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THE UBIQUITIN SYSTEM FOR PROTEIN MODIFICATION AND DEGRADATION

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Ubiquitin participates in several fundamental metabolic events in eukaryotic cells. The ubiquitylation of target proteins is a prerequisite in the expression of ubiquitin biological activity. Ubiquitylation may be considered as one form of reversible post-translational covalent modification of proteins. The target proteins can be linked to one or a few ubiquitin molecules, resulting in a modification of the protein function. Multi-ubiquitylation marks proteins selectively for degradation.

The ubiquitin-mediated proteolytic pathway plays an important role on the intracellular selective protein breakdown, being responsible for the catabolism of many regulatory proteins. In this pathway, a multi-enzyme system catalyses the ATP-dependent covalent ligation of ubiquitin molecules to proteins destined for catabolism that bear unknown degradation signals (or degrons) recognized by this system. The resulting large molecular mass ubiquitin-protein conjugates are then specifically degraded by a very large (~ 2 MDa) ATP-dependent protease, the 26S proteasome. This protease consists of the previously characterized 20S core proteasome and a complex containing multiple ATPases (the 19S cap) at both ends of the 20S proteasome. The ubiquitin pathway has been studied mostly in animal cells and yeast, but all components and reactions of this system occur in plants as well.

RESUMO

O SISTEMA DA UBIQUITINA PARA MODIFICAÇÃO E DEGRADAÇÃO DE PROTEÍNAS

A ubiquitina participa em vários processos metabólicos fundamentais das células eucariotas. A ubiquitilação de proteínas-alvo é um pré-requisito na expressão da actividade biológica da ubiquitina. A ubiquitilação pode ser

considerada como uma forma de modificação covalente e reversível, pós-tradução, de proteínas. As proteínas-alvo podem ser ligadas a uma ou a algumas moléculas de ubiquitina, o que resulta numa alteração da função da proteína. A multi-ubiquitilação marca selectivamente as proteínas para degradação.

A via proteolítica mediada pela ubiquitina desempenha um papel importante no catabolismo intracelular e selectivo de proteínas, sendo responsável pela degradação de muitas proteínas com funções de regulação. Nesta via, um sistema multi-enzimático catalisa a ligação covalente, dependente da hidrólise de ATP, de moléculas de ubiquitina a proteínas destinadas a sofrer proteólise. Estas proteínas são portadoras de sinais desconhecidos de degradação (ou degrões), reconhecidos por este sistema. Os conjugados de ubiquitina – proteína de massa molecular elevada assim formados são subsequentemente degradados especificamente por uma protease muito grande (2 MDa), dependente da hidrólise de ATP, o proteassoma 26S. Esta protease é constituída por um núcleo (o já anteriormente caracterizado proteassoma 20S), contendo um complexo que engloba múltiplas ATPases (a tampa 19S) em cada extremidade. A via da ubiquitina tem sido principalmente estudada em células animais e de levedura. Contudo, as células vegetais possuem todos os componentes e reacções deste sistema.

INTRODUCTION

It has long been realised that proteins are continually being synthesized and degraded in the cells, and that some proteins turn over more rapidly than others. However, while protein synthesis has been relatively well elucidated for almost 30 years, the cellular breakdown of these polymers back to amino acids is only poorly understood.

Intracellular protein degradation plays an important role in the biology of cells. This process can be highly selective, so that some proteins are degraded within minutes while others are pratically stable. It is generally accepted that damaged proteins and regulatory enzymes, including those that control the flow of metabolites through the metabolic pathways, are subjected to intense turnover.

Eukaryotic cells possess two types of proteolytic pathways: lysosomal (vacuolar in the case of yeast and plant cells) and nonlysosomal pathways. While lysosomal degradation of intracellular proteins occurs mostly under stressed conditions, nonlysosomal mechanisms are responsible for the highly selective turnover of intracellular proteins that takes place under basal

metabolic conditions and also for some aspects of proteolysis under stress (Ciechanover, 1994). An important nonlysosomal proteolytic pathway is the ubiquitin (Ub) system, in which proteins are degraded by a 26S protease following conjugation by multiple ubiquitin-molecules.

It was shown that protein degradation occurs in reticulocytes (the precursors of erythrocytes) which lack lysosomes. Therefore, rabbit reticulocytes became model cells to study nonlysosomal intracellular proteolysis. A cytosolic ATP-dependent proteolytic system was discovered in reticulocytes by Etlinger & Goldberg (1977). Subsequent fractionation of the reticulocyte lysate generated two fractions (I and II) which were required for the ATP-dependent proteolysis of some test proteins. The active factor in fraction I was purified, named APF-I, and found to be covalently conjugated to proteins in the presence of ATP and fraction II (Ciechanover *et al.*, 1980). APF-I was then shown to be identical to Ub (Wilkinson , Urban & Haas, 1980).

