Basic Medical Research Award

The ubiquitin system

AVRAM HERSHKO, AARON CIECHANOVER & ALEXANDER VARSHAVSKY

Ubiquitin-mediated protein degradation: The early days

AVRAM HERSHKO

It has been often stated that until recently the ubiquitin system was thought to be mainly a 'garbage disposal' for the removal of abnormal or damaged proteins. This statement is certainly not true for those who have been interested in the selective and regulated degradation of proteins in cells. The dynamic turnover of cellular proteins was discovered in the pioneering studies of Rudolf Schoenheimer in the 1930s, when he first used isotopically labeled compounds for biological studies[1]. Between 1960 and1970 it became evident that protein degradation in animal cells is highly selective, and is important in the control of specific enzyme concentrations[2]. The molecular mechanisms responsible for this process, however, remained unknown. Some imaginative models have been proposed to account for the selectivity of protein degradation, such as one suggesting that all cellular proteins are rapidly engulfed into the lysosome, but only short-lived proteins are degraded in the lysosome, whereas long-lived proteins escape back to the cytosol[3].

I became interested in the mechanisms of intracellular protein breakdown when I was a post-doctoral fellow in the laboratory of Gordon Tomkins 30 years ago (1969–1971). At that time, the main subject in that laboratory was the mechanism by which corticosteroid hormones cause the increased synthesis of the enzyme tyrosine aminotransferase. I found this subject a bit crowded, so I chose to study a different process that also regulates tyrosine aminotransferase concentration: the degradation of this enzyme. I found that the degradation of tyrosine aminotransferase in cultured hepatoma cells is completely arrested by inhibitors of cellular energy production, such as fluoride or azide[4]. These results confirmed and extended the previous observations of Simpson on the energy dependence of the release of amino acids from liver slices[5]. Similar energy requirements for the degradation of many other cellular proteins were subsequently found in a variety of experimental systems[6].

I was very impressed by the energy dependence of intracellular protein breakdown, because proteolysis itself is an exergonic process that does not require energy. I assumed that there was an as-yet-unknown proteolytic system that uses energy for the highly selective degradation of proteins. After returning to Israel in 1971 and setting up my laboratory at the Technion, my main goal was to identify the energy-dependent system responsible for the degradation of cellular proteins. It took a bit of faith to base my entire research project on the effects of energy 'poisons', because these inhibitors could affect protein breakdown rather indirectly. For example, I remember that when Racker, a great biochemist, visited my laboratory in Haifa in the mid-1970s, he dismissed these observations as being secondary to the inhibition of the proton pump, which maintains the acidic environment in lysosomes. I was convinced, however, that lysosomal autophagy cannot account for the selectivity and regulation of intracellular protein breakdown. I was also convinced that the best way to identify a new system was that of classical biochemistry: to reproduce ATP-dependent protein breakdown in a cell-free system and then to fractionate such a system and to find the mode of action of its components.

An ATP-dependent proteolytic system from reticulocytes was first described by Etlinger and Goldberg[7], and then was analyzed by our biochemical fractionation–reconstitution studies. In this work, I was greatly helped by Aaron Ciechanover, who was then my graduate student. Substantial support and advice were provided by Irwin Rose, who hosted me in his laboratory in Fox Chase Cancer Center for a sabbatical year in 1977–1978 and many times afterwards. Initially, reticulocyte lysates were fractionated on DEAE-cellulose into two crude fractions: fraction 1, which was not adsorbed, and fraction 2, which contained all proteins adsorbed to the resin and eluted with high salt. The original aim of his fractionation had been to remove hemoglobin (present in fraction 1), but we found that fraction 2 lost most of ATP-dependent proteolytic activity. Activity could be restored by combining fractions 1 and 2. The active component in fraction 1 was a small protein that we purified

