The ubiquitin–proteasome pathway of intracellular proteolysis

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Abstract

Intracellular proteins are targeted for degradation by the covalent attachment of chains of the small protein ubiquitin; a process known as ubiquitylation. Many proteins are phosphorylated prior to ubiquitylation, and therefore ubiquitylation and degradation of these proteins is regulated by kinase activity and signalling cascades. Many ubiquitylated proteins are degraded by the 26 S proteasome complex, which is found in the cytosol and nucleus. The 26 S proteasome consists of a 20 S core with proteolytic activity and 18 S regulatory complexes containing ATPases and ubiquitin-chain-binding proteins. Proteins degraded by the ubiquitin–proteasome pathway include cyclins and other regulators of the cell cycle, and transcription factors. Abnormal polypeptides are also degraded by the ubiquitin pathway, including abnormal polypeptides in the endoplasmic reticulum, which are translocated back out of the endoplasmic reticulum prior to ubiquitylation and degradation by the proteasome. The ubiquitin–proteasome pathway is implicated in numerous diseases including cancer and neurodegenerative diseases.

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Introduction

The proteasome is a large multi-subunit protease found in eukaryotic cells and archaeabacteria, which appears to be responsible for the majority of the degradation of intracellular proteins. Most proteins are targeted for destruction by the proteasome by being covalently tagged with the small protein ubiquitin. The attachment of ubiquitin to target proteins, ubiquitylation, is itself a complex multi-step process and is responsible for much of the selectivity of proteasome-mediated proteolysis. The ubiquitin–proteasome pathway is implicated in numerous cell processes through the regulated degradation of key proteins, often in response to signalling events. In addition, the ubiquitin–proteasome pathway is involved in the removal of damaged or abnormal proteins. Given the important role of this pathway, it is not surprising to find that it is involved in several disease processes.

Ubiquitin: marking proteins for destruction

The crystal structure of ubiquitin [1], which is only found in eukaryotes, reveals a small compact ‘peardrop-like’ protein with stretches of interspersed α-helices and β-sheets (Figure 1a). The ‘business end’ of the protein extends into the aqueous space and can be used in the formation of an isopeptide bond (ubiquitylation) between the carboxyl group of the C-terminal glycine and the side-chain amino group of a lysine residue in target proteins, or indeed in another copy of ubiquitin (Figure 1b). Ubiquitin contains seven lysine residues, many of which can form isopeptide bonds to give rise to families of distinct ubiquitin chains. Ubiquitin Lys^48^-linked chains (between the side-

![Figure 1. Ubiquitin and the ubiquitin–protein isopeptide bond](image-url)
chain amino group of Lys^{48} of one ubiquitin and the C-terminal group of another ubiquitin and with at least four ubiquitins in the chain) conjugated to target proteins are signals for degradation of the isopeptide-linked target protein. Chains of ubiquitins that are linked through other lysine residues may be involved in other cellular pathways.

A number of ubiquitin-like proteins (ubiquitin paralogues) have been discovered, including SUMO (small ubiquitin-like modifier). Sumoylation of proteins may prevent their degradation by preventing ubiquitylation [2].

**Ubiquitylation and de-ubiquitylation**

Ubiquitylation of target proteins is a multi-step process that starts with ubiquitin activation. The ubiquitin-activating enzyme (E1 in Figure 2) promotes the formation of ubiquitin–adenylate. The activated ubiquitin then forms a thiolester with the activating enzyme, with the release of AMP. Ubiquitin is then transferred to one of a family of conjugating enzymes (E2) in the form of a thiolester [3]. Another class of enzymes, E3s or ubiquitin–protein ligase, is necessary for the transfer of the ubiquitin to the target protein, with the associated formation of an isopeptide bond.

E3 proteins or protein complexes recognize target proteins and ubiquitin-charged E2 proteins, and bring them together for ubiquitylation of the target protein. Therefore, it appears that the specificity of the ubiquitin-mediated proteolytic machinery depends upon the E3 proteins. Ligases must recognize features of the target protein, and a number of these degradation signals, or degrons, have been identified. Many proteins must be phosphorylated prior to ubiquitylation and, therefore, the phosphorylated residue forms part of the degron.