UBIQUITIN

Ubiquitin (Ub) is one of the most conserved proteins known to date. After its initial discovery in lymphocytes by Gideon Goldstein and his colleagues (Goldstein *et al.*, 1975), it has been detected in all eukaryotic cells examined so far. Because the antibodies prepared against Ub reacted with similar proteins in organisms as diverse as mammals, yeast and plants, the protein was initially named ubiquitous immunopoietic peptide.

Ub is a compact globular protein with a C-terminus that extends away from the main body of the protein into the aqueous space. This polypeptide is composed by 76 amino acid residues (Table I) and possesses a molecular mass of 8,565 Da. There are no Cys or Trp residues in the molecule. It has a neutral isoelectric point (6.7) and little appreciable charge below pH 9.0. It is extremely resistant to thermal denaturation, showing a reversible denaturation transition at about 85°C (Wilkinson, 1988). Ub occurs in cells both as a free monomer and covalently attached to itself and to other proteins.

Ub has several fundamental functions in intracellular metabolism that are a result of its ability to become covalently ligated to a wide variety of other cellular proteins (Hershko, 1988). The covalent ligation of one or more Ub molecules to a target protein is termed ubiquitylation. Indeed, Ub whose C-terminal (Gly 76) carboxyl group is covalently linked to another compound is called the ubiquityl moiety, the derivative terms being ubiquitylation and ubiquitylated (Varshavsky, 1997). This terminology is

TABLE I

Amino acid sequence of ubiquitin from a variety of animal sources is given in the top line.
For the other entries, a dash indicates identity with the animal sequence (Wilkinson, 1988)

								5					10					15					20					25					30
Animal	M	Q	I	F	V	K	T	L	T	G	K	T	I	T	L	E	V	E	P	S	D	T	I	E	N	V	K	A	K	I
Oat	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	S	–	–	–	–	D	–	–	–	–	–	–
Yeast	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	S	–	–	–	–	D	–	–	–	S	–	–
								35					40					45					50					55					60
Animal	Q	D	K	E	G	I	P	P	D	Q	Q	R	L	I	F	A	G	K	Q	L	E	D	G	R	T	L	S	D	Y	N
Oat	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	A	–	–	–
Yeast	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
								65					70					75													
Animal	I	Q	K	E	S	T	L	H	L	V	L	R	L	R	G	G														
Oat	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–														
Yeast	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–														

also recommended by the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (1992).

Ub is typically conjugated to other proteins through an amide bond, termed isopeptide bond, formed between the a-carboxyl group of the C-terminal residue of Ub (Gly 76) and the ∈-amino group of a Lys residue in the acceptor protein. In the case of multi-Ub chains, containing two or more Ub moieties, the acceptor protein is another Ub molecule. However, Ub has seven Lys residues. The first multi-Ub chain to be discovered had its Ub moieties conjugated through the Lys 48 residue of Ub. Therefore, multi-Ub chains can be typically built up on a single Lys of the target protein, by isopeptide bond formation between the carboxyl group of Gly 76 of one Ub with the amino group of the side chain of Lys 48 of the preceding Ub (Chau *et al.,* 1989). Substitution of Lys at position 48 with Cys results in a mutant Ub which does not support multi-Ub chains and is incapable of targetting proteins for proteolysis (Chau *et al.,* 1989). Other multi-Ub chains involve Lys 63 or Lys 29. A chain linked through Lys 63 appears to have a distinct role in a pathway of DNA repair (Spence *et al.*, 1995) and under stress conditions (Amasson & Ellison, 1994). Multi-Ub chains containing isopeptide bonds Gly 76-Lys 11 are generated in keratinocytes, and these chains are also able to target proteins for degradation by the 26S proteasome (Baboshina & Haas, 1996).

Multi-Ub chains occur either as isolated free forms, linked to trypsinated Ub (des-Gly-Gly-Ub) or linked to target proteins at a specific Lys residue (Jennissen, 1995).

