The Nobel Prize in Chemistry 2004

'Ubiquitous' Quality Control of Life

C S Karigar and K R Siddalinga Murthy

The Nobel Prize in Chemistry for 2004 is shared by Aaron Ciechanover, Avram Hershko and Irwin Rose, who made fundamental discoveries concerning how cells regulate the breakdown of cellular proteins with extreme specificity. The three biochemists discovered ubiquitin-mediated proteolysis, a process where an enzyme system tags unwanted proteins with many molecules of a small protein called ubiquitin and then sends then to the proteasome where they are broken down.

Protein Degradation in the Cell

Eukaryotic cells, from yeast to humans, contain some 6000 to 30000 protein-encoding genes and at least as many proteins. While much attention and research has been devoted to how proteins are synthesized, the reverse process, i.e. how proteins are degraded, has received little attention. Mammalian cells contain two distinct proteolytic pathways that are involved in different aspects of protein breakdown. Proteins that enter the cell from the extracellular milieu (such as receptor-mediated endocytosed proteins) are degraded in lysosomes under stress conditions. Nonlysosomal mechanisms are responsible for the highly selective turnover of intracellular proteins that occurs under both basal metabolic, as well as stress conditions. An important nonlysosomal proteolytic pathway is the energy requiring process involving the 'ubiquitin system' in which proteins are degraded by a 26S protease complex following conjugation by multiple molecules of 'ubiquitin'. The 'catalytic core' of the complex is a 20S protease complex also known as the proteasome.

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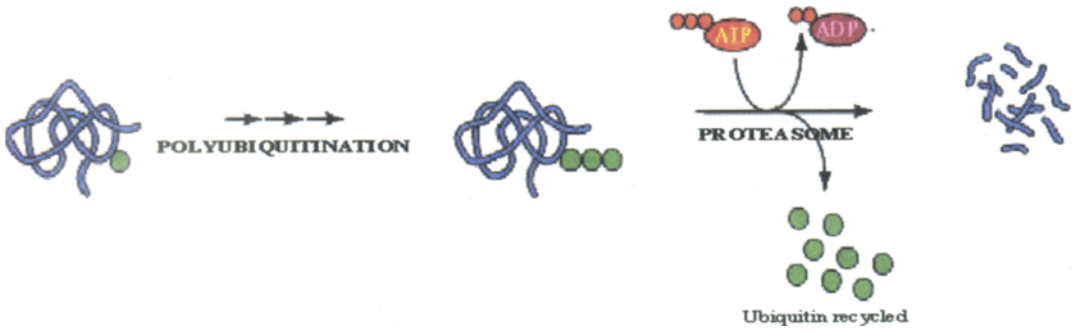

The Core Particle (CP): The core particle is made of 2 copies of each of 14 different proteins.

These are assembled in groups of 7 forming a ring.

The 4 rings are stacked on each other (like 4 donuts).

The Regulatory Particle (RP): There are two identical RPs, one at each end of the core particle.

Each is made of 14 different proteins (none of them the same as those in the CP).

Six of these are ATPases.

Some of the subunits have sites that recognize the small protein ubiquitin.

Ubiquitin: A small protein (76 amino acids).

Conserved throughout all the kingdoms of life; that is, virtually identical in sequence whether in bacteria, yeast, or mammals.

Used by all these creatures to target proteins for destruction.

STRUCTURE OF THE PROTEASOME

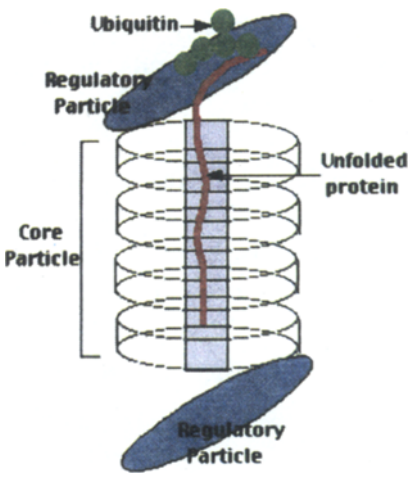

Figure 1. Polyubiquitination and proteolysis.

