KUNGL. VETENSKAPSAKADEMIEN
THE ROYAL SWEDISH ACADEMY OF SCIENCES

Information Department, P.O. Box 50005, SE-104 05 Stockholm, Sweden
Phone: +46 8 673 95 00, Fax: +46 8 15 56 70, E-mail: info@kva.se, Website: www.kva.se

## Ubiquitin-mediated proteolysis

The Nobel Prize in Chemistry for 2004 is shared between three scientists who have made fundamental discoveries concerning how cells regulate the breakdown of intracellular proteins with extreme specificity as to target, time and space.
Aaron Ciechanover, Avram Hershko and Irwin Rose together discovered ubiquitin-mediated proteolysis, a process where an enzyme system tags unwanted proteins with many molecules of the 76-amino acid residue protein ubiquitin. The tagged proteins are then transported to the proteasome, a large multisubunit protease complex, where they are degraded. Numerous cellular processes regulated by ubiquitin-mediated proteolysis 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.

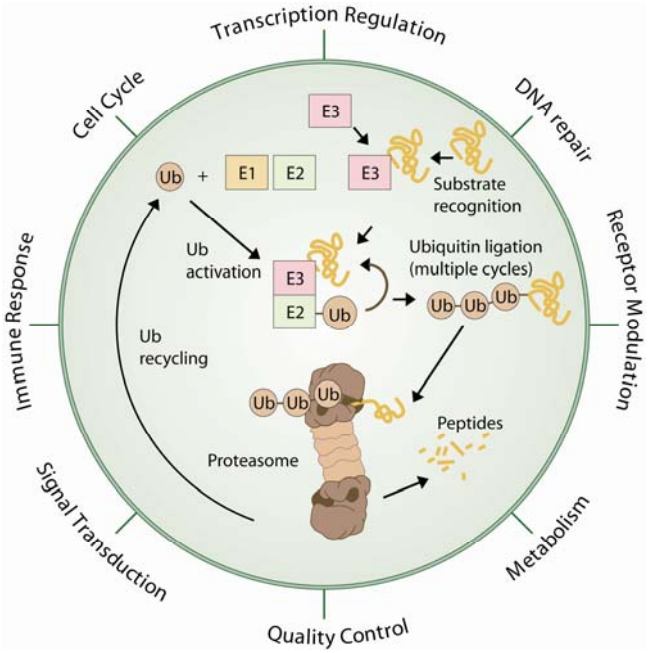



Fig. 1 Ubiquitin-mediated proteolysis and its many biological functions

## Introduction

Eukaryotic cells, from yeast to human, contain some 6000 to 30000 protein-encoding genes and at least as many proteins. While much attention and research had been devoted to how proteins are synthesized, the reverse process, i.e. how proteins are degraded, long received little attention. A pioneer in this field was Schoenheimer, who in 1942 published results from isotope tracer techniques indicating that proteins in animals are continuously synthesized and degraded and therefore are in a dynamic state (Schoenheimer, 1942). A majority of enzymes that degrade proteins are not energy-dependent. One of these is trypsin, which degrades the proteins in our food in the small intestine. In contrast, most intracellular protein degradation requires metabolic energy and this was first detected by Simpson, who measured the release of amino acids from cultured liver slices and found that this process was energy-dependent (Simpson, 1953). The attention given to the apparently non-regulated proteolysis of endocytosed proteins in the lysosomes held back progress in the field of energy-dependent proteolysis. What finally led to the discovery of the central pathway of ubiquitin-mediated proteolysis was Hershko´s great devotion to understanding the paradox of why intracellular proteolysis required energy.

As a postdoc, Hershko worked in G. Tomkins's laboratory on the energy-dependent degradation of the enzyme tyrosine aminotransferase in cultured hepatoma cells. Hershko and Tomkins suggested that ATP participates as an energy source in an early phase of enzyme degradation (Hershko and Tomkins, 1971). It is interesting that Ciechanover much later showed that tyrosine aminotransferase degradation is indeed ubiquitin-mediated (Gross-Mesilaty et al., 1997).