Several types of ubiquitin-protein conjugates may be formed: (1) proteins that are ubiquitylated with one or a few Ub molecules, often termed stable Ub conjugates, where ubiquitylation does not serve the function of marking the proteins for destruction. Rather, this covalent modification of the proteins may alter the target protein's function. These conjugates are not, in fact, stable, since they can be split by isopeptidases. (2) The labile Ub conjugates (monoconjugates or diconjugates) formed by thioester linkage between Ub and E1 or E2 enzymes (See Section 4). These conjugates are easily destroyed by exposure to reducing agents. (3) Proteins that are monoubiquitylated at several Lys residues, i.e. multisite ubiquitylation, are designated by pluri-ubiquitylated proteins. (4) Proteins that are conjugated to a multi-Ub chain are termed multi-ubiquitylated proteins (Jennissen, 1995). Apparently, the 26S proteasome shows higher affinity for multi-ubiquitylated substrates than for mono-ubiquitylated species. It seems that four copies of Ub in a chain may be required to target proteins to the

26S proteasome, as these Ub_4 chains exhibit certain structural characteristics that may be recognized by the protease. On the other hand, multi-or pluri-ubiquitylation of the target proteins enhances the degradation rate between 2 and 10 fold as a function of the degree of ubiquitylation (Jennissen, 1995).

The ubiquitylation of proteins has been implicated in a variety of processes such as selective protein degradation and determination of steady-state levels of regulatory proteins, response to heat and other stresses (including the immune response), DNA repair, control of cell cycle, biogenesis of ribosomes, regulation of cell surface receptors, biogenesis of peroxisomes, regulation of transcription, viral infection, programmed cell death, cellular differentiation, embryogenesis, signal transduction, transmembrane and vesicular transport, function of the nervous system and the possible pathogenesis of some neurodegenerative diseases (Hershko & Ciechanover, 1992; Kampen, Wettern & Schulz, 1996). Ub itself has been shown to be a heat shock protein (Bond & Schlesinger, 1985). Other roles attributed to Ub include a chaperonin function (Finley, Bartel & Varshavsky, 1989) and a signal for endocytosis (Hicke & Riezman, 1996).

Ub has been shown to play a role in the response to both biotic and abiotic stresses. In what plants are concerned, there is evidence for the involvement of Ub in plant-virus and plant-pathogenic fungus interactions (Kampen, Wettern & Schulz, 1996). Furthermore, various abiotic environmental conditions are known to affect the Ub system. These conditions include heat, drought, salt, cold, heavy metal and irradiation stresses.

Ub and the various components of this proteolytic pathway have been detected in all eukaryotes so far investigated, including mammals, yeast and higher plants (Finley & Varshavsky, 1985; Hershko & Ciechanover, 1992). Despite an earlier report on the presence of ubiquitin in bacteria (Goldstein *et al.*, 1975), it became subsequently accepted that prokaryotes do not possess Ub (Hershko & Ciechanover, 1992). Chloroplasts have many features in common with prokaryotes, which lead to the endosymbiont theory for the origin of these organelles (Alberts *et al.*, 1983). On the other hand, it is known that most chloroplast proteins are encoded in the nucleus and synthesized on cytosolic ribosomes (Ellis, 1981). Nevertheless, chloroplasts have been shown to contain ATP-stimulated proteolytic processes capable of degrading their own protein constituents (Liu & Jagendorf, 1984; Malek *et al.*, 1984). These observations raise the question of whether chloroplasts contain Ub and a functional Ub-dependent proteolytic pathway. Some controversy exists concerning the presence of Ub in plant chloroplasts. Several

studies suggest the presence of Ub, Ub-protein conjugates and/or Ub conjugating activity in plant and algal chloroplasts (Wettern *et al.*, 1990; Hoffman *et al.*, 1991; Veierskov, Ferguson & La-Yee, 1992; Wolf, Schulz & Schnabl, 1993). However, a recent work failed to detect free Ub, Ub-protein conjugates and Ub conjugating activity in *Lemna minor* and *Spinacea oleracea* chloroplasts and suggested that the presence of Ub-protein conjugates in some chloroplast preparations is due to cytosolic contamination (Ramos *et al.*, 1995). Nevertheless, the Ub pathway was reported to function in the cyanobacteria *Anabaena variabilis* (Durner & Böger, 1995), after being detected in the archaebacterium *Thermoplasma acidophilum* (Wolf, Lottspeich & Baumeister, 1993).

One of the most surprising recent findings was the identification of genes in animal and plant viruses encoding Ub-like proteins and fusion proteins of unknown function (Kampen, Wettern & Schulz, 1996). Thus, ubiquitylated proteins have been found associated with the tobacco mosaic, barley stripe mosaic, brome mosaic, cowpea mosaic, cowpea severe mosaic and satellite panicum mosaic viruses (Hazelwood & Zaitlin, 1990).