Ubiquitin

Ubiquitin, a monomeric protein comprising 76 amino acids, is involved in the nonlysosomal protein degradation process. The structure of ubiquitin reveals an extended carboxyl terminus that is activated and linked to other proteins. Lysine residues on the molecule are important, including lysine 48, the major site for linking additional ubiquitin molecules. Ubiquitin derives this name owing to its ubiquity and abundance in various cells and is one of the most highly conserved eukaryotic proteins

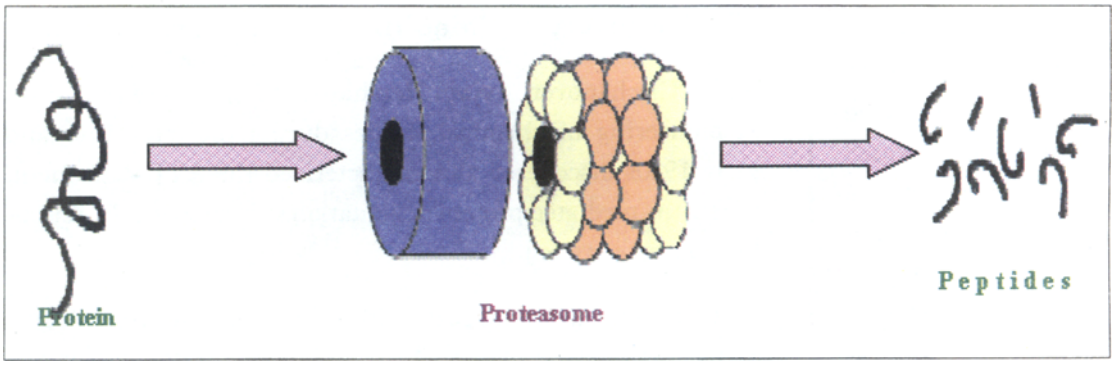

Figure 2. Role of proteasome in protein.

known (it is identical in such diverse organisms as humans, trout, and fruit fly) suggesting that it is uniquely suited for an essential cellular function.

Ubiquitination

There are two main steps involved in the protein ubiquitination: the covalent attachment of an ubiquitin chain to the substrate (*Figure* 1), and specific recognition and degradation of the tagged protein by the 26S proteasome (*Figure* 2).

Ubiquitination is a multiple step process. Ubiquitin is initially activated in the presence of ATP to a high-energy thiol ester intermediate by the ubiquitin-activating enzyme (E1), which transfers ubiquitin via a thiol ester linkage to ubiquitin-conjugating enzymes (E2s). E2s and/or ubiquitin-protein ligases (E3s), play an important role in the selection of proteins for conjugation. They bind the first ubiquitin molecule to protein substrates via an isopeptide bond between the activated C-terminal glycine residue of ubiquitin and the ε-amino group of a lysine residue of the substrate. The resulting monoubiquitinated protein is usually not targeted for degradation by the proteasome. Alternatively, E2s and/or E3s catalyze the formation of polyubiquitinated conjugates.

The ubiquitin-conjugating system in mammals consists of a single E1, at least 20-30 E2s, and several dozens of E3s. Ubiquitinated proteins are dynamic entities with ubiquitin molecules being rapidly attached and removed.

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The Discovery of Ubiquitin-Mediated Proteolysis

Aaron Ciechanover, Avram Hershko and Irwin Rose in a series of pioneering biochemical studies during the late 1970s and early 1980s discovered and characterized the ATP-dependent, ubiquitin-mediated protein degradation system using the reticulocyte lysate system.

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The first breakthrough came in 1978 when the reticulocyte extract was passed over a DEAE-cellulose column to remove hemoglobin. The proteins separated into two fractions, each one individually inactive, but recombination of the two fractions restored the ATP-dependent proteolysis. The first fraction, a heat-stable protein with a molecular weight around 9000 (called APF-1) was identified as ubiquitin by Wilkinson and his colleagues. The following year Hershko, Ciechanover and Rose reported the separation of the second fraction by salt precipitation into an ATP-stabilized protein of around 450 kDa, containing the proteasome, but nobody followed up the observation for almost a decade. The E1-E3 enzymes were later isolated from this fraction. All three fractions were required to degrade ^{125}I-labelled albumin as substrate.

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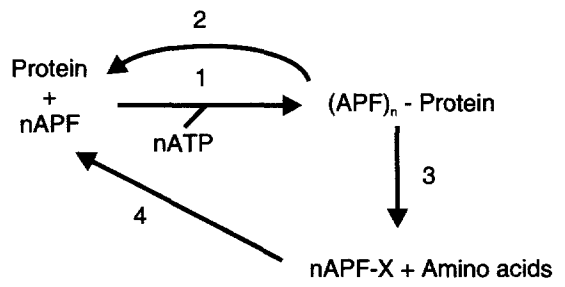
The second breakthrough came in 1980 and was described in two papers communicated to the journal, *Proceedings of the National Academy of Sciences*, USA. One was the unanticipated discovery of multiple ubiquitin conjugate to the same substrate molecule. Further, a deubiquitinating enzyme activity was also found in the lysate that could release the conjugated ubiquitin from substrate molecules. The paper demonstrated two novel enzymatic activities; an ubiquitin-protein amide synthetase and an amidase. A scheme (*Scheme* 1) was presented showing the proposed sequence of events in ATP-dependent protein degradation:

These totally unexpected discoveries completely changed the direction of the work. Instead of looking for an ATP-dependent protease, the group now sought to identify the enzyme system



1. APF-1-protein amide synthetase;
2. Amidase;
3. Peptidases;
4. Amidase for APF-1-X where X is lysine or a small peptide.

