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Ubiquitin as an Obtainable Medium for Proteolysis

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ABSTRACT

Between the 1960s and 1980s, most life scientists focused their attention on studies of nucleic acids and the translation of the coded information. Protein degradation was a neglected area, considered to be a nonspecific, dead-end process. Although it was known that proteins do turn over, the large extent and high specificity of the process, whereby distinct proteins have half-lives that range from a few minutes to several days, was not appreciated. The discovery of the complex cascade of the ubiquitin pathway revolutionized the field. It is clear now that degradation of cellular proteins is a highly complex, temporally controlled, and tightly regulated process that plays major roles in a variety of basic pathways during cell life and death as well as in health and disease. With the multitude of substrates targeted and the myriad processes involved, it is not surprising that aberrations in the pathway are implicated in the pathogenesis of many diseases, certain malignancies, and neurodegeneration among them. Degradation of a protein via the ubiquitin/proteasome pathway involves two successive steps such as the conjugation of multiple ubiquitin moieties to the substrate and degradation of the tagged protein by the downstream 26S proteasome complex. New techniques allow for the determination of individual protein turnover on a global scale. This will enable the generation of a comprehensive annotation of turnover rates as a function of experimental perturbations or disease states, opening the door to a systems-level understanding of protein degradation.

Keywords: Ubiquitin, proteolysis, enzymes (E), Protein turnover and neurodegeneration.

INTRODUCTION

Like all macromolecular components of an organism, the proteome is in a dynamic state of synthesis and degradation. During proteolysis, the peptide bonds that link amino acids are hydrolyzed, and free amino acids are released [1]. The process is carried out by a diverse group of enzymes termed proteases. During proteolysis, the energy invested in the synthesis of the peptide bond is released. Distinct proteolytic mechanisms serve different physiological requirements and allow the organism to accommodate to changing environmental and pathophysiological conditions. One should distinguish between destruction of "foreign" and "self" proteins. Foreign dietary proteins are degraded "outside" the body, in the lumen of the gastrointestinal tract. To avoid triggering an immune response, the epithelial lining of the digestive tract does not allow absorption of intact proteins into the body, and they are degraded to nonantigenic amino acids that are absorbed by the body and serve as building blocks for synthesis of its own proteins [2]. Self proteins can also be classified into extracellular and intracellular; the two groups of proteins are degraded via two distinct mechanisms. Extracellular proteins such as the blood coagulation factors, immunoglobulins, albumin, cargo-carrying proteins [such as the core protein of the low-density lipoprotein (LDL)], and peptide hormones (such as insulin) are taken up via pinocytosis or receptor-mediated endocytosis [3]. They are then carried via a series of vesicles (endosomes) that fuse with primary lysosomes where they are degraded.

During this process, the extracellular proteins are never exposed to the intracellular environment (the cytosol) and remain "extracellular" (topologically) throughout. Degradation of proteins in lysosomes is not specific, and all engulfed proteins exposed to lysosomal proteases are degraded at approximately the same rate. Several observations lead to the prediction that degradation of intracellular proteins must be carried out by completely distinct mechanisms [4]. The process is highly specific, and different proteins have half-life times that vary from a few minutes (e.g., the tumor suppressor p53) to several days (e.g., the muscle proteins actin and myosin) and up to a few years (crystalline). Furthermore, inhibitors of lysosomal degradation, weak bases such as chloroquine, for example, do not have any effect on degradation of intracellular proteins under basal metabolic conditions. These compounds titrate the normal acidic intralysosomal pH and bring it to a point that does not allow activity of the lysosomal proteases [5]. These findings led to the hypothesis that degradation of intracellular proteins must be carried out by a nonlysosomal proteolytic system that is endowed with a high degree of specificity toward its substrates [6]. Also, the fact that the proteolytic enzymes and their substrates reside in the same cellular compartment predicted a requirement for tightly regulated machinery that uses metabolic energy for control. The discovery of the ubiquitin-proteasome proteolytic pathway has resolved these enigmas.