Ubiquitin was first isolated from bovine thymus but later found in many different tissues and in many organisms (Goldstein et al., 1975). It is a protein of only 76 amino acids, and it was originally assumed to participate in the differentiation of lymphocytes. The first observation of its covalent attachment to another protein was made by H. Busch and collaborators. Busch named a mono-ubiquitinated form of histone H2A "protein A24". In 1977, Goldknopf and Busch demonstrated that protein A24 consists of histone H2A and a non-histone protein joined by an isopeptide linkage; this odd molecule had two N-terminals but only one C-terminal! (Goldknopf and Busch, 1977). On the basis of its amino acid sequence, the non-histone protein was in the same year identified as ubiquitin by Hunt and Dayhoff (1977). Surprisingly, the function of mono-ubiquitinated histone H2A is still unknown but it appears to have nothing to do with protein degradation.

An important methodological step in the study of energy-dependent protein degradation was the development by Etlinger and Goldberg of a cell-free system capable of degrading abnormal proteins in an ATP-dependent way (Etlinger and Goldberg, 1977). Using a lysate from rabbit reticulocytes they found that the proteolytic activity had a pH optimum of around 7.8, excluding any major contribution from lysosomes where most proteases show optimal activity in the acid range.

**The discovery of ubiquitin-mediated proteolysis**

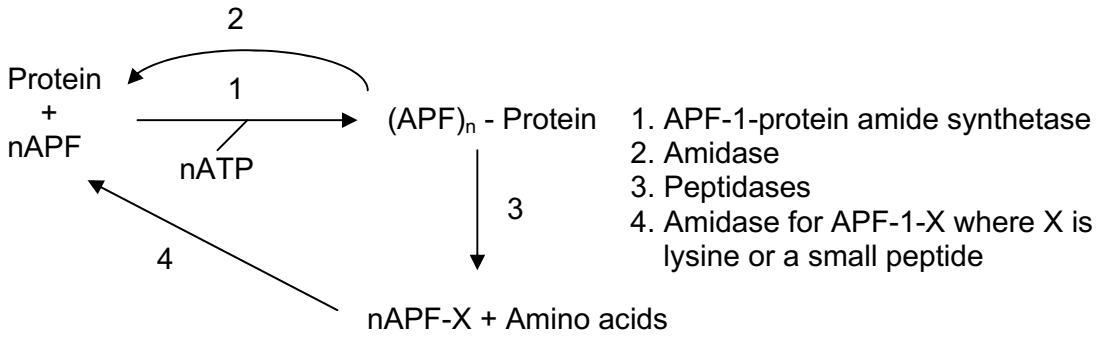
Using the reticulocyte lysate system, Aaron Ciechanover, Avram Hershko and Irwin Rose in a series of pioneering biochemical studies in the late 1970s and early 1980s discovered and characterized the ATP-dependent, ubiquitin-mediated protein degradation system. A major part of the work was done during a series of sabbatical leaves when Hershko and Ciechanover worked in Rose's laboratory at the Fox Chase Cancer Center in Philadelphia.

The first step came in 1978 when the reticulocyte lysate was passed over a DEAE cellulose column to remove the hemoglobin. Surprisingly, this separated the lysate into two fractions, each one individually inactive; but after recombination of the two fractions ATP-dependent proteolysis was reconstituted (Ciechanover et al, 1978). A heat-stable protein, later called APF-1 (active principle of fraction 1), with a molecular weight around 9000, was identified as the active component in the first fraction – this protein was later identified as ubiquitin by Wilkinson, Urban and Haas (1980).

The following year Hershko, Ciechanover and Rose reported that the second fraction of the reticulocyte lysate could be subdivided by salt precipitation into an ATP-stabilized protein of around 450kDa – most probably this fraction contained the proteasome but nobody followed up the observation for almost 10 years – and a fraction which contained the E1-E3 enzymes that were later isolated (Hershko, Ciechanover and Rose, 1979). All three fractions were required to degrade $^{125}$I-labelled albumin, a new substrate that showed much less ATP-independent "background" proteolysis than the substrate globin used earlier.

