis by the 26S proteasome have been reported. The best documented case is that of ornithine decarboxylase (ODC), the rate-limiting enzyme in polyamine biosynthesis (Coffino, 1998). Accumulation of polyamines stimulates synthesis of antizyme protein. Antizyme negatively regulates ODC by binding to it and

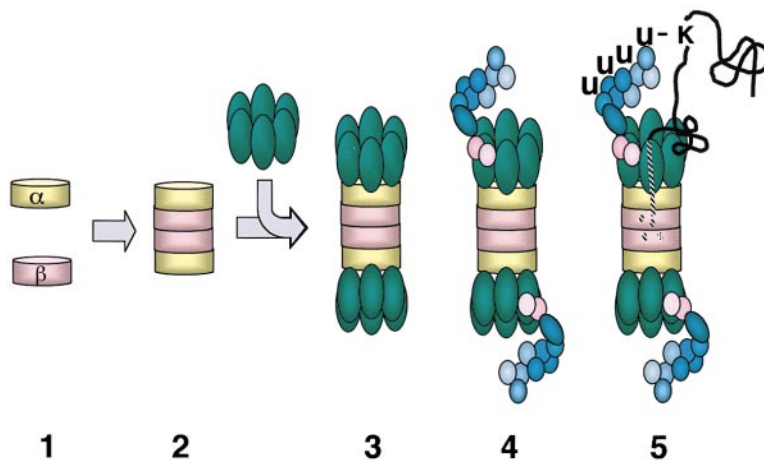


Figure 1. Summary of Proteasome Architecture

(1) In both prokaryotes and eukaryotes, 6 or 7 α and 6 or 7 β subunits assemble to form rings. The proteolytic sites are contained within the β rings, and face inward. (2) α and β rings stack to form the 20S proteasome. There are some variations on this theme among the compartmentalized proteases—for example, ClpP-based complexes contain only two stacked rings of ClpP subunits. (3) A ring of ATPases binds to the ends of the 20S barrel. The ClpA ATPase of *E. coli* forms hexameric rings, and by analogy the ATPases of the eukaryotic 26S proteasome are presumed to form a hexameric ring. (4) Two additional non-ATPase proteins (shades of purple) assemble with the Rpt (ATPase) subunits of the eukaryotic 19S cap to form the “base.” An additional set of eight Rpn proteins

(shades of blue) assemble together to form the “lid,” which sits on top of the “base.” (5) A hypothetical scheme for how the 26S proteasome works. A ubiquitinated substrate is tethered to the 26S proteasome via tight interaction between the ubiquitin chain and components of the lid. The tethered substrate is unfolded by the ATPase ring and threaded into the inner chamber of the 20S, where proteolysis takes place.

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enabling its destruction by the 26S proteasome in the absence of ubiquitination. Whereas both antizyme and ODC contain domains that mediate ODC's induced turnover, neither protein is degraded rapidly by itself, suggesting that two signals must conspire to lure ODC to the 26S proteasome. It remains unclear, however, whether ODC is a unique example or a prototype of an undiscovered class of 26S proteasome substrates.

Recently, Sheaff et al. reported that the Cdk inhibitor p21^{Cip1} is degraded by the 26S proteasome in a ubiquitin-independent manner (Sheaff et al., 2000). These authors demonstrated that p21^{Cip1} is dramatically stabilized by proteasome inhibitors, resulting in accumulation of p21-ubiquitin conjugates. Surprisingly, when all six lysines of p21^{Cip1} are eliminated, the mutant protein is nonetheless degraded rapidly by the proteasome. Moreover, overexpression of a mutant lysine-free ubiquitin (UbR7) that "chain-terminates" multiubiquitin synthesis blocks ubiquitin-dependent turnover of cyclin E, but has no effect on degradation of wild type or lysine-free p21^{Cip1} (B. Clurman and J. Roberts, personal communication). Although their case is compelling, Sheaff and coworkers' contention that p21^{Cip1} is eliminated by the proteasome without prior ubiquitination would be strengthened by showing that p21^{Cip1} (but not cyclin E) destruction persists in mutant cells that harbor temperature-sensitive (ts) E1.