Ubiquitin is a common demoninator in the targeting of substrates to all three major protein degradation pathways in mammalian cells: the proteasome, the lysosome, and the autophagosome [7]. The factors that direct a substrate toward a particular route of degradation likely include ubiquitin chain length and linkage type, which may favor interaction with particular receptors or confer differential susceptibility to deubiquitinase activities associated with each pathway [8].

Mechanism of Ubiquitin Pathway

Degradation of a protein via the ubiquitin-proteasome pathway involves two discrete and successive steps:

1. Tagging of the substrate by covalent attachment of multiple ubiquitin molecules
2. Degradation of the tagged protein by the 26S proteasome complex with release of free and reusable ubiquitin [9].

This last process is mediated by ubiquitin recycling enzymes [deubiquitinating enzymes (DUBs)]. Conjugation of ubiquitin, a highly evolutionarily conserved 76-residue polypeptide, to the protein substrate proceeds via a three-step cascade mechanism as shown in Figure. 1. Initially, the ubiquitin-activating enzyme E1 activates ubiquitin in an ATP-requiring reaction to generate a high-energy thiol ester intermediate, E1-S ubiquitin [10]. One of several E2 enzymes [ubiquitin-carrier proteins or ubiquitin-conjugating enzymes (UBCs)] transfers the activated ubiquitin moiety from E1, via an additional high-energy thiol ester intermediate, E2-S ubiquitin, to the substrate that is specifically bound to a member of the ubiquitin-protein ligase family, E3. There are a number of different classes of E3 enzymes as seen in figure 1 and 2. For the HECT (homologous to the E6-AP COOH terminus) domain E3s, the ubiquitin is transferred once again from the E2 enzyme to an active site Cys residue on the E3, to generate a third high-energy thiol ester intermediate, ubiquitin S-E3, before its transfer to the ligase-bound substrate [11]. RING finger-containing E3s catalyze direct transfer of the activated ubiquitin moiety to the E3-bound substrate. E3s catalyze the last step in the conjugation process: covalent attachment of ubiquitin to the substrate. The ubiquitin molecule is generally transferred to an ε-NH2 group of an internal Lys residue in the substrate to generate a covalent isopeptide bond. In some cases, however, ubiquitin is conjugated to the NH2-terminal amino group of the substrate. By successively adding activated ubiquitin moieties to

internal Lys residues on the previously conjugated ubiquitin molecule, a polyubiquitin chain is synthesized (Fig. 1) [12]. The chain is recognized by the downstream 26S proteasome complex. Thus E3s play a key role in the ubiquitin-mediated proteolytic cascade since they serve as the specific recognition factors of the system.

