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The ubiquitin–proteolytic pathway: From obscurity to the patient bed

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Traditionally, researchers in the field of proteolysis have tried to characterize and purify a single protease while pursuing the activity they are studying. I used a similar approach at the beginning of my studies as a graduate student with Avram Hershko, when we initiated our efforts to purify the non-lysosomal, ATP-dependent proteolytic activity that Etlinger and Goldberg[7] and we[20] had characterized earlier in reticulocyte lysate. An obvious first step during the purification of proteins from red blood cells is to remove hemoglobin, the main protein in the extract, by anion exchange chromatography. When we fractionated the lysate, we could not recover the proteolytic activity in either the flow-through material that contained hemoglobin (fraction I) or in the adsorbed, high-salt-desorbed material (fraction II). The proteolytic activity could be recovered, however, after the addition of both fractions I and II to the reaction mixture[8] (Table 1). This was a critical experiment, as it taught us that this complex system contained at least two (and if two, possibly more, as was later shown to be true) complementary factors, and not a single, 'traditional' protease that was also ATP-dependent. From then on, we used the power of 'classical' biochemistry and a 'complementing-add-and-subtract' approach to initially purify from fraction I ATP-dependent proteolytic factor-1 (APF-1; ref. 21). We called the protein APF-1, as it was the first factor we characterized. At the same time we started to identify additional complementing factors, and looked for a simple terminology to enable convenient communication in the laboratory.

APF-1 was later identified as ubiquitin[22], a known protein of previously unknown function. Keith Wilkinson and Arthur Haas were post-doctoral fellows with Irwin A. Rose at the Fox Chase Cancer Center in Philadelphia, where Avram Hershko spent a sabbatical in 1977–1978 and, later, his summers. I joined him during the summers of 1978–1981. Wilkinson and Haas were fascinated by the new type of post-translational modification by APF-1 and, along with Michael Urban, who worked in a neighboring laboratory studying histones, identified APF-1 as ubiquitin. It was known that ubiquitin generates a single modified adduct with histones H2A and H2B; however, the function of these adducts has unexpectedly remained obscure to these very days. We adopted the existing terminology, and APF-1 became ubiquitin. In parallel, we showed that multiple molecules of the protein are conjugated covalently to the target substrate and suggested that they serve as a degradation signal[12,13]. Initially we thought that each ubiquitin moiety attaches to a single internal Lys in the target molecule. Later Hershko[23] and then Varshavsky[24] and their colleagues demonstrated the formation of a poly-ubiquitin chain anchored to a single internal Lys residue. In the conjugation reaction, the C-terminal group of ubiquitin (Gly) generates a high-energy, isopeptide bond with an ε-amino group of an internal Lys residue of the substrate. Later we showed that the first conjugation event can also involve the N-terminus residue of the protein. We then went on to dissect the mechanism that underlies the activation of ubiquitin that must precede the generation of the high-energy bond with the target protein. Here, we made use of the known mechanism of amino-acid activation during protein synthesis, but mostly of the mechanism shown by Fritz Lipmann and his colleagues when they reconstituted a cell-free, non-ribosomal biosynthesis of the bacterial deca-peptide antibiotic gramicidin S (ref. 25). The similarities between the three mechanisms of activation of animo acids for protein and peptide synthesis and ubiquitin were strikingly similar. Dissection of the activation mechanism of ubiquitin paved the road to purification, by reaction-based, 'covalent' affinity chromatography over immobilized ubiquitin, of the three sequentially acting, conjugating enzymes: the ubiquitin-activating enzyme E1, the ubiquitin carrier protein E2 and the ubiquitin protein ligase E3, which accomplishes the last and most-essential step in the conjugation reaction, specific ligation of ubiquitin to the target protein[16,26]. In 1980, before the identification of APF-1 as ubiquitin and the dissection of the conjugation mechanism, we had already proposed a model[13] (Fig. 2a) according to which conjugation of multiple APF-1 molecules targets the substrate to degradation by an unknown, yet conjugate-specific, downstream protease. Intact APF-1 that can be re-used is recycled through the activity of isopeptidases. The protease, the 26S proteasome complex, was characterized and purified later by Martin Rechsteiner and colleagues[18]. In 1982, a more-detailed model was described, and the system began to be placed in its appropriate biological context[6]. In particular, its functions were analyzed and compared to those of the lysosomes involved mostly in the degradation of extracellular proteins taken up through pinocytosis and receptor-mediated endocytosis[6].