The Case for and against Ubiquitin

ODC has long been regarded as an unusual exception to the ubiquitin targeting hypothesis. The observations of Sheaff and colleagues now beg the question: how many "unusual exceptions" exist? Two major lines of evidence buttress the view that most, if not all, proteins must be ubiquitinated before they can be degraded by the 26S proteasome. First, thermal inactivation of ts E1 diminishes turnover of short-lived proteins by 80%–90% (Ciechanover et al., 1984). It is unclear whether the ATP-dependent protein turnover that persists results from residual E1 activity, or represents ubiquitin-independent proteolysis. These experiments measured bulk protein turnover, and thus the results may be skewed by a small number of abundant proteins.

The second line of evidence derives from the strategy most commonly employed to establish ubiquitin's complicity in a protein's destruction. As was shown for p21^{Cip1}, many unstable proteins accumulate as multiubiquitinated species upon inhibition of the proteasome's proteolytic activity. Although the ubiquitinated species are presumed to represent essential intermediates in the destruction process, these data establish correlation but not causation. A common problem is that only a small fraction of stabilized protein accumulates in a ubiquitinated form, thereby raising the specter that ubiquitin is an innocent bystander rather than serving as the proteasome's accomplice.

In the jurisdiction of molecular biology, logic dictates that a labile protein that is destroyed via the ubiquitin pathway should be stabilized if its ubiquitination is blocked by either chemical inhibitors or mutations in ubiquitination enzymes, or by mutations in *cis* that eliminate ubiquitination sites. In many instances, these criteria are not met. A major limitation is a lack of drugs that inhibit ubiquitination in animal cells. (In yeast, however, unstable proteins can often be stabilized by mutation

of specific E2s or E3s.) Although the ts E1 mutant can be employed to address this point, it is seldom used. Ubiquitin chain assembly can also be blocked by overexpression of UbR7. However, UbR7's effects have not been characterized extensively, and it has not been ruled out that UbR7 does not influence ubiquitin-independent turnover (e.g., of ODC). Even if a labile protein is stabilized upon inhibiting multiubiquitination in *trans* (with ts E1 or UbR7 in animal cells, or with ts E2 and E3 mutants in yeast), it is difficult to exclude that the target is stabilized indirectly by activation/accumulation of an inhibitor of its destruction.

Mutation of lysine residues in *cis* is the most rigorous approach to test whether ubiquitination is an essential step in a protein's destruction. Even if a particular lysine(s) is required, it is important to establish that it is ubiquitinated, as opposed to serving in some other capacity to promote destruction. In contrast to most protein kinases, ubiquitination enzymes engage in careless gunplay, and are thought to be indiscriminate in choosing target lysines. Thus, eliminating ubiquitination can require substituting many (if not all) of the target's lysines. Because lysine residues are common in proteins, this approach requires constructing a multiply mutated protein of dubious character. With p21^{Cip1}, however, Sheaff and coworkers established that the lysine-free mutant retains its Cdk inhibitor activity, yet is still degraded by the proteasome.

Aside from p21^{Cip1}, there are few examples in which the causal role of ubiquitination has been rigorously proven by systematic mutation of lysines. The clearest example is β -galactosidase that is engineered to be a substrate for the N-end rule pathway (Gln- β -gal). Gln- β -gal is ubiquitinated on lysines 15 and 17, and is dramatically stabilized by mutation of these lysines (Chau et al., 1989). On the other hand, lysine-free mutants of both unassembled T cell receptor α chain (Yu and Kopito, 1999) and a constitutively unstable domain of I κ B (Krappmann et al., 1996) are, like p21^{Cip1}, degraded by the proteasome with normal kinetics. Lysine-free TCR α is modestly stabilized upon thermal inactivation of ts E1 or overexpression of K48R-ubiquitin, thus raising the question of whether ubiquitination plays a direct role in turnover of unassembled TCR α chains.

Some lysine-free proteins can be targeted for degradation via N-terminal ubiquitination. MyoD is one example (Breitschopf et al., 1998), and TCR α may be another. Sheaff et al. (2000) suggested that p21^{Cip1} is not targeted for degradation in this manner, as ubiquitinated forms of p21^{Cip1} were not detected (although absence of evidence is not necessarily evidence of absence).

Besides the investigations on lysine-free proteins mentioned above, there are numerous reports that 26S proteasomes can degrade unfolded proteins *in vivo* and unmodified or monoubiquitinated proteins *in vitro* (Lam et al., 1997; consult Sheaff et al., 2000 for additional references). However, the physiological significance of the latter observations remains unclear.