**De-ubiquitylation — an important regulatory mechanism**

There are many de-ubiquitylating enzymes (DUBs), which implies that de-ubiquitylation may have an important regulatory role in protein degradation [4]. There are two classes of DUBs. Ubiquitin C-terminal hydrolases cleave ubiquitin from the fusion proteins that are the products of ubiquitin genes, thereby releasing ubiquitin monomers and the unrelated C-terminal extension proteins. Ubiquitin-specific proteases (UBPs) cleave ubiquitin from multi-ubiquitin chains. The activities of UBPs may, therefore, function by saving proteins from degradation. The proteasome contains a UBP activity, which trims long multi-ubiquitin chains on target proteins. If one considers the large numbers of DUBs (19 in *Saccharomyces cerevisiae*), it may be that they have an important role in regulating intracellular proteolysis. Several DUBs are known to be involved in specific cell processes and some are involved in disease.

**The big mean proteolytic machine: the proteasome**

Once a target protein is marked by multi-ubiquitylation, it appears to have a short half-life in the cell as it is degraded rapidly by the proteasome. The
The multi-subunit proteasome is a compartmentalized protease: the active sites (β-subunits) are within the 20 S proteolytic core which consists of 28 subunits (α7, β7, β7, α7) [5] (Figure 3). An intracellular protease should not expose catalytic sites to the cellular milieu, as this would result in the inappropriate degradation of proteins.

The proteasome is a threonine protease, with the threonine residue at the N-terminus of at least three of the β-subunits that are involved in nucleophilic attack [6,7]. This threonine can be replaced by serine with no loss of activity [7].

Ubiquitylated proteins cannot gain access to the central 20 S cavity that contains the catalytic sites: in yeast, and presumably higher organisms, the ends of the 20 S cylinders are closed by the N-termini of the α-subunits [8]. These termini must be opened to enable target proteins to gain access to the catalytic sites. Furthermore, the opened pore or hole is still too small for globular proteins. Therefore, the heptameric α-subunit rings at each end of the 20 S cylinder can bind to a 19 S regulator, giving rise to the 26 S proteasome that is

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Figure 2. Ubiquitin activation and conjugation

E1 (or UbA in yeast) is the ubiquitin-activating enzyme, which initially forms a ubiquitin–adenylate complex (not shown) and then forms a thiol ester between the carboxyl group of the C-terminal glycine of ubiquitin and a cysteine residue of E1. The ubiquitin is then transferred to one of a large family of ubiquitin-conjugating enzymes (E2; Ubc in yeast), the ubiquitin forming a thiolester as before with a cysteine residue of the enzyme. Finally, ubiquitin is transferred to the target protein, forming an isopeptide bond between the C-terminus of ubiquitin and an ε-amino group in the side chain of a lysine residue of the target protein. The transfer of ubiquitin to the target can occur directly from a charged E2, with E3 (ubiquitin ligase) recognizing the target and bringing it and charged E2 together to form a complex. Alternatively, some E3s act as ubiquitin acceptors and ubiquitin is transferred to the E3 to form a thiolester before being transferred to the target protein, which is also recognized by this class (HECT; homologous with E6-associated protein carboxy terminus) of E3/ligases. Further copies of ubiquitin can then be conjugated to ubiquitin attached to the protein to form a multi-ubiquitin chain. Ubiquitin–ubiquitin isopeptide bonds are between the C-terminal carboxyl group of the distal ubiquitin and the ε-amino group of Lys48 or Lys63 of the proximal ubiquitin. Lys48-based multi-ubiquitin chains of at least four ubiquitin molecules are necessary to target proteins to the 26 S proteasome.
responsible for degrading ubiquitylated proteins [9]. Each 19 S regulator consists of a ‘base’, which binds to each α-subunit heptameric ring, and a ‘lid’. The base contains six non-identical related ATPases, which are presumed to form a hexameric complex [9] as in prokaryotic ATP-dependent proteases (Figure 3). The ATPases are molecular chaperones, which, like the ATPase subunits of prokaryotic protease complexes, unfold target proteins. The lid contains proteins such as S5a (otherwise known as Rpn10 and Mcb1), which appears to be involved in the recognition of multi-ubiquitylated proteins [10]. The ATPases may also be involved directly or indirectly in recognizing multi-ubiquitylated proteins. There are protein substrates that are degraded by the 26 S proteasome that do not have to be ubiquitylated. An example is ornithine decarboxylase, which, following association with a protein known as antizyme [11], is degraded by the 26 S proteasome. Other substrates, such as the cyclin-dependent kinase inhibitor, p21, can be degraded by the 20 S core proteasome lacking the 26 S regulator, after binding to a 20 S subunit [12].