The cellular location of Ub-dependent proteolysis is indicated by the presence of Ub conjugates in the cytosol, cytoskeleton, nucleus, plasma membrane and, under certain conditions, in lysosomes (Jennissen, 1995). Like in chloroplasts, the presence of Ub in mitochondria remains subject to controversy.

THE UBIQUITIN-MEDIATED PROTEOLYTIC PATHWAY

In 1978, Ciechanover, Hod & Hershko reported the discovery of a cytosolic, non-lysosomal, ATP-dependent proteolytic system from reticulocytes which depended on a heat-stable protein factor (APF-I), later identified as Ub. In this pathway, Ub serves as a marker for targeting a protein for its subsequent degradation. Thus, degradation of a protein via the Ub pathway involves two distinct but sequential steps: signalling of the protein by covalent attachment of multiple Ub molecules and degradation of the targeted protein with the release of free and reutilizable Ub (Ciechanover, 1994).

The Ub-dependent proteolytic pathway, schematically presented in Fig.1, is composed of four major components: Ub, the Ub-conjugating system (ATP-dependent; which includes three types of enzymes, designated by E1, E2 and E3), the protein-substrates and the 26S proteasome (ATP-dependent;

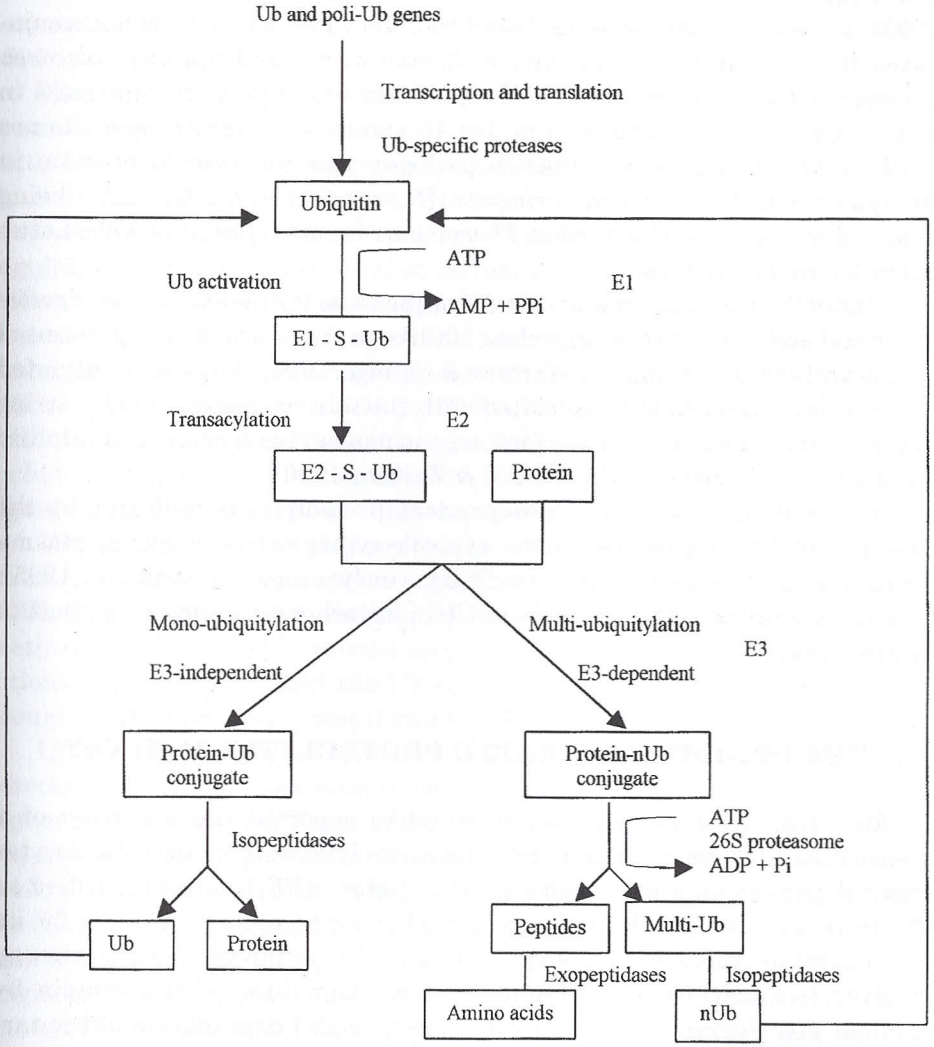

Fig. 1. Proposed sequence of events in the formation of Ub conjugates and subsequent deubiquitylation, with or without proteolysis.

with isopeptidase activity). A fifth major component, essentially unknown at present, includes the degradation signals, present on the protein substrates, that lead to their ubiquitylation.