The physiological relevance of our initial findings remained unknown, as until that time (1981) we did all our studies in a cell-free reconstituted system, using secretory (and not intracellular; as these were available in large amounts and low cost) proteins as model substrates. The first evidence that the system is involved in degradation of proteins *in vivo* came from immunochemical analysis of ubiquitin adducts in cells[27]. Using antibodies raised against ubiquitin, we showed that, after incubation of cells in the presence of amino-acid analogs, the resulting abnormal proteins are short-lived. Their rapid degradation is accompanied by a transient, yet substantial increase in the level of ubiquitin adducts, strongly indicating that they serve as essential intermediates in the proteolytic process. Later, stronger and more-direct proof of the involvement of the ubiquitin system in the degradation cellular proteins came from the observation Alexander Varshavsky, Daniel Finely and I made that a cell-cycle arrest mutant that contains a thermolabile E1 enzyme is also defective in the degradation of short-lived abnormal proteins at the non-permissive temperature[28,29].

An important yet unresolved problem at that time involved the identification of the specific signaling motifs that target proteins for degradation. My initial entry into this fascinating area

Table 1 Anion exchange chromatographical resolution of reticulocyte lysate into unadsorbed (fraction I), and adsorbed, high-salt-desorbed (fraction II) complementing proteolytic activities.

Enzyme fraction used	Percent degradation of labeled globin	
	(–) ATP	(+) ATP
Complete lysate	1.5	10.0
Fraction I (flow-through material)	0	0
Fraction II (high salt desorbed eluate)	1.5	2.7
Fraction I + Fraction II	1.6	10.6

Adapted with modifications from ref. 8.

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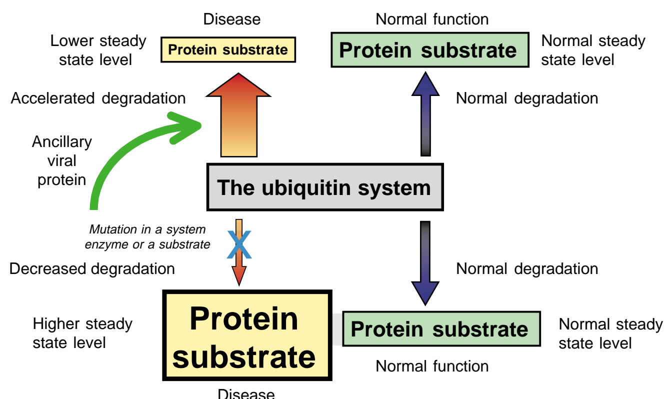

Fig. 3 Accelerated or decreased rates of ubiquitin-mediated proteolysis can underlie the pathogenesis of human diseases. Protein substrates are degraded each at a distinct and specific rate that may vary in different conditions (blue arrows), and maintain a steady-state level (green boxes) that enables them to function properly. Accelerated degradation (thick red arrow), as occurs in HPV E6-targeted degradation of p53, results in a low steady-state level of the target substrate (small beige box) and exposure of the cell to malignant transformation. Decreased degradation (narrow red arrow with a blue X) can occur when the signaling motif in the substrate is mutated (mutations in the phosphorylation sites in β-catenin in certain cases of malignant melanoma or colorectal carcinoma, or mutations in the NEDD4 E3 recognition motif of the kidney epithelial sodium channel in Liddle hypertension syndrome); or when E3 is mutated (as in Angelman syndrome, in which E6-AP is mutated). In all these examples, the excess accumulated substrate (large beige box) is 'toxic'.

was unanticipated. A large-scale purification of APF-1 carried out in Haifa in 1980 showed a substantial discrepancy between its high dry weight and low protein content, as determined by a Lowry assay. Hershko and I thought that the protein was probably a ribonucleoprotein complex, and the excess non-protein mass was due to the RNA component. Treatment of the APF-1 preparation with RNase A led to abrogation of its stimulatory activity towards bovine serum albumin (BSA), but not lysozyme. Not appreciating at that time the extent of the high specificity of the system toward its different substrates, we could not explain the 'selective' RNase effect, and did not pursue the study. The discrepancy between weight and protein measurement was resolved later with the finding that ubiquitin has a low content of the aromatic amino acids that are the basis of every known method for protein measurement. Obviously, ubiquitin is a pure protein and not a ribonucleoprotein complex. Later, our studies showed that nuclease added to the ubiquitin preparation destroyed $tRNA^{Arg}$ that was necessary, along with arginyl–tRNA protein transferase, to convert the N-terminal acidic (Asp) residue of BSA to Arg (refs. 30,31). Only the modified BSA and not the wild-type BSA can bind to the 'basic' N-terminal binding site of E3α, the ubiquitin ligase involved in recognition via the N-terminal residue (the N-end rule pathway ligase; ref. 15 and see below commentary by A. Varshavsky). Lysozyme, with a Lys at the N-terminal residue, does not undergo this post-translational modification and is recognized directly by E3α, and its degradation, therefore, is not sensitive to RNase. This finding became part of the more thorough and systematic mode of recognition identified through genetic tools by Alexander Varshavsky and his colleagues, and is known as the N-end-rule pathway[32]. Earlier, Hershko also noted the importance of an exposed N-terminal residue in targeting certain model proteins for degradation[33], but at that time, the mechanistic relevance of this finding was not apparent. Although most known substrates of the ubiquitin system are targeted through different recognition motifs[34], the N-end-rule pathway was the first well-defined signal of substrate recognition (see commentary by A. Varshavsky).