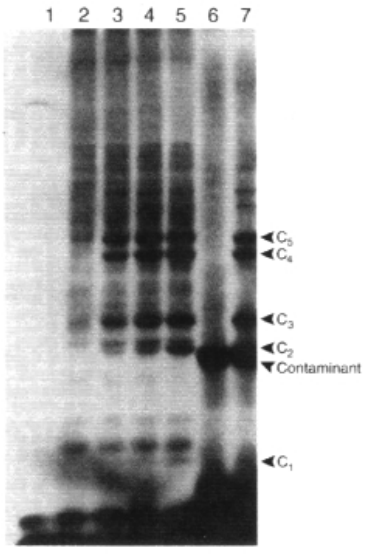

Fig. 1. Discovery of the ligation of ubiquitin to lysozyme, a substrate of the proteolytic system. Reaction products were separated by SDS–PAGE. Lane 1, incubation of ^{125}I-labeled ubiquitin with fraction 1 in the absence of ATP; ubiquitin remains free and migrates at the front. Lanes 2–5, incubation of ^{125}I-labeled ubiquitin with fraction 1 in the presence of ATP. Lane 2, ubiquitin becomes covalently linked to many high-molecular-weight derivatives, presumably endogenous protein substrates present in fraction 2. Lanes 3–5, several new labeled bands appear (C1–C5), which increase with increasing concentrations of lysozyme. Lanes 6 and 7, incubation of ^{125}I-labeled lysozyme with fraction 2 in the absence of ATP (lane 6) or with ATP and unlabeled ubiquitin (lane 7); bands C1–C5 contain the label of ^{125}I-labeled lysozyme and consist of increasing numbers of ubiquitin molecules ligated to lysozyme. Reproduced from ref. 13, with permission.

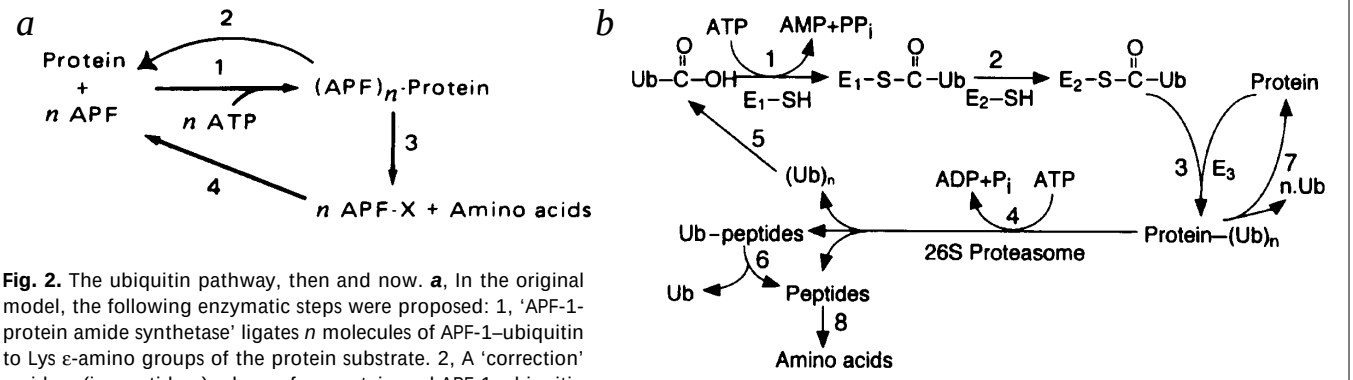

Fig. 2. The ubiquitin pathway, then and now. ***a***, In the original model, the following enzymatic steps were proposed: 1, 'APF-1-protein amide synthetase' ligates *n* molecules of APF-1–ubiquitin to Lys ε-amino groups of the protein substrate. 2, A 'correction' amidase (isopeptidase) releases free protein and APF-1–ubiquitin from erroneous ligation products. 3, An endopeptidase (protease) specifically acts on proteins ligated to several molecules of APF-1 and cleaves peptide bonds with the liberation of APF-1 still linked to Lys or a Lys-containing peptide (APF-1-X). 4, Amidase (isopeptidase) cleaves the bond between APF-1 and the ε-amino group of Lys residues and thus liberates reusable APF-1–ubiquitin. Reproduced from ref. 13, with permission. ***b***, Current information on the enzymatic reactions of the ubiquitin system. Steps 1, 2 and 3, accomplished by E1, E2 and E3, correspond to step 1 of the original model. Step 4, accomplished by the 26S proteasome, corresponds to step 3 of the original model. Steps 5, 6 and 7, accomplished by ubiquitin–carboxy-terminal hydrolases (isopeptidases), correspond to steps 2 and 4 of the original hypothesis. Reproduced from ref. 15, with permission.

by taking advantage of its remarkable stability to heat treatment[8]. It was first called APF-1, for ATP-dependent proteolysis factor 1. The identification of APF-1 as ubiquitin was later made by Wilkinson and co-workers[9], after our discovery of its ligation to proteins. Ubiquitin was first thought to be a thymic hormone, but subsequently was found to be present in many tissues and organisms, hence its name[10]. It was found to be conjugated to histone 2A (ref. 11), but its functions remained unknown. Although we did not know at that time that APF-1 was ubiquitin, I will use the term ubiquitin here to facilitate the discussion.