The breakthrough came in 1980. It was described in two papers that were both communicated on 10 December 1979 to the journal Proceedings of the National Academy of Sciences of the USA. Up to this point the role of APF-1 was unknown; unexpectedly, the results reported in the first paper clearly demonstrated that $^{125}$I-labeled APF-1 bound covalently to a number of proteins in the lysate (Ciechanover et al., 1980).

In the second paper, three substrate proteins, lysozyme, α-lactalbumin and globin were labelled instead of the APF-1 protein. This led to the second unanticipated discovery: multiple APF-1 polypeptides could be conjugated to the same substrate molecule (Hershko et al., 1980). Resistance to alkali and hydroxylamine suggested an amide bond via the ε-amino groups of lysines. Further, a deubiquitinating enzyme activity found in the lysate was capable of releasing conjugated ubiquitin from substrate molecules. Thus the paper demonstrated two novel enzymatic activities; an APF-1-protein amide synthetase and an amidase. A scheme 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 that conjugated APF-1 to the substrate.

In a later publication, in 1980, the APF-1 protein was shown to be identical to ubiquitin. In the Discussion in the paper, the authors speculated that since ubiquitin has a highly conserved amino acid sequence and is widely spread within different eukaryotes, it is likely that ubiquitin-mediated ATP-dependent proteolysis is of general importance. Further, Wilkinson et al. proposed that ATP is required in an exothermic reaction because it allows control and specificity.

Between 1981 and 1983, Ciechanover, Hershko and Rose worked out the multi-step ubiquitin-tagging hypothesis by isolating and characterizing three separate enzyme activities, E1, E2 and E3. This work is still the basis for the description of the ubiquitin system in all text books.

They first identified and purified the ubiquitin-activating activity E1 (Ciechanover et al., 1981). Inhibition of ATP-dependent proteolysis by pyrophosphate in reticulocyte lysates indicated an adenylyl-transfer type of activation mechanism. Other reactions of this type e.g. in the biosynthesis of aminoacyl tRNAs, involve the carboxyl group as the site of adenylation, and earlier data indicated that the carboxyl group in the isopeptide linkage belongs to ubiquitin. Therefore, ubiquitin rather than the substrate protein was most likely the target of the activating reaction. The C-terminal glycine of ubiquitin was indeed identified, by reductive cleavage of the intermediate by labelled sodium borohydride, as the activated amino acid residue (Hershko et al. 1981).

Adenylation is a reversible reaction and consequently Ciechanover, Hershko and Rose set out to detect a ubiquitin-dependent ATP-pyrophosphate exchange activity in the reticulocyte extract. Such an activity was extensively purified and also shown to catalyse ubiquitin-dependent ATP-AMP exchange. This indicated that the activated group is transferred to an acceptor, liberating AMP. The bond between the new enzyme and ubiquitin, unlike the conjugate between ubiquitin and substrate protein, was sensitive to alkali and destroyed by the reducing agent sodium borohydride, pointing to a high-energy thioester bond. All the data supported the notion that the ubiquitin-activating enzyme catalysed a two-step reaction: first the C-terminal carboxyl group of ubiquitin is activated with the formation of an adenylate, a reaction which consumes ATP and releases

pyrophosphate. Secondly, the adenylate is transferred to an acceptor sulfhydryl on the enzyme with the release of AMP. Later work indicated that the ubiquitin-adenylate formed in the first reaction is stably bound to the enzyme (Haas et al, 1982).