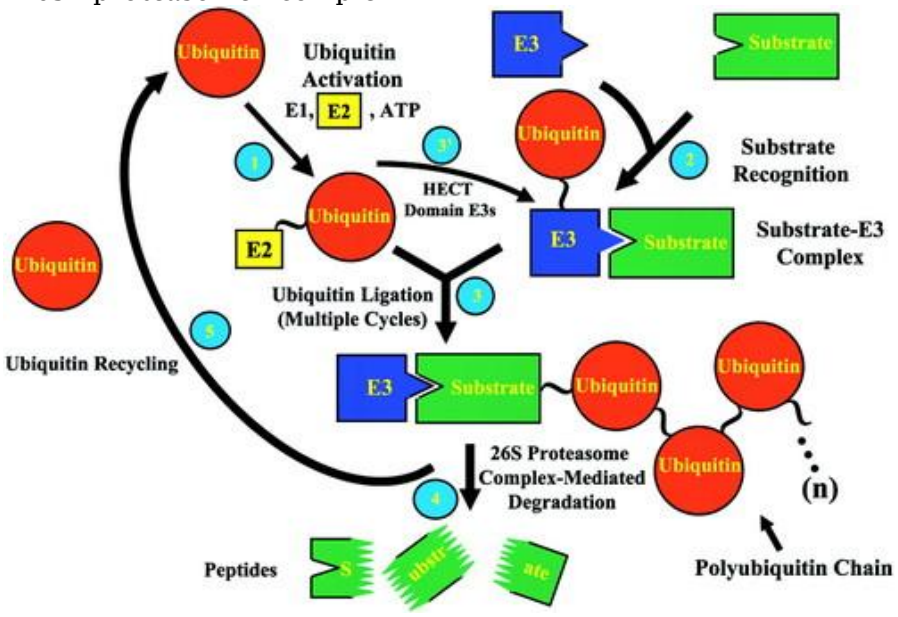

Figure 1: The Ubiquitin Proteolytic Pathway. The activation of ubiquitination process is mediated by the addition of ubiquitin moiety to E1 enzyme, and E1 transfers ubiquitin to E2 enzyme that subsequently interacts with E3 leading the formation of polyubiquitin chain. Ultimately, substrate proteins tagged with a multiple-ubiquitin chain are then degraded by the 26S proteasome that is composed of a 20S catalytic core and two 19S subunits [12].

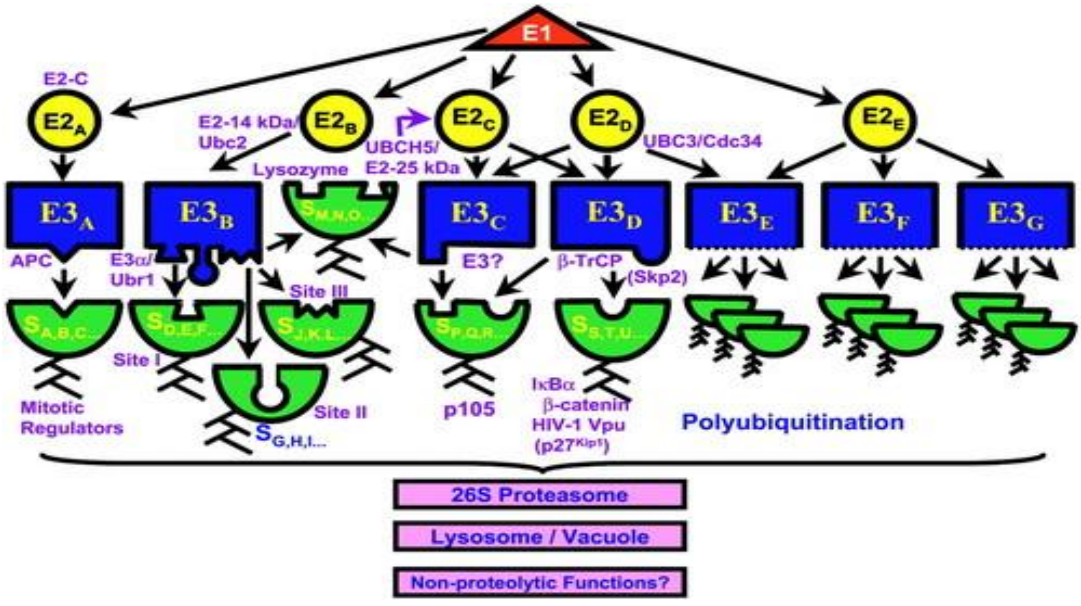

Figure 2: The hierarchical structure of the Ubiquitin system. The simplified view of the hierarchical structure of the ubiquitin conjugation machinery is that a single E1 (red)

activates ubiquitin for all conjugation reactions. E1 interacts with all E2s (yellow). Typically, each E2, exemplified by $E2_c$, interacts with several E3s ($E3_c$, $E3_c$, and $E3_c$; blue). Each E3 targets several substrates (green). The interactions of the conjugating enzymes among themselves and with many of the target substrates may differ from this "classical" cascade. For example, a single E3 can interact with 2 distinct E2s.