The Ub catabolic route is supposed to be the major pathway for the hydrolysis of short-lived, denatured, or abnormal proteins, as well as of certain cellular regulators (Kampen *et al.*, 1996). Many of these proteins serve regulatory functions in the cells, so that the Ub pathway itself is a vital regulatory system of eukaryotic cells controlling the concentration of key proteins by selective degradation. This means the Ub system selectively recognizes some proteins via some features in their structure, which in turn may be regulated. The extremely high selectivity of the Ub system is illustrated by the fact that Ub-dependent proteolysis can destroy a subunit of an oligomeric protein selectively, leaving intact the rest of the protein's subunits (Johnson, Gonda & Varshavsky, 1990). This capability of the Ub system accounts for large differences in the *in vivo* half-lives of subunits of many regulatory proteins.

Ub plays an important role in limited proteolysis as well. For example, the precursor of the transcription factor NF-*K*B, IF-*K*B, is subjected to partial degradation by the Ub system, resulting in the active NF-*K*B (Palombella *et al.*, 1994).

The multi-ubiquitylated substrates appear to have a kinetic advantage in degradation. On the other hand, the reversible ligation of a single or few Ub molecules seems to have a regulatory function, causing alterations in the protein structure and function. In this sense, Ub conjugation constitutes a post-translational modification of proteins and may be an important modulator of a protein's function. This has been suggested for calmodulin-tetraubiquitylation, catalysed by the Ca^{2+}-dependent uCAM synthetase (Majetschak, Laub & Jennissen, 1993).

In spite of the importance of Ub in eukaryotic protein metabolism, there is no convenient assay for this protein. Two different approaches are commonly employed to detect the biological participation of this protein in the proteolytic system: one methodology involves raising antibodies to Ub and the combined use of gel electrophoresis, transfer to membrane and immunoblot analysis to illustrate the distribution of Ub among different molecular mass protein species (Fig. 2A). The other approach measures Ub conjugating activity by the ATP-dependent, *in vitro* synthesis of ^{125}I-Ub-protein conjugates followed by autoradiography (Fig. 2B). For this purpose, previously labelled ^{125}I-Ub is conjugated to endogenous proteins present in a total cell extract, in the presence of ATP.

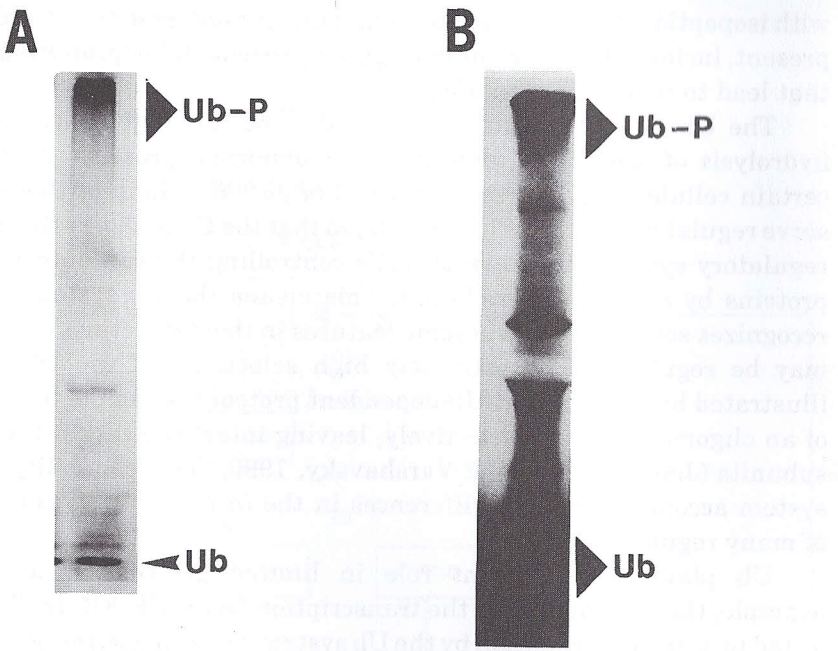

Fig. 2. Detection of ubiquitin biological activity. (A) Immunoblot showing the distribution of Ub among the different molecular species present *in vivo* in *Lemna minor* cells. A total protein extract was prepared from L. minor, fractionated by SDS-PAGE, transferred to a membrane and probed with specific anti-Ub antibodies. (B) Autoradiogram obtained after *in vitro* conjugation of ^{125}I-Ub to *L. minor* endogeneous proteins in the presence of ATP. Ub, Ub-P: free Ub and high molecular mass Ub-protein conjugates, respectively.