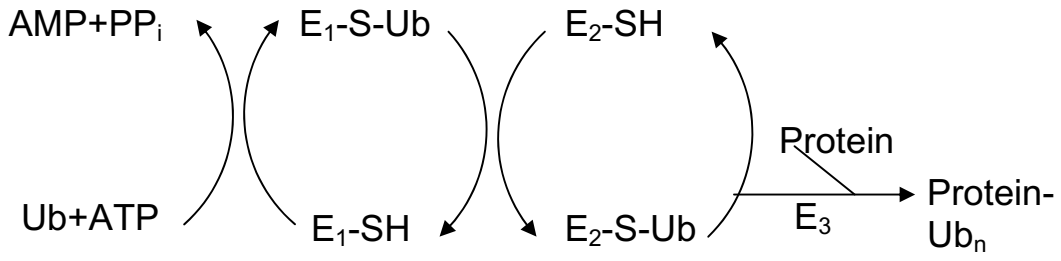
During the purification of the ubiquitin-activating enzyme E1, an elegant covalent affinity chromatography method was developed (Haas et al., 1981; Ciechanover et al., 1982). This method was crucial for the subsequent purifications of the E2 and E3 enzymes. When a reticulocyte lysate fraction containing the activating enzyme in the presence of ATP was applied to a Sepharose column containing covalently attached ubiquitin, 50% of the ubiquitin-dependent ATP-pyrophosphate exchange activity was retained on the column. In the absence of ATP, no enzyme was bound. The bound enzyme was not eluted by 1M KCl but could be eluted by raising the pH to 9 in the presence of dithiothreitol. It also eluted with AMP together with pyrophosphate. With this method, the activating enzyme was purified to homogeneity and demonstrated to be a homo-dimer of 210 kDa.

The pioneer phase of the study of ubiquitin-mediated proteolysis ended with the identification of the ubiquitin-conjugating enzyme E2, and the ubiquitin protein ligase E3 (Hershko et al., 1983). Realizing that the ubiquitin activating enzyme, E1, could not form ubiquitin-protein conjugates by itself, the group had used the newly-developed covalent affinity chromatography to purify two further enzymes, E2 and E3. They noticed that passage of the reticulocyte lysate over the ubiquitin-Sepharose column also in the absence of ATP led to a considerable decrease in proteolytic activity. Since the ubiquitin-activating enzyme bound to the column only in the presence of ATP, the results indicated that other factors of the proteolytic system were retained on the column. By first loading the column with lysate in the presence of ATP and then eluting with high-salt (E3), AMP plus pyrophosphate (E1) and dithiothreitol (E2) they recovered three fractions which fully reconstituted ATP-dependent proteolysis when mixed with ubiquitin, ATP and the unadsorbed fraction.

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 300kDa. Importantly, the gel filtration profiles of the ubiquitin conjugation activities of E1, E2 and E3 were identical to their protein degradation activities. Therefore, the three enzymes of the ubiquitin-protein ligase system were identical with the three factors participating in protein degradation.

The binding of E2 to the ubiquitin-Sepharose column required E1 and ATP, but the binding of E3 to the column required neither. Taken together, the results indicated that E2 bound covalently to the column like E1 did, while E3, which could be eluted by salt, bound non-covalently. The inhibition of E2 activity with iodoacetamide, which could be prevented by preincubation with E1 plus ubiquitin and ATP, indicated the presence of an iodoacetamide-sensitive thiol site in E2. The hypothesis was now tested that E1 might transfer ubiquitin to E2 and that E2 might participate in the transfer of ubiquitin from E1 to the substrate. The thiol ester of E1-ubiquitin did not dissociate during SDS-

polyacrylamide gel electrophoresis, and this stability allowed a search for a possible transfer of $^{125}$I-ubiquitin from E1 to E2 on gels. Such a transfer was indeed observed and the transfer of ubiquitin from E1-ubiquitin to E2 was also observed when ATP was removed by hexokinase before the addition of E2. No ubiquitin transfer from E1-ubiquitin to E3 was detected. Instead, E3 catalysed the transfer of labelled ubiquitin from E2 to the protein substrate, forming amide bonds. These bonds were resistant to β-mercaptoethanol – in contrast to the E2-ubiquitin conjugates which released ubiquitin when boiled in the presence of β-mercaptoethanol, indicating a thiol ester. The full conjugation pathway was now clear: activated ubiquitin bound via its COOH terminus to the thiol site of E1 is first transferred 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:




The existence of multiple, biochemically distinct, E2s was later demonstrated by Pickart and Rose (1985). We now know that mammalian cells contain only one or a few E1s, several different E2s and several hundred different E3s.