As for the myriad cellular substrates and regulatory functions of the ubiquitin system now known, it was only in the early 1990s that scientists started to unravel these secrets. The discovery of oncoproteins, tumor suppressors, transcriptional factors and cell-cycle regulators have shown that all these proteins are short-lived and their stability is tightly regulated. This previously unknown mode of regulation through destruction, which, unlike phosphorylation, is irreversible and may have evolved to secure directionality, has attracted many scientists to study the systems and signals that govern the stability (and hence the activity) of many different proteins[35–38]. Without even knowing the underlying mechanisms, Varshavsky, Finley and I predicted that the ubiquitin system may be involved in the regulation of the cell cycle[28]. The prediction was based on the observation that the ts85 mutant cell is defective in both E1 that inactivates the ubiquitin system and in transition along the S/G2 boundary of the cell cycle. This prediction has been corroborated by many studies demonstrating involvement of the ubiquitin system in programmed degradation of a broad array of cell cycle regulators.

Given the many processes and substrates involved, recent indications of the involvement of the system in the pathogenesis of many inherited as well as acquired diseases are not unexpected. Drug companies are trying to target the aberrations in the system that underlie these pathologies. The common denominator shared by all these diseases is a change in the steady-state level of a particular protein substrate or set of protein substrates. In general, the diseases belong to two classes, resulting from accelerated or decreased rates of degradation of different substrates (Fig. 3). The second class can be further divided into diseases due to mutations in enzymes of the system or to mutations in recognition motifs of substrates (Fig. 3). Although it is not possible to systematically review here all these diseases (reviewed recently in ref. 39), a few salient examples follow. An example of accelerated degradation involves the degradation of p53 induced by the human papillomavirus (HPV) E6 oncoprotein, which probably underlies the pathogenesis of human uterine cervical carcinoma, a very prevalent and severe malignant disease. E6 associates with p53 and targets it for rapid degradation mediated by the ubiquitin ligase E6-associated protein (E6-AP), which does not recognize the free tumor suppressor. Inactivation of the cellular DNA damage-control machinery exposes the cell to malignant transformation. Mutations in E6-AP lead to Angelman syndrome, an inherited disease associated with severe mental retardation and motor disorders. Here, accumulation of unidentified native substrate(s) of E6-AP (p53 is not the native substrate of the enzyme and is normally targeted by Mdm2) is probably toxic to the developing brain. In another example, mutations in the phosphorylation-targeting motif of the transcription factor β-catenin, or in adenomatosis polyposis coli (APC) that is part of the β-catenin degradation complex, lead to stabilization and accumulation of the protein, accompanied by its uncontrolled activity. These mutations may be involved in the pathogenesis of many forms of colorectal carcinomas and malignant melanomas. Viruses such as HPV have evolved different mechanism that enable them to evade the normal mode of

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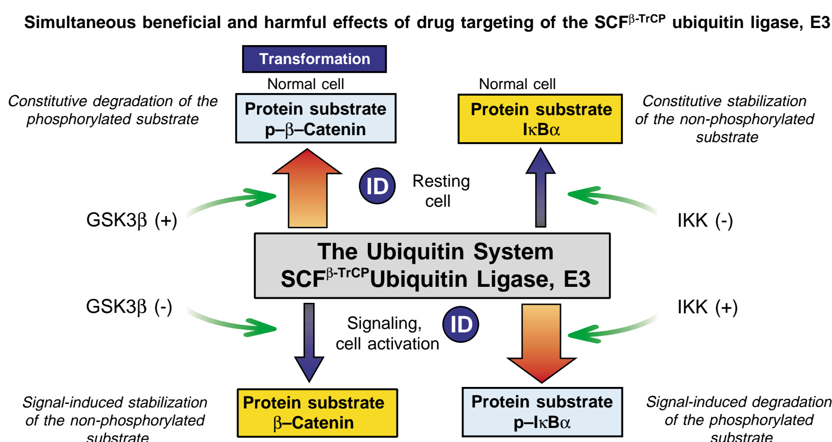