The Ubiquitin Conjugating Machinery: E1, E2, And E3

A. The Ubiquitin-Activating Enzyme, E1

E1 activates ubiquitin, via a two-step intramolecular and ATP-dependent reaction, to generate a high-energy E1-thiol-ester~ubiquitin intermediate (Fig. 1). The activated ubiquitin moiety is then transferred to E2 [13]. The yeast genome encodes for a single ubiquitin-activating enzyme, UBA1. Inactivation of this gene is lethal. The protein contains a nuclear localization signal [14]. The enzyme is phosphorylated, a modification that was suggested to play a role in its cell cycle-dependent nuclear localization. However, the physiological relevance of this modification has not been further substantiated.

B. Ubiquitin-Carrier Proteins (Ubiquitin-Conjugating Enzymes), E2s

E2s catalyze covalent attachment of ubiquitin to target proteins, or, when acting along with HECT domain E3s, transfer of the activated ubiquitin moiety to a high-energy E3~ubiquitin intermediate. They all share an active-site ubiquitin-binding Cys residue and are distinguished by the presence of a UBC domain required for binding of distinct E3s. In a few cases, they can also interact with the substrate [15]. The physiological significance of this interaction is not known. Eleven ubiquitin conjugating enzymes (Ubc1–8, 10, 11, 13) have been identified in the yeast genome. Two additional enzymes, Ubc9 and Ubc12, are members of the UBC family, although they conjugate the ubiquitin-like proteins Smt3 and Rub1, respectively, and not ubiquitin. Many more E2s have been described in higher organisms. Typically, each E2 interacts with a number of ligases, thus being involved in targeting numerous substrates.

C. Ubiquitin-Protein Ligases, E3s

The E3s, which are responsible for the specific recognition of the multitude substrates of the ubiquitin system, are the least defined components of the pathway and display the greatest variety among its different components [16]. The ubiquitin ligase is a protein or a protein complex that binds to both the E2 and the substrate. Interaction with the substrate can be direct or via ancillary proteins. In most cases (i.e., RING finger domain E3s, see below), the E3 serves as a scaffold that brings together the E2 and the substrate to the proximity that allows for efficient transfer of the activated ubiquitin moiety from E2 to the substrate. In other cases (HECT domain E3s), the activated ubiquitin is transferred from E2 to an internal Cys residue on E3 before conjugation of ubiquitin to an NH2 group in the target. Here, the E3 has a catalytic role [17]. An additional subset of E3s (U-box domain) termed also E4s serves as scaffold to aid in transfer of ubiquitin from the E2 to a previously conjugated ubiquitin moiety, in effect elongating polyubiquitin chains. Sites of ubiquitination vary among different substrates. For most proteins, the first ubiquitin moiety is conjugated to an ε-NH_2 group of an internal Lys residue. For at least three substrates, the transcription factor MyoD, the latent membrane protein 1 (LMP1) of the Epstein-Barr virus (EBV), and the E7 oncoprotein of the human papillomavirus (HPV) [18], it has been shown that the first ubiquitin moiety is attached to the free α-NH_2 terminus of the protein. In either class, additional ubiquitin moieties are then conjugated to an ε-NH_2 group of an internal Lys residue in the previously conjugated ubiquitin. As for the location of the Lys residues that are tagged in the target protein, no rules can be formulated. For signal-induced degradation of IκBα, it has been shown that the polyubiquitin chain is conjugated specifically to either Lys-21 or Lys-22 [19]. The same residues can also be sumoylated, possibly protecting the

inhibitor from ubiquitination and subsequent degradation. For p53, multiple Lys residues that reside in a limited region in the COOH-terminal domain (K372, K373, K381, and K382) are targeted by the E3 enzyme Mdm2 [20]; substitution of all of them decreased ubiquitination significantly. Interestingly, the same Lys residues are also targeted by acetyl groups, and it appears that acetylation and ubiquitination play opposite roles in governing the stability of the tumor suppressor [21]. In contrast, for cyclin B and the ζ-chain of the T cell receptor (TCR), mutagenic analyses indicate that there is no specificity as for the Lys residue targeted, and no single residue serves as a specific anchor for the polyubiquitin chain.