THE UBIQUITIN CONJUGATING SYSTEM

Ubiquitin is activated and conjugated to other cellular proteins by the sequential action of three enzymes: E1, E2 and E3 (Fig. 1). The Ub-activating enzyme (E1) catalyses the ATP-dependent formation of a high-energy thioester bond between the C-terminal residue of Ub (Gly 76) and a specific Cys residue of E1. A Ub adenylate is formed as an intermediate step, with the displacement of PPi from ATP and the subsequent release of AMP. The E1-linked Ub moiety is then transferred, in a transacylation reaction, from E1 to a Cys residue of one of the members of the family of Ub-conjugating enzymes (E2; previously called Ub carrier proteins). These enzymes can transfer Ub directly to substrate proteins. This pathway apparently serves to modify the

function of proteins (e.g. histones). Ub can be subsequently recycled by an isopeptidase and reenter the conjugation pathway. However, in some cases, an additional enzyme is required for ubiquitylation of some target proteins. This route of the pathway involves a Ub-protein ligase or recognin (E3), which forms a complex with a target protein and a Ub-charged E2. This enzyme selects the protein-substrate through an interaction with its degradation signal and participates in moving Ub from E2 to an ∈-amino group of a Lys residue in the target protein, yielding a multi-Ub-protein conjugate. The E3 enzyme, initially called APF-1-protein amide synthetase, was classified as EC 6.3.2.19. An E3 enzyme that is specific for a natural substrate has been termed ubiquitin-calmodulin ligase or ubiquityl-calmodulin synthetase (uCAM synthetase;EC 6.3.2.21). Thus, Ub-protein ligation may occur by direct transfer of Ub from E2 to the target protein (E3-independent ubiquitylation) or by a process in which the target protein is first bound to specific sites on E3 (E3-dependent ubiquitylation). The multi-ubiquitylated target protein is then a substrate for the 26S proteasome. A subunit of the 19S regulator specifically recognizes multi-Ub chains and the Ub moieties are removed from the substrate. The protein is subsequently hydrolysed by the 20S core complex of the 26S proteasome.

Ubiquitin is activated by a Ub-activating enzyme or E1. Although only one E1 enzyme has been found in yeast or mammals, plants seem to encode multiple E1s. For example, in *Triticum aestivum* and *Chlamydomonas reinhardtii*, three and four distinct E1 enzymes have been identified, respectively (Vierstra, 1993; Kampen, Wettern & Schulz, 1996). The functional significance of the existence of heterogeneous forms of E1 enzymes in plants remains to be elucidated.

There is evidence for the existence of phosphorylated (Cook & Chock, 1995) and ubiquitylated (Arnold & Gevers, 1990) forms of E1. Phosphorylation of E1 enzymes apparently regulates their ability to transfer Ub to E2; phosphorylation by protein kinase C to a stoichiometry of 0.64 mol phosphate/mol is accompanied by a doubling of activity (Kong & Chock, 1992). Covalent modification of E1 with Ub has also been reported (Arnol & Gevers, 1990).

E1 enzymes have been purified from various sources. They are homodimers with apparent molecular masses of 210 kDa, composed of two identical 105 kDa subunits (Hershko & Ciechanover, 1992). E1 has been strongly conserved during evolution. The human and the yeast proteins are 53% identical. On the other hand, plant, yeast, human and mouse proteins possess five conserved Cys residues. The Cys residue at position 626 of the

yeast enzyme is the residue involved in catalysis. E1 contains also a nucleotide binding site. Genes encoding this enzyme have been cloned from yeast, wheat and humans. E1 is found in the nucleus and cytosol and associated with the cytoskeleton.

All E2 enzymes contain a conserved domain of approximately 16 kDa (the UBC domain), containing a Cys residue essential for thioester formation with Ub. This Cys residue accepts Ub from E1. Substitution of this Cys abolishes E2 activity (Sullivan & Vierstra, 1991). The E2 enzymes exhibit heterogenous molecular masses. Some of the E2 enzymes are small proteins (14-18 kDa) that consist almost entirely of the conserved domain (UBC4, 5 and 6), while others have C-terminal extensions, which are either neutral (UBC1) or highly acidic (UBC2 and 3) or N-terminal extensions.

Ten genes that encode different species of E2s have been isolated from yeast. Several E2 enzymes have been identified in plants, such as *Arabidopsis thaliana* and *T. aestivum*, although little is known about their exact functions. The different E2 enzymes appear to exhibit distinct substrate specificities and functions.