Fig. 4 Drug targeting of an E3 enzyme can become a 'double-edged sword'. In the resting cell (top), IκBα (blue arrow and beige box, upper right) is not phosphorylated and degraded slowly, whereas β-catenin is phosphorylated constitutively by GSK3β and is degraded rapidly following ubiquitination by the SCF$^{β\text{-}TrCP}$–E3–ubiquitin–ligase complex (heavy red arrow and light blue box, upper left). In the signaled cell (bottom), IκBα is phosphorylated by IκB kinase (IKK), rapidly ubiquitinated by the same SCF–TrCP complex and degraded (thick red arrow and light blue box, lower right). Also, in signaled cells, GSK3β is inhibited, and the non-phosphorylated β-catenin is stabilized, translocated into the nucleus and stimulates transcription (blue arrow and beige box, lower left). In general, IκB-containing cells are distinct from β-catenin-containing cells and so are the signals that activate the two pathways. However, the phosphorylated recognition motifs of the two proteins are similar and they seem to be targeted by the same TrCP ubiquitin ligase. An E3 inhibitor (inhibitory drug, ID) can lead to inhibition of degradation of IκB in stimulated cells, and consequently to suppression (beneficial effect) of NF-κB-induced inflammatory processes that may occur in autoimmune diseases, for example. At the same time, ID treatment will result in suppression of degradation of phosphorylated β-catenin in resting cells, with resultant accumulation of the transcription factor and possible subsequent malignant transformation (harmful effect).

activity of the ubiquitin system and allow them to continue their replication and propagation. Epstein Barr nuclear antigen 1 (EBNA-1) persists in healthy carriers for life, and its persistence contributes to some of the virus-related pathologies. Unlike all other Epstein Barr viral proteins, EBNA-1 cannot elicit a cytotoxic T lymphocyte (CTL) response. A long, C-terminal Gly-Ala repeat inhibits ubiquitin-mediated degradation and subsequent major histocompatibility complex (MHC) class I antigen presentation of EBNA-1. The human cytomegalovirus (CMV) encodes two endoplasmic reticulum (ER) resident proteins, US2 and US11, that bind to MHC class I molecules in the ER and escort them to the translocation machinery. After retrograde transport to the cytoplasm, they are ubiquitinated and degraded by the proteasome. Removal of the MHC molecules enables the virus to evade the immune system. A completely different case involves Liddle syndrome. In this disorder, a mutation in the recognition motif that targets the kidney epithelial sodium channel (ENaC). to ubiquitination by the Nedd4 E3 leads to accumulation of the channel, excessive reabsorption of sodium and water, with resulting severe hypertension.

Because of the central function of the ubiquitin system in many basic cellular processes, development of drugs that modulate 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. An attractive possibility is the development of small molecules that inhibit specific E3 molecules. For example, specific phospho-peptide derivatives can inhibit the β-TrCP ubiquitin ligase, E3 complex (β-Transducin repeat-Containing Protein[40]). However, this approach can turn into a 'double-edged sword' (Fig. 4). Ideally, small molecules should be developed that bind to specific substrates or to their ancillary proteins, and thus inhibit a specific process. Peptide aptamers (small molecules/peptides that bind to active/association sites of proteins and inhibit their native interactions) that bind specifically to HPV E6 and probably prevent its association with p53, have been shown to induce apoptosis and reverse certain malignant characteristics in HPV-transformed cells, probably by interfering with p53 targeting[41]. Unfortunately, because of the rarity of proteins targeted by similar mechanisms, this approach may be currently limited to a small number of cases.

Discovering the functions and degrons of the ubiquitin system

ALEXANDER VARSHAVSKY

Through preparation, help from friends and a lot of luck I was able to leave the former Soviet Union in the fall of 1977, and ended up in Boston. A month later I was a faculty member of the Biology Department of the Massachusetts Institute of Technology (MIT), before I knew what exactly grants were (and before the colleagues who hired me became aware of that fact). In Moscow, I studied chromosome structure and regulation of gene expression, and looked forward to continuing this work.

There were few similarities between my earlier milieu and the astonishing new life. The libraries were one of them. They were just as quiet and pleasant in Cambridge as in Moscow, and a library at MIT soon became my second home. Reading there I came across a curious 1977 paper by Harris Busch, Ira Godknopf and their colleagues. They found a DNA-associated protein that had one C-terminus but two N-termini, an unprecedented structure. The short arm of that Y-shaped protein was joined, through its C-terminus, to an internal Lys of histone H2A. The short arm was soon identified, by Margaret Dayhoff, as ubiquitin, a 76-residue protein of unknown function that was described (as a free protein) by Gideon Goldstein and colleagues in 1975 (ref. 10).

I became interested in this first ubiquitin conjugate, UbH2A. Back in Russia, I had begun to develop a method for high-resolution analysis of nucleosomes. These DNA–protein complexes were subjected to electrophoresis in a low-ionic-strength polyacrylamide gel (a forerunner of the gel-shift assay), followed by second-dimension electrophoresis of either DNA or proteins. We located UbH2A in a subset of the nucleosomes, succeeded in separating these nucleosomes from those lacking UbH2A, and eventually showed that UbH2A-containing nucleosomes were enriched in transcribed genes and excluded from the inactive (heterochromatic) parts of the chromosomes[9].

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