If Not Ubiquitin, Who Is the Proteasome's Accomplice?

How might the 26S proteasome recognize its substrates if they are not ubiquitinated? To find clues to this puzzle, we need look no further than prokaryotes and archaea. Organisms from both of these kingdoms contain ATP-dependent proteases that share remarkable structural

and functional similarity with the eukaryotic 26S proteasome (Voges et al., 1999). The most intensively studied of these are the Clp complexes of *Escherichia coli*. For example, ClpY (HslU), which is a member of the "AAA+" superfamily and is distantly related to the ATPases of the 19S cap (Bochtler et al., 2000), forms hexameric rings that sandwich two stacked hexameric rings of ClpQ (HslV). ClpQ is an N-terminal hydrolase related to the proteolytically active β subunits of the eukaryotic 20S core. The conserved "sensor and substrate discrimination" (SSD) domain of ClpY directly binds sequences within substrates that serve as degradation-targeting signals (Smith et al., 1999). The structure of ClpY, however, suggests that substrates may bind elsewhere on the molecule (Bochtler et al., 2000). Nevertheless, direct interactions between substrates and Clp ATPases enable selective proteolysis. The 26S proteasome may employ a similar modus operandi to snare its victims. Unfolded (but not folded) citrate synthase binds to the base of the 19S cap (Braun et al., 1999). Proteasomes composed of the 20S core topped by the base subcomplex (but lacking the lid) degrade casein in vitro in an ATP-dependent, ubiquitin-independent manner (Glickman et al., 1998), indicating that the proteasome can recognize and degrade proteins that lack a multiubiquitin chain. Thus, what works for bacterial proteasomes may work for 26S proteasomes as well.

If Selective Proteolysis Can Be Achieved without Ubiquitin, Why Bother with It?

Prokaryotes and archaea contain multiple compartmentalized proteasome-like proteases and employ selective proteolysis as a regulatory strategy, but nevertheless they thrive without ubiquitin. Why did ubiquitination achieve such a central role during the evolution of proteolytic pathways in eukaryotes? Below, we develop four interrelated arguments: ubiquitin-mediated targeting (i) expands the range of substrates that can be selectively degraded, (ii) enables greater flexibility in regulating proteolysis, (iii) increases the specificity of substrate targeting, and (iv) enhances the biochemical repertoire of the proteasome.

Expanded Substrate Range. For a protein to be targeted for degradation, it must presumably dock onto either a Clp ATPase (prokaryotes), or a ubiquitin pathway enzyme (eukaryotes). Because both E2 and E3 enzymes can, in theory, contribute to substrate selection, there is potential to achieve diversification of the substrate repertoire via combinatorial mixing of E2s and E3s. It will be interesting to see if this theoretical capacity is exploited. An alternative argument (suggested by M. Hochstrasser) rests on the design of the specificity-determining component. In prokaryotes, substrate recognition must be accommodated within the conserved architecture of the AAA+ ATPase domain, which may limit the range of both the ligand binding motifs that can be displayed by Clp proteasomes, and the substrates that can be recruited. In contrast, the remarkable lack of target lysine specificity commonly evinced by E2/E3 enzymes suggests a flexible design that can tolerate a broad range of both docking platforms and substrates.

Enhanced Regulatory Flexibility. If ubiquitination expands the repertoire of available substrate-targeting motifs and pathways, it would create enhanced opportunity to differentially regulate the turnover of proteins in

response to a myriad of signals. Intriguingly, protein turnover via the ubiquitin pathway is rife with examples of regulated destruction, including regulation at the level of both substrates (phosphorylation-triggered ubiquitination of Sic1, I κ B α), and E3s (mitotic activation of APC) (Zachariae and Nasmyth, 1999).

Increased Selectivity. Assembly of a multiubiquitin chain is a discrete process interposed between substrate recognition and destruction that can be used as a timer to measure the rate of dissociation of substrates from ubiquitination enzymes, such as GTP hydrolysis serves as a timer to enhance fidelity during translation via "kinetic proofreading." Moreover, the requirement for a multiubiquitin chain allows for additional fine-tuning of substrate selection, in that a protein's stability can be manipulated via changes in the rate of either ubiquitination or deubiquitination. Other arguments along these lines have been discussed previously (Thrower et al., 2000).