Scheme 1.

that conjugated ubiquitin to the substrate. Between 1981 and 1983, Ciechanover and his colleagues worked out the multi-step ubiquitin-tagging hypothesis by isolating and characterizing three separate enzyme activities, E1, E2 and E3. During the purification of the ubiquitin-activating enzyme E1, an elegant covalent affinity chromatography method was developed. This method was crucial for the subsequent purification of the E2 and E3 enzymes. When a reticulocyte lysate fraction containing the activating enzyme in the presence of ATP was applied to a Sepharose column containing covalently attached ubiquitin and then eluted with high-salt (E3), AMP plus pyrophosphate (E1) and dithiothreitol (E2), the three fractions of E3, E1 and E2, respectively were recovered, which fully reconstituted ATP-dependent proteolysis when mixed with ubiquitin, ATP and unadsorbed fraction. The activating enzyme E1 was purified to homogeneity and is a homo-dimer of 210 kDa. The E2 and E3 fractions were further purified with gel filtration chromatography where the E2 activity eluted as a single peak with an apparent molecular weight of 35 kDa and the E3 activity as a peak with an apparent molecular weight of 300 kDa. Importantly, the gel filtration profiles of the ubiquitin conjugation activities of E1, E2 and E3 were identical to their protein

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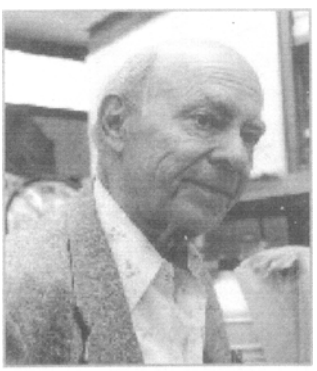
Aaron Ciechanover

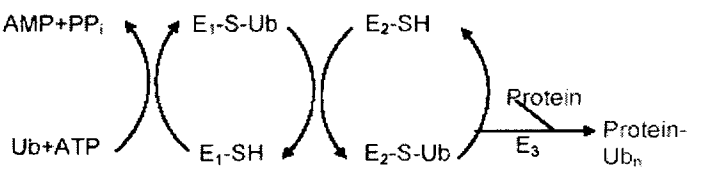
Avram Hershko



Irwin Rose

degradation activities. Therefore, the three enzymes of the ubiquitin-protein ligase system were identical with the three factors participating in protein degradation.

They hypothesized that E1 transfers ubiquitin to E2 and E2 participates in the transfer of ubiquitin finally to the substrate. No ubiquitin transfer from E1-ubiquitin to E3 was detected. Instead, E3 catalysed the transfer of labeled ubiquitin from E2 to the protein substrate, forming amide bonds. The full conjugation pathway involves binding of activated ubiquitin via its COOH terminal glycine to the thiol site of E1 followed by its transfer to another sulfhydryl site on E2. Ubiquitin is then further transferred from E2-bound thiol esters to stable protein conjugates in the presence of E3. The E3 catalysed reaction is iterated on the original substrate, resulting in polyubiquitination. All the above studies were made in a cell-free system.



To study the physiological functions of ubiquitin-mediated proteolysis, Hershko *et al* devised an immunochemical method for isolating ubiquitin-protein conjugates from intact cells. Cells were first pulse-labeled with tryptophan, an amino acid that is absent in ubiquitin. Using an antibody against ubiquitin, they could then specifically measure proteolysis of the protein substrate of ubiquitin conjugates. The results showed a marked increase in labeled ubiquitin-protein conjugates during the formation of abnormal proteins in reticulocytes induced by adding amino acid analogues. The ubiquitin-protein conjugates were degraded more rapidly than general cell proteins. The phenomenon was not restricted to reticulocytes, but was also observed in Ehrlich ascites cells. These data were consistent with the interpretation that the degradation of abnormal intracellular proteins is carried out by ubiquitin-mediated proteolysis. However,

the results "do not exclude the participation of the ubiquitin pathway in some other types of protein breakdown". There is now experimental evidence that up to 30% of the newly synthesized polypeptides in a cell are selected for rapid degradation in the proteasome because they do not pass the quality control system of the cell.