All studies described up to now 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 (Hershko et al., 1982). Cells were first pulse-labelled with tryptophan, an amino acid which is missing 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 labelled 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 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, although 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 (Alberts et al., 2002).

## The proteasome

What about the proteasome? With hindsight, this ATP-driven proteolytic machine has been discovered many times and has been given many different names. A brief history of the proteasome field is given by Baumeister et al. (1997) and the Annual Review of Biochemistry reviewed the field in 1999 (Voges et al., 1999). The active sites of the proteasome are protected from the cellular environment in the interior of the barrel-shaped 20S structure, and proteasomes are found both in the nucleus and in the cytoplasm. Poly-ubiquitinated proteins are recognized by the regulatory 19S complexes of the proteasome. Together with the 20S core particle they form the intact 26S proteasome of about 2.5 MDa. 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 processively until peptides of 7-9 amino acid residues remain. The 19S complex also contains an isopeptidase that removes ubiquitin from the substrate protein.

While poly-ubiquitination of substrate proteins is the signal leading to proteolysis in the proteasome, mono-ubiquitination of proteins has other functions. The first example of mono-ubiquitination was Busch´s A24 protein or ubiquitinated histone H2A (Goldknopf and Busch, 1977), but, ironically, the function of this modification is still not understood. A non-proteolytic function of ubiquitin was discovered by Finley et al. who identified three yeast ubiquitin genes that encode fusions of ubiquitin to ribosomal proteins. This transient presence of ubiquitin in front of the ribosomal proteins is required for efficient biogenesis of ribosomes (Finley et al., 1989). 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 (Hicke and Riezman, 1996). There are now many examples of a non-proteolytic function of ubiquitination, or modification by the small ubiquitin-related modifier (SUMO) (Pichler and Melchior, 2002).

## Later developments

While the multi-step ubiquitin-tagging pathway was resolved around 1983, its full physiological significance was not realized at that time. A role in the removal of abnormal intracellular proteins was already clear but to further explore the physiology of ubiquitin-mediated proteolysis, a mutant cell, defective in this pathway, was needed. Such a useful mutant cell line was isolated 1980 by Masa-atsu Yamada and collaborators. They studied chromosome condensation as a function of cell cycle progression and their hypothesis was that histone phosphorylation was of importance in the process. They mutagenized mouse cells and selected temperature-sensitive mutants that were unable to grow at the non-permissive temperature. In this way, a mutant cell line, ts85, was isolated which at the non-permissive temperature arrested in the $G_2$ phase of the cell cycle, i.e. after DNA replication but before mitotic division (Matsumoto et al., 1980).

The ts85 mutant showed abnormal chromosome condensation and a deficiency in histone phosphorylation. This indicated that the defect might be an alteration in chromatin structure. Most importantly, Marunouchi et al. observed that ubiquitinated histone H2A, Busch´s protein A24, rapidly disappeared from ts85 cells at the non-permissive temperature and reappeared again after shift to the permissive temperature (Marunouchi et al., 1980). This disappearance was not observed in wild type cells or in a revertant of the ts85 cells. Finally, the Japanese group showed that ATP was required for the synthesis of ubiquitinated H2A and that the disappearance at the non-permissive temperature was most likely due to a lower rate of synthesis rather than increased degradation (Matsumoto et al., 1983). Considering the peculiar behaviour of H2A in the ts85 cells, they suggested that the mutation may affect an enzyme in the ubiquitin pathway. This was soon confirmed by A. Varshavsky and co-workers, who demonstrated that the thermo-labile component of the ts85 cells was indeed the ubiquitin-conjugating enzyme E1 (Finley et al., 1984). The result, together with the earlier work of Yamada, suggested a role for the ubiquitin pathway not only in the degradation of abnormal proteins but in cell cycle control, DNA replication and chromosome structure.

**Consequences**

Since the late 1980s, a rapidly growing number of physiological substrates of the ubiquitin-mediated proteolysis system have been identified. Here only a few of the most important examples will be given.