Modes of Substrate Recognition and Regulation of the Ubiquitin Pathway

Targeting of a protein via the ubiquitin system must involve specific binding of the protein to the appropriate ubiquitin ligase, E3. Despite recent progress in our understanding of modes of recognition and regulation of the system, it is only in a handful of cases where the E3 recognition motif has been identified precisely [22]. In principle, recognition can be mediated via several mechanisms: either the substrate is modified so as to be recognized or not by the appropriate E3, or the activity of the E3 can be modulated. Although the number of cases is still too low to make sweeping generalizations, the mode of regulation appears to correlate with the class of E3. Ubiquitination by SCF complexes requires phosphorylation of the substrate; APC activity is modulated by the presence of ancillary substrate-binding factors and/or by phosphorylation of the complex subunits, and activity of the HECT-domain proteins may also depend on ancillary proteins that bridge between the enzyme and the substrate [23]. E3α/Ubr1 appears to be constitutively active toward certain substrates and allosterically regulated toward others.

A. General Regulation

The ubiquitin-proteasome pathway can be regulated at the level of ubiquitination or at the level of proteasome activity. Because conjugation and proteasomal degradation is required for a multitude of cellular functions, regulation must be delicately and specifically tuned [24]. In two cases however, it was reported that general rather than specific components of the pathway could be modulated by physiological signals. One is the upregulation of the pathway that is observed during massive degradation of skeletal muscle proteins. This occurs in mammals under normal fasting, but also under pathological conditions such as cancer cachexia, severe sepsis, metabolic acidosis, or after denervation [25]. It occurs also during specific developmental processes, such as insect metamorphosis; the massive breakdown of larval tissue before the development of the imago is accompanied by upregulation of the ubiquitin pathway. A different case in which degradation can be regulated is by changing the specificity of the proteasome cleavage sites within the frame of its function in antigen presentation [26]. It was shown that in mammalian cells, three components of the 20S proteasome, two that are encoded within the MHC locus and one that is encoded by a different region, are upregulated after interferon-γ treatment. They replace three other proteasomal subunits that confer to the proteasome a different peptide cleavage specificity, presumably favoring the types of peptides that are better bound to the major histocompatibility complex (MHC) class I molecules of the presenting cell and the T cell receptor of the cytotoxic T cell (CTL) [27].

B. Specific Regulation

There are several modes for which specific substrate recognition is obtained such as:

1. Recognition via the NH2-terminal residue: the N-end rule pathway where the substrates bind directly to the ligase via their NH_2-terminal residue.
2. Peptide binding-mediated allosteric activation.

3. Phosphorylation of substrates and/or ubiquitination enzymes.
4. Recognition in trans.
5. Abnormal/mutated/misfolded proteins.
6. Recognition via specific sequences.
7. Regulation by ubiquitin-like proteins.
8. Regulation by masking of a degradation signal.
9. Regulation by specificity factors [28].

Recent Advances of Ubiquitin System

For many years, the accepted model for ubiquitination had been an E1/E2/E3-catalyzed cascade reaction in which the first ubiquitin moiety is anchored via its COOH-terminal Gly residue to an ε-NH2 group of an internal Lys residue in the target substrate. This is followed by generation of a polyubiquitin chain in which additional ubiquitin moieties are linked to one another via a Gly-76-Lys-48 isopeptide bonds. During the years, many "exceptions" to this "rule" have been discovered that can be classified into two groups namely reactions catalyzed by a different set of enzymes and reactions that utilize different anchoring sites whether on the substrate or on the ubiquitin moiety [29].