Role of Proteasome in Protein Degradation

The second major step in the ubiquitin-proteasome pathways is the degradation of polyubiquitinated proteins by the 26S proteasome, which is formed by the binding of two 19S regulatory complexes with the 20S proteasome. Proteasomes are found both in the nucleus and in the cytoplasm, and the active sites of the proteasome are protected from the cellular environment in the interior of the barrel-shaped 20S structure. The 19S complex is an activator that stimulates both peptidase and proteolytic activities of the 20S proteasome. This complex contains at least 18 different subunits and can be topologically defined by two subcomplexes called the base and the lid. The base contains six ATPases, and two non-ATPase subunits in yeast. The ATPases provide energy for the assembly of the 26S proteasome and the breakdown of ubiquitinated proteins into peptides, for the gating of the proteasome channel, and presumably the unfolding of protein substrates, and their injection into the catalytic chamber of the proteasome. The binding of the 20S proteasome to the base alone supports ATP-dependent peptide hydrolysis. In contrast, both the base and the lid are required for ubiquitin-dependent proteolysis. The lid contains at least eight non-ATPase subunits. Subunit S5a binds tightly to the polyubiquitin degradation signal. Another subunit or several additional subunits may also act as a polyubiquitin-conjugate receptor. The 19S regulatory subcomplexes of the proteasome unfold the protein substrates and assist in their translocation through a narrow gate into the 20S core particle where degradation takes place. The protein substrates are degraded progressively until peptides of 7-9 amino acid residues remain. The 19S complex also contains an isopeptidase that removes ubiquitin from the substrate

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Terms and Meanings

Proteasome: Molecular machine that degrades proteins that are synthesized within the cell.

Ubiquitination: The process of covalent attachment of an ubiquitin chain to the protein for degradation.

Reticulocyte lysate: This extract or lysate contains the components required for translation and degradation of proteins for *in vitro* studies.

DEAE-cellulose column: Ion exchange chromatography matrix for separation of charged molecules.

APF-1: Active Protein Fraction - I.

Salt precipitation: A laboratory method for precipitation of proteins.

Deubiquitination: Release of the conjugated ubiquitin from protein molecules by deubiquitinating enzyme found in the lysate.

Polyubiquitination: Poly-ubiquitination of substrate proteins is the signal for proteolysis by the proteasome.

Monoubiquitination: Signal for endocytosis of a plasma membrane-embedded receptor proteins.

C-terminal glycine: Attachment site for ubiquitin.

Pulse-label: Addition of radioactive [^{35} S]methionine to the reticulate lysate for translation analysis.

Ehrlich ascites cells: Epithelial mouse carcinoma cells of use in comparative testing of anti tumour agents for *in vivo* and *in vitro* studies.

ATPases: Adenosine triphosphatases are enzymes essential for deriving cellular energy. They hydrolyse ATP to release its chemical energy.

Multiple myeloma: A cancer of the plasma cell.

Transcription factors: A protein needed to initiate the transcription of a gene. Some transcription factors bind to specific sequences of DNA (promoters and enhancers); others bind to each other; many bind both to DNA as well as to other transcription factors.

Cell cycle: An eukaryotic cell divides by doubling of its genome and halving of the genome into daughter cells. It consists of: G_1 = growth and preparation of the chromosomes for replication, S = synthesis of DNA, G_2 = preparation for mitosis, and M = mitosis.

protein. Surprisingly, the base of the 19S complex has also chaperone-like activity and is able to refold a denatured protein in vitro, a function opposite to its presumed role in proteolysis. Poly-ubiquitination of substrate proteins is the signal leading to proteolysis in the proteasome. However, monoubiquitination of a plasma membrane-embedded receptor was later found to signal its endocytosis, indicating that ubiquitination of proteins also has important targeting functions in endocytosis and secretion.

Ubiquitination of proteins also has important targeting functions in endocytosis and secretion.

A non-proteolytic function of ubiquitin was discovered by Finley and coworkers. They identified three yeast ubiquitin genes that bring about fusions of ubiquitin to ribosomal proteins, which is required for efficient biogenesis of ribosomes. There are now many examples of a non-proteolytic function of ubiquitination, or modification by the small ubiquitin-related modifier (SUMO).

Ubiquitin System, a Target for Drug Development

Since the late 1980s, a rapidly growing number of physiological substrates of the ubiquitin-mediated proteolysis system have been identified. They include the cell cycle, DNA repair and transcription, protein quality control and the immune response. Defects in this proteolysis have a causal role in many human diseases, including a variety of cancers. The ubiquitin system has become an interesting target for the development of drugs against various diseases. Such drugs may be directed against components of the ubiquitin-mediated proteolysis system to prevent degradation of specific proteins or the reverse; drugs may trigger the system to destroy unwanted proteins. One drug already in clinical trials is the proteasome inhibitor Velcade (PS341), that is approved for treatment of multiple myeloma.

Suggested Reading

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