Other regulators of the 20 S proteasome exist. The most studied is the 11 S regulator or PA28. This heptameric assembly can attach to one end of a 20 S cylinder with a 19 S regulator at the other end. The binding of 11 S to the 20 S core appears to facilitate the cleavage of substrate proteins to give rise to peptides which are more suited to the binding of MHC Class I (MHC I) molecules [13]. Generation of peptide antigens is one of the key roles of the proteasome.
As might be anticipated, proteins which are not part of these complexes can interact with the complexes to modulate the proteolytic activity. Proteins that bind to individual ATPases in the putative hexameric ring of the 19 S base include physiological regulators like gankyrin (which targets retinoblastoma protein for degradation by the proteasome) [14] and proteins from pathogens, e.g. human papilloma virus E7 protein, which also targets pRb for degradation [15]. It is likely that many other proteins that interact with different subunits of both the proteasomal 19 S regulator and the 20 S core will be discovered in due course.

**Intracellular pathways controlled by the ubiquitin–proteasome system**

Numerous cellular processes involve regulated proteolysis of key components by the ubiquitin–proteasome system. Phosphorylation events often trigger degradation of a protein by the ubiquitin–proteasome pathway. It is not possible in this review to cover many examples of protein degradation by this system; a few are included here.

**Cell cycle**

Each step in the cell cycle is controlled by cyclin-dependent protein kinases (Cdks) and, in turn, by the degradation of cyclins and kinase inhibitors by the ubiquitin–proteasome pathway. The accumulation of a cyclin, and therefore the activation of a Cdk, will promote entry into a particular stage of the cell cycle. These factors will also inhibit entry into the next stage of the cycle until, for instance, the cyclin is degraded. This ensures that the cell cycle is composed of distinct stages. Mutations in kinases, kinase inhibitors and components of ubiquitin protein ligases are found in cancer cells. The E3 proteins involved in these degradative events are the multi-component SCF (Skip–cullin–F-box) type ligase and phosphorylation of the target protein often triggers ubiquitylation. A related complex, the anaphase-promoting complex (APC; cyclosome), which ubiquitylates mitotic cyclins, contains a RING-finger protein (Apc11), an E2 and a cullin (Cdc20/Hct1), but Skp1 is replaced by multiple APC subunits [16]. The APC is involved in the degradation of Pds1/Cut1, factors involved in the regulation of daughter chromatid separation. Regulation of the cell cycle is highly complex, with many components of the cycle control mechanism subjected to rapid degradation by the ubiquitin–proteasome pathway (Figure 4).

**Transcription**

Transcription factors that control the expression of eukaryotic genes are essential but potentially lethal molecules. Aberrant transcription is a very significant feature of cancer. Transcription factors are very short-lived and are degraded quickly by the ubiquitin pathway. One of the best studied examples is slightly different in that an inhibitor of a transcription factor is degraded, thus
releasing the transcription factor to cause gene expression. The transcription factor is nuclear factor κB (NFκB), which regulates the expression of many genes involved in inflammation and immunity. The inhibitor of NFκB (IκB), binds to NFκB and prevents the transcription factor from entering the nucleus and, therefore, binding to DNA. Once again, phosphorylation of the target protein, IκB, triggers ubiquitylation by an SCF ligase. IκB is phosphorylated by IκB kinase, which in turn is activated by the kinase tumour necrosis factor-activated kinase 1 (TAK1), which is activated by association with the self-ubiquitylated protein tumour necrosis factor-associated factor 6 (TRAF6) [17]. The multi-ubiquitin chain formed on TRAF6 is formed between Lys63 residues of ubiquitin, rather than the Lys48 residues that lead to degradation by the proteasome (Figure 5). This is an example of ubiquitylation as a regulatory covalent modification with a function distinct from proteolysis. Interestingly, a form of NFκB where the IκB inhibitor is incorporated into a NFκB precursor is partially degraded following phosphorylation and ubiquitylation, to generate active transcription factor. This is a situation where ubiquitylation does not lead to complete degradation of the ubiquitylated protein [18], another example being the release of a membrane-bound transcription factor resulting from limited proteolysis following ubiquitylation [19].
Protein folding versus degradation