Drug Development for Targeting Aberrant Activity of the Ubiquitin System

Because of the central role the ubiquitin system plays in such a broad array of basic cellular processes, development of drugs that modulate the activity of the system may be difficult. Inhibition of enzymes common to the entire pathway, such as the proteasome, may affect many processes nonspecifically, although a narrow window between beneficial effects and toxicity can be identified for a short-term treatment. Recent experimental evidence strongly suggests that such inhibitors may indeed be beneficial in certain pathologies, such as in cancer, asthma, brain infarct, and autoimmune encephalomyelitis [30]. In malignancies, the drugs may act via inhibition of degradation of different cell cycle inhibitors, whereas in neuroprotection they may act via inhibiting activation of NF-κB, which elicits an inflammatory response. In autoimmune diseases, they may act by inhibiting presentation of self peptides, but also by interfering with signal transduction along cellular immune cascades [12]. A completely different approach to drug development can be, however, the development of small molecules that bind and inhibit specific E3s. For example, specific phosphopeptide derivatives that span the phosphorylation targeting domains in different substrates can serve as "baits" to the respective E3s [16]. This approach can turn, however, into a double-edge sword. In the case of p27 and IκBα, where phosphorylation destabilizes negative regulators, inhibition of the E3 can control disregulated cell cycle and decrease untoward activity of the immune system. Thus compounds that exert such activity can be thought of as potential drugs for the treatment of certain forms of malignancies and uncontrolled inflammatory states, respectively. However, the similarity between the phosphorylation sites of IκBα and β-catenin may lead also to stabilization of β-catenin, which is an activator, and its excessive transcriptional activity can result in malignant transformation of benign cells [6]. A better approach may be the development of small molecules that are substrate specific and bind, preferably, to specific substrates or to their ancillary proteins rather than to an E3. When accelerated degradation of a tumor suppressor results in exposure of cells to malignant transformation, selective inhibition of the recognition machinery can potentially reverse the malignant phenotype. Peptides that bind specifically to HPV-E6 and prevent its association with p53 can interfere with p53 targeting [10]. They were able to induce p53 in HPV-transformed cells with subsequent reversal of certain malignant characteristics or induction of apoptosis [1]. Treatment directed at increasing the level of p27Kip1resulted in regression of the malignant phenotype in experimental models. Although it is not clear that they

act via the ubiquitin system, interleukin-6 and phenylacetate, for example, lead to G1 arrest by increasing the level of p27 [31].

CONCLUSION

Evidently, no part of the cell is out of reach of the ubiquitin-proteasome regulatory system. Levels of proteins in the nucleus, cytoplasm, ER lumen, as well as membrane proteins, are all kept in check by the ubiquitinating enzymes and the proteasome. Ubiquitin tagging is common to the three major cellular pathways for protein degradation. Herein lies a conundrum: how is a given substrate targeted to a particular pathway? Variable parameters include location, chain length, and linkage type. A clear bias of the endosomal pathway toward K63-linked chains has emerged. This may simply reflect the subcellular localization of specific E3 ligases in combination with a high local concentration of ubiquitin-binding proteins, which couple to the ESCRT-machinery rather than the proteasome. New techniques allow for the determination of individual protein turnover on a global scale. This will enable the generation of a comprehensive annotation of turnover rates as a function of experimental perturbations or disease states, opening the door to a systems-level understanding of protein degradation.

What we can certainly expect in the near future is the identification of an ever-increasing number of substrates of the ubiquitin system and their specific E2/E3 complexes. It remains to be seen whether all the new E3s will belong to the known classes or whether new types of E3s will be identified. Consequently, but also in parallel, we can expect to see the development of an exciting area, that of specific modulators/drugs that can interfere with specific substrate recognition at different levels of the system in a variety of pathological states.

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