The existence of so many E2s may be attributed to the fact that at least some of the specificity of ubiquitylation appears to be dependent on E2s. On the other hand, the functions of E2 enzymes in plants may be somewhat different from those in other organisms. For example, UBC4, a E2 enzyme from yeast, is strongly inducible by heat stress, whereas some homologous E2 enzymes from *A. thaliana* are not (Girod *et al.*, 1993). Closely related genes encoding E2 enzymes in *A. thaliana* are controlled by different regulatory mechanisms (Genschik *et al.*, 1994). Also, UBC6, another E2 enzyme from *A. thaliana*, is organ-specifically expressed (Watts *et al.*, 1994).

An E2 enzyme, with a molecular mass of 32 kDa, has been shown to be phosphorylated by protein-tyrosine kinase to a stoichiometry of 2 mol phosphate/mol, leading to a 2.4-fold activation (Kong & Chock, 1992).

E2 enzymes may be classified into three groups on the basis of their structure: Class I – these proteins comprise simply the UBC domain. UBC4 and 5 of *Saccharomyces cerevisiae* and UBC1 of *A. thaliana* are examples of this class of E2 and are known to be important in the ubiquitylation of many short-lived and abnormal proteins prior to degradation. Class II – these enzymes contain C-terminal extensions of the UBC domain. The extensions are different in type but very acidic extensions, as found in UBC2 of *S. cerevisiae*, appear to mediate interaction with protein targets that results in protein modification rather than degradation. Other C-terminal extensions may be involved in E2 localisation – the UBC6 *of S. cerevisiae* is found

anchored to the ER membrane with an active site facing the cytosol. The 95 residue C-terminal extension of UBC6 includes a hydrophobic signal-anchor sequence. Class III – N-terminal extensions are present in this class of E2s. The function of the extensions is unknown. At least one E2 enzyme does not fit into any of the three classes – a large, 230 kDa, E2 from reticulocytes.

The E3 enzymes select and bind to the target protein. This implies that E3s recognize a motif in the substrate protein (a degron) that targets it for ubiquitylation. E3 enzymes are therefore called recognins. To date, two classes of E3s have been identified: (1) E3s which do not form thiol esters with Ub. These E3s act as recognins, forming complexes with Ub-charged E2s and target proteins to facilitate ubiquitylation. They do not form thiol esters with Ub but merely serve to identify and sequester suitable targets for ubiquitylation. (2) E3s which do form thiol esters with Ub. These E3 enzymes act as intermediates in the ubiquitylation pathway, in that Ub is transferred from a charged E2 to a Cys in the E3, and from there to the target protein.

Several E3 proteins are known: the mammalian E6AP, the yeast (*Schizosaccharomyces pombe*) Pub1p and the E3 of the N-end rule pathway Ubr1p, which recognizes proteins as a function of their N-terminal residue. This enzyme binds the protein-substrate and E2, and Ub is subsequently transferred to the target protein (Varshavsky, 1992). Scheffner, Nuber & Huibregtse (1995) showed that E6AP forms, like E1 and E2, a thioester bond with Ub, which is subsequently transferred to the substrate. Girod *et al.* (1993) identified an E3 enzyme in *T. aestivum* which requires a special E2 enzyme and does not recognize the N-terminal residue in the target protein.

E3 enzymes were reported to be dimers (350 kDa) composed of 180 kDa subunits.

DE-UBIQUITYLATION

Essential to the operation of the Ub system is the recycling of free and functional ubiquitin. The covalent bond formed between Ub and other proteins can be enzymatically cleaved – in fact, there are multiple, ATP-independent proteases (*S. cerevisiae* contains over 20) whose common property is the ability to recognize a Ub moiety and cleave at the Ub junction.

The terminology utilized in the classification of enzymes involved in de-ubiquitylation considers the nature (Ub or other protein) and size (protein or small compound) of the leaving group and the amino group in the protein (α-NH_2 or ϵ-NH_2) to which Ub is linked. These enzymes are collectively

designated Ub C-terminal hydrolases, i.e. enzymes that hydrolise the linkage between the C-terminal Gly residue of Ub and various adducts. When the bond cleaved is a Ub-$\in$-NH_2-protein isopeptide linkage, the term isopeptidase is employed. The two main groups of isopeptidases, those splitting multi-Ub chains (N^{ϵ}-ubiquityl-ubiquitin bonds) and Ub from proteins (N^{ϵ}-ubiquityl-proteins bonds) are differentiated from other peptidases involving Ub such as those splitting poli-Ub chains (N^{α}-ubiquityl-ubiquitin bonds) (Jennissen, 1995).