The purification of ubiquitin from fraction 1 was the key to the elucidation of the mode of its action in the proteolytic system. At first I thought that it could be an activator, or a regulatory subunit of a protease or other enzyme component of the system present in fraction 2. To examine this possibility, purified ubiquitin was radioiodinated and incubated with crude faction 2 in the presence or absence of ATP. There was substantial ATP-dependent binding of ^{125}I-labeled ubiquitin to high-molecular-weight proteins by gel filtration chromatography[12]. However, a covalent amide linkage was unexpectedly formed, as shown by the stability of the 'complex' to treatment with acid, alkali, hydroxylamine or boiling with SDS and mercaptoethanol[12]. Analysis of the reaction products by SDS–PAGE showed that ubiquitin was ligated to many high-molecular-weight proteins. Because crude fraction 2 from reticulocytes contains not only enzymes but also endogenous substrates of the proteolytic system, we began to suspect that ubiquitin might be linked to protein substrates, rather than to an enzyme. In support of this interpretation, we found that proteins that are good (although artificial) substrates for ATP-dependent proteolysis, such as lysozyme, form several conjugates with ubiquitin[13]. In the original experiment that convinced us that ubiquitin is ligated to the protein substrate, similar high-molecular-weight derivatives were formed when ^{125}I-labeled ubiquitin was incubated with unlabeled lysozyme (Fig. 1, lanes 3–5), and when ^{125}I-labeled lysozyme was incubated with unlabeled ubiquitin (Fig. 1, lane 7). Analysis of the ratio of radioactivity in ubiquitin and lysozyme indicated that the various derivatives consisted of increasing numbers of ubiquitin molecules linked to one molecule of lysozyme. On the basis of these findings, we proposed a model in 1980 (Fig. 2*a*) in which several molecules of APF-1–ubiquitin are linked to Lys ε-amino groups of the protein substrate by an 'APF-1-protein amide synthetase' (Fig. 2*a*, step 1). We proposed that proteins ligated to several ubiquitins were broken down by a specific protease that recognizes such conjugates (Fig. 2*a*, step 3). Thus, the protein would be broken down to free amino acids and to APF-1–ubiquitin still linked by isopeptide linkage to Lys or a small peptide (APF-1-X). Finally, free APF-1–ubiquitin is released for re-use by the action of a specific amidase/isopeptidase (Fig. 2*a*, step 4). Based on a suggestion by Ernie Rose, we added a hypothetical 'correcting' isopeptidase to this scheme, which would release free ubiquitin and substrate protein from products of erroneous ubiquitin–protein ligation (Fig. 2*a*, step 2). An isopeptidase that may have such correction function was described recently[14].

Comparison of the original model with our current knowledge of the reactions of the ubiquitin pathway[15] (Fig. 2*b*) shows that the original model was essentially correct, but much further detail provides explanation for the high selectivity of ubiquitin-mediated protein degradation. Thus, we have found that 'APF-1-protein amide synthetase' is actually composed of three types of enzymes: a ubiquitin-activating enzyme E1, a ubiquitin-carrier protein E2 and a ubiquitin-protein ligase E3 (ref. 16). Specific E3 enzymes recognize specific structural features in specific protein substrates, and thus account for substrate selectivity[17]. Proteins ligated to multi-ubiquitin chains are degraded by a 26S proteasome complex discovered by Rechsteiner and co-workers[18]. ATP is needed not only for the ubiquitin–protein ligation reaction, as originally proposed, but also for the action of the 26S proteasome[18]. Finally, free and reusable ubiquitin is released by the action of a large variety of ubiquitin C-terminal hydrolases[15].

As indicated before[19], the main lesson from our story is the continued importance of the use of biochemistry in modern biomedical research. Without biochemistry, it is doubtful whether an entirely new system could have been discovered. On the other hand, molecular genetics has been essential in discovering the many functions of this system in processes such as cell cycle control, signal transduction and the immune response[15].