Regulation of the cell cycle**.** The first indication that the ubiquitin system was involved in the regulation of the cell cycle came from work on the ts85 cell line as already mentioned. Another indication came when a previously identified yeast factor, Cdc34, known to regulate the cell cycle, was discovered to belong to the evolutionarily-conserved ubiquitin-conjugating E2 enzymes (Goebl et al., 1988). A direct proof of the importance of ubiquitin-mediated proteolysis in controlling the cell cycle came from the laboratory of M. Kirschner, who showed that ubiquitin-mediated proteolysis of cyclin is essential for cells to exit mitosis (Glotzer et al., 1991). They also identified a target signal, the “destruction box” in the N-terminal part of cyclins. The E3 enzyme responsible for this degradation was later identified as the anaphase-promoting complex, a multisubunit enzyme, which also has a key role in the separation of chromosomes during mitosis and meiosis (Nasmyth, 2001).

Mis-segregation of chromosomes during meiosis or mitosis results in cells with altered numbers of chromosomes, and this is the leading cause of spontaneous miscarriages in humans. An extra chromosome 21 in humans leads to Down´s syndrome (trisomy 21). Most malignant solid tumour cells also have altered numbers of chromosomes as a result of repeated mis-segregation of chromosomes during mitosis.

DNA repair, cancer and apoptosis**.** The levels of the tumour suppressor protein p53 in a normal cell are kept low by continuous synthesis and degradation. This degradation is regulated by ubiquitin-mediated proteolysis in which the responsible E3 enzyme, which forms a complex with p53, is Mdm2 (Honda et al., 1997). After DNA damage, the p53

protein is phosphorylated, reducing the interaction with Mdm2. Proteolysis is prevented and the levels of p53 increase. The p53 protein is a transcription factor that binds to specific sequences on the promoter regions of genes regulating cell-cycle arrest, DNA repair and programmed cell death (apoptosis). p53 is induced in cells after DNA damage, leading first to an arrested cell cycle to allow DNA repair and later, if the damage is too extensive, to apoptosis. The p53 protein has been called the "guardian of the genome" and it is mutated in up to 50% of all human cancers.

Human papilloma virus infection is strongly correlated to human uterine cervical cancers. The virus escapes the p53 control function by using a viral protein to activate another cellular E3 enzyme, E6-AP. This ubiquitinates the p53 protein leading to proteolysis. As a result, an infected cell can no longer repair DNA damage in a normal way or go into apoptosis; mutations accumulate and finally a cancer may be formed (Scheffner et al., 1993).

Immune and inflammatory responses**.** A transcription factor called NF-κB regulates many cellular genes that play essential roles in immune and inflammatory responses. NF-κB exists as an inactive complex with an inhibitor protein IκB in the cytoplasm. When cells are exposed to infecting bacteria or some local signalling substances, IκB is phosphorylated and this is the signal for ubiquitin-mediated degradation in the proteasome. Once IκB is degraded, NF-κB translocates to the nucleus where it activates gene expression (Chen et al., 1995).

The ubiquitin-proteasome system also generates the peptides that are presented by MHC-class I molecules to T lymphocytes as an important part of our defence against virus infections.

Both the p53 and NFκB examples show how ubiquitin-mediated proteolysis, often together with phosphorylation, regulates transcription by controlling the stability of different transcription factors.

Cystic fibrosis (CF)**.** The hereditary disorder CF is caused by the functional absence of a plasma membrane chloride channel called CFTR, cystic fibrosis transmembrane conductance regulator. Most CF cases have one single genetic lesion, deletion of a phenylalanine (ΔF508) in the CFTR protein. This mutation in the CFTR causes misfolding of the protein, which leads to its total retention in the endoplasmic reticulum of the cell. Finally, the protein is retrotranslocated to the cytosol where it is rapidly degraded by ubiquitin-mediated proteolysis in the proteasome (Ward et al., 1995).

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) which is approved for treatment of multiple myeloma.

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Lars Thelander

Professor of Medical and Physiological Chemistry, Umeå University
Member of the Nobel Committee for Chemistry