Enhanced Functional Repertoire. The 26S proteasome handles demanding tasks with ease. For example, we have shown that it selectively extricates ubiquitinated Sic1 from S phase cyclin/Cdk complexes, degrades it, and releases active S phase cyclin/Cdk. How does the proteasome accomplish this remarkable gymnastic feat? The destruction of ubiquitinated proteins appears to be kinetically limited by their prior unfolding (Thrower et al., 2000). Thus, selective elimination of proteins from multisubunit complexes may require stable, low off-rate interaction between substrate and the degradation machinery. Energy expended in substrate ubiquitination may be harnessed to convert a dynamic protein-protein interaction (substrate-E3) into a slowly dissociable interaction between ubiquitinated substrate and 26S proteasome. This would allow the 19S ATPases ample time to unfold and translocate the substrate before it dissociates. In contrast, the off-rate for substrate-ATPase interaction in the Clp system may be too rapid to allow for efficient unfolding of multidomain polypeptide chains (which are more prevalent in eukaryotic cells), and disassembly of multisubunit complexes. The homodimeric phage lambda replication protein RepA is partitioned to alternative fates of disassembly and degradation upon incubation with ClpA/ClpP proteasomes (Pak et al., 1999). Partitioning of RepA may result from dissociation of disassembled RepA monomers from ClpA prior to a rate-limiting unfolding step. Many of the 26S proteasome's substrates (e.g., cyclins, Cdk inhibitors, securin, I κ B) reside in heteromeric assemblies, and must be efficiently degraded for the proper operation of a regulatory switch. A probabilistic partitioning between disassembly and degradation, as is observed for RepA's processing by ClpA/ClpP, would most likely compromise the effectiveness of such proteolysis-driven switches.

What's Next on the Docket?

The results of Sheaff et al. raise many intriguing questions. How many ubiquitin-independent targets of the 26S proteasome exist? Is the ubiquitin-independent degradation of p21^{Cip1} mediated by its direct affiliation with the ATPase subunits of the 19S cap? Perhaps a more important question to consider is, if a multiubiquitin chain is not necessary for proteolysis, is it really even sufficient? Fragmentary data suggest that in some

instances, it is not (Dai et al., 1998; Klotzbucher et al., 1996; Thrower et al., 2000). An intriguing possibility is that unstable prokaryotic proteins contain a single class of signal (class I degron) that enhances their turnover by ATP-dependent proteases. Degron I may modulate substrate targeting and/or downstream processing events (e.g., unfolding). Unstable eukaryotic proteins may typically contain an additional signal that promotes ubiquitination (class II degron). The two signals operating in parallel may increase the rate and specificity of protein destruction. In most cases (especially for proteins that are large, tightly folded, or assembled into multisubunit complexes), neither degron may be sufficient to specify efficient turnover. In some instances (e.g., small and/or loosely folded eukaryotic proteins such as p21^{Cip1}), a potent degron I signal by itself may administer the coup de grace, rendering ubiquitination dispensable.

The sequences and structures of ClpY/Q, *Thermoplasma acidophilum*, and budding yeast proteasome subunits suggest that eukaryotic, prokaryotic, and archaeal cells contain proteasomes that originate from a common ancestor (Voges et al., 1999; Bochtler et al., 2000). We speculate that proteasomes in all three branches of life can recognize linear peptide stretches within proteins (class I degrons), but that during evolution, the eukaryotic proteasome acquired the additional capacity to recognize a multiubiquitin chain. According to this view, the mode of substrate recognition mediated by degron I is evolutionarily ancient, and we believe it is likely to be important in the turnover of eukaryotic proteins. It is ironic that the "atypical" ODC, which is targeted for degradation by what may correspond to a compound class I degron, is more reminiscent of the archetypal mode of targeting that occurs in prokaryotes than is the more commonly studied ubiquitin signal. ODC may be a eukaryotic counterpart to the RssB-activated turnover of RpoS in *E. coli* (Becker et al., 1999). No doubt, the mystery of *how* the proteasome *dunit* will continue to satisfy our morbid curiosity as more and more cases of proteins slain by the proteasome are prosecuted.

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