Folding of newly synthesized polypeptides in the cytosol is often helped by molecular chaperones, e.g. Hsp70 (heat-shock protein 70). But what happens if a protein cannot fold or misfolds? The co-chaperone CHIP (C-terminal Hsp70-interacting protein) contains a modified RING-finger (the U-box) and promotes the ubiquitylation of misfolded proteins, which are then degraded by the proteasome [20]. The term ‘defective ribosomal products’ (DRIPs) has been coined for misfolded newly synthesized proteins. It is likely that repeated binding of a partially folded polypeptide with exposed hydrophobic residues to a Hsc70 (heat-shock cognate 70 stress protein) chaperone family member increases the likelihood of ubiquitylation by CHIP. Polypeptides that fold quickly may escape this fate. There is increasing evidence that a substantial fraction of all newly synthesized proteins are degraded to produce antigenic peptides for MHC I presentation (see later). Chaperones and CHIPs may be needed for this process. It is suggested, therefore, that CHIP is involved in ‘protein triage’ decisions in the cell, favouring degradation of target proteins over chaperone-mediated folding processes.
Endoplasmic reticulum (ER)-associated degradation system

All membrane-associated and secreted proteins are synthesized on membrane-bound ribosomes and pass into the lumen of the ER. The lumen of the ER is, therefore, a very ‘busy’ place, filled with chaperones and enzymes involved in protein folding and post-translational modifications, e.g. glycosylation.

What happens to misfolded and misglycosylated proteins in the ER? Remarkably, they are extracted from the ER through the same channel by which they enter the lumen (the translocon), ubiquitylated and degraded by the proteasome (Figure 6). Both soluble luminal ER proteins, e.g. a mutant carboxypeptidase in yeast, and ER membrane proteins, e.g. cystic fibrosis transmembrane conductance regulator [21], are degraded after extraction from the ER. Molecular chaperones interact with the misfolded proteins, and prevent them from aggregating in the ER and permit their dislocation to the cytosol for ubiquitylation and degradation. The presence of abnormal proteins in the ER triggers the unfolded protein response, which upregulates a number of genes, including some of those coding for components of the ubiquitin–proteasome pathway [22]. It would not be surprising if viruses did not exploit the ER degradation pathway. Cytomegalovirus and HIV have proteins that bind to MHC I molecules in the ER and force their ejection from the membrane, which is followed by their degradation by the ubiquitin pathway in order to reduce the MHC I immune response (see later) to viral proteins [23].

Figure 6. ER-associated degradation: ubiquitin–proteasome degradation of ER proteins in S. cerevisiae

Misfolded or misglycosylated proteins are recognized by Cne1 (calnexin in mammalian cells) or Kar2 (BiP, the ER-resident 70 kDa chaperone/heat-shock protein in mammals), which probably prevents aggregation and presents the protein to the translocon for reverse translocation (dislocation) to the cytoplasm. A cytosolic glycosidase activity appears to trim or remove carbohydrate moieties and the protein is ubiquitylated by a complex including a RING-finger E3/ligase (Hrd1) which recruits the E2 Ubc7. Cue1 also seems to be involved in E2 recruitment. The membrane E2 (Ubc6) may also be involved in ubiquitylation in ERAD. Hrd3, which extends into the ER lumen, serves to regulate Hrd1 activity.
Antigen processing
The immune surveillance system in vertebrate cells involves the presentation of small peptides (8–10 amino acids) at the cell surface bound to the MHC I. The peptide–MHC I complex can then be recognized by cytotoxic T-cells. MHC I binds peptides that are derived by proteolysis of both cellular and viral proteins. These peptides are generated from ubiquitylated proteins by the proteasome in the cytoplasm and are transported into the ER by TAP (transporter associated with antigen processing) where they bind to MHC I and are trafficked to the cell surface. The proteolytic generation of peptides for presentation requires a special proteasome complex, the immunoproteasome, which generates peptides of the right length with the basic or hydrophobic C-terminal residues required for binding to MHC I. In the immunoproteasome 20 S core, catalytic subunits β1, β2 and β5 are replaced by interferon-inducible subunits β1i, β2i and β5i. In addition, the 20 S proteasome core is capped at one end by a 19 S regulator complex, but at the other by the proteasome activator PA28 (11 S) regulator complexes. The presence of the 11 S regulator and the interferon inducible 20 S subunits regulates the immunoproteasome in such a way that it generates the peptides required for antigen presentation [24]. Defective ribosomal products (see above) may provide a pool of self peptide antigens via the ubiquitin–proteasome pathway. Some viruses code for proteins that interact with the proteasome, but the effect of these interactions on antigen processing is not clear.

Ubiquitin pathway and disease
Central regulatory pathway involved in disease
It should be apparent by now that the ubiquitin pathway of intracellular proteolysis, not to mention the other functions of ubiquitin and ubiquitin paralogues, add a new layer on the cake of cellular physiology. Therefore, as already indicated, abnormalities in the pathway can lead to diseases like cancer, and pathogens such as viruses can exploit the pathway in many ways. Mutations in a HECT-domain E3 result in Angelman’s syndrome [25], a neurological developmental disorder, and mutations in the RING-finger E3 Parkin cause juvenile onset recessive parkinsonism [26]. In addition, neurodegenerative diseases, e.g. Alzheimer’s disease, Parkinson’s disease, motor neuron disease and the spongiform encephalopathies, are characterized by the accumulation of aggregates of insoluble proteins [27]. These protein deposits contain ubiquitylated proteins and may be the consequence of failed attempts by the cell to dispose of abnormal proteins [28]. Indeed, in the aging brain, post-transcriptional ‘molecular misreading’ leads to increased quantities of a mutant C-terminal-extended ubiquitin that can still be incorporated into ubiquitin chains and is a potent inhibitor of the 26 S proteasome [29]. This impairment of the ubiquitin system may help to explain why these diseases are age-related. Once protein aggregates start to form, they can result in further
inhibition of the proteasome [30], leading to accelerated aggregate formation as abnormal proteins fail to be degraded.

**Conclusion**

Protein degradation is flexible, adaptable and participates in diverse cellular regulatory mechanisms. Undoubtedly, the major role for ubiquitin itself is in targeting proteins for degradation. However, recent research has shown that ubiquitylation targets proteins for degradation by both major cellular proteolytic systems, the 26 S proteasome and the lysosomal apparatus. Non-degradative roles for ubiquitin in protein regulation and vesicular trafficking are also emerging. The roles of the ubiquitin-like proteins SUMO and Nedd8 are being elucidated, but the roles of the various other ubiquitin-like proteins still remain to be determined. Every week sees the identification of new substrates for ubiquitin–proteasome-mediated degradation, and the ever-expanding repertoire for this pathway in the control of cellular processes by regulated proteolysis looks set to continue for some time. It is likely that every cellular pathway feels the bite of the proteasome.

**Summary**

- **Ubiquitylation is a reversible modification that targets proteins for a variety of fates, including degradation by the 26 S proteasome.**
- **The 26 S proteasome is a large multi-subunit threonine protease, consisting of a 20 S catalytic core and 19 S regulator complexes, that degrades ubiquitylated proteins**
- **Many cell surface proteins are ubiquitylated and targeted to the lysosome for degradation. Mono-ubiquitylation can also serve as a signal for endocytosis.**
- **Signals in target proteins are recognized by ubiquitin ligase enzymes. Many ligases are complexes, with substrate recognition and ubiquitin transfer activities provided by different subunits. Phosphorylation precedes ubiquitylation in many cases.**
- **A system with similarities to the ubiquitylation pathway is responsible for controlling autophagic sequestration of cytoplasmic proteins for lysosomal degradation.**

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References