Several Ub-C-terminal hydrolases have been described. However, in most cases, their functions have not been satisfactorily established. The possible cause for the multiplicity of Ub-specific processing proteases is the diversity of their targets which include linear Ub fusions (DNA – encoded), Ub adducts with small molecules such as glutathione and free or substrate-linked multi-Ub chains (Varshavsky, 1997). Rose (1988) pointed out that the reaction of E1-Ub or E2-Ub thiol esters with intracellular nucleophiles (such as glutathione or polyamines) would very quickly deplete the pool of free Ub unless such side products are rapidly cleaved by appropriate Ub C-terminal hydrolases. In the protein degradation pathway, Ub-C-terminal hydrolases or isopeptidases are required to release Ub from isopeptide linkage with Lys residues on the protein substrate or to disassemble multi-Ub chains during or after degradation of the substrate protein (Hershko & Ciechanover, 1992).

Another classification of isopeptidases has been used to group these enzymes according to the nature of the compound hydrolysed: Isopeptidases that hydrolyse small molecules conjugated to Ub (such as residual peptides attached to Ub) have been termed Ub carboxyl-terminal esterases/hydrolases. These enzymes have typical molecular masses of approximately 30 kDa. Isopeptidases capable of splitting larger ubiquityl-protein conjugates are commonly known as ubiquityl-protein hydrolases and possess molecular masses in the range of 100-400 kDa. A third type of isopeptidase cleaves free homooligomers and homopolymers of Ub (e.g. di-Ub and multi-Ub). These enzymes, with molecular masses around 100 kDa, have been termed ubiquityl-ubiquitin hydrolases. Recently, ATP-dependent C-terminal hydrolases and ATP-dependent isopeptidases have been reported (Jennissen, 1995).

The ubiquitylation of several cellular proteins with non-catabolic functions (see below) demonstrates the physiological importance of isopeptidases. In this case, de-ubiquitylation reverses the modification of protein function. Such appears to be the case with the ubiquitylated histones H2A and H2B, which are rapidly de-ubiquitylated during mitosis and re-ubiquitylated shortly afterwards (Mueller *et al.*, 1985).

Recent evidence suggests that some Ub-specific processing proteases function in association with the 26S proteasome, as editing enzymes; their ability to de-ubiquitylate an already targeted (proteasome-bound) Ub-substrate conjugate can modulate the rate of proteolysis. The Ub-specific processing proteases may also bind specifically to a protein that bears a degradation signal, blocking the formation of multi-Ub chains and preventing the degradation of the protein.

Additional roles attributed to Ub C-terminal hydrolases include a proof-reading function to release free protein from incorrectly ubiquitylated derivatives, the trimming of abnormal multi-Ub chains and the processing of precursors in the biosynthesis of Ub (all Ub genes are either arranged in linear poli-Ub arrays or are fused to ribosomal proteins). Therefore, processing functions include the release of Ub from linear poli-Ub precursors, the cleavage of Ub from ribosomal proteins and the removal of extra amino acid residues that are at the C-terminal of some poli-Ub genes (Hershko & Ciechanover, 1992).

THE 26S PROTEASOME

20S proteasomes (EC 3.4.99.46) are widely distributed among living organisms, being present in virtually all eukaryotes (including animal, plant and yeast cells), in archaebacteria and in eubacteria (Kampen, Wettern & Schulz, 1996). Immunological studies revealed that this protease is evenly distributed in the nucleus and cytoplasm of cells. The 20S proteasome also commonly referred to as the multicatalytic proteinase, MCP, 20S cylinder particle, ingensin, macropain and prosome, has been given about 25 different names in recent years (Peters, 1994). The term proteasome was initially proposed when the identity of the 20S particle and the multicatalytic proteinase was discovered. The term was subsequently modified to 20S proteasome to avoid confusion with the larger 26S proteasome.

The 20S proteasome exhibits a highly organized and evolutionary conserved structure (Peters, 1994). This protease is a cylindrical shaped particle (17 nm×11 nm), composed of 4 adjacent ring structures (Löwe *et al.*, 1995). In its simplest form, the archaebacterial proteasome, the 4 rings are made up by two different polypeptides, α and β. Each of the inner rings is formed by 7 β polypeptides and each of the outer rings is composed of 7 α polypeptides. Thus, the molecular organization of the particle is $\alpha_7\beta_7\beta_7\alpha_7$. The eukaryotic proteasome has a more complex structure with the rings built by two similar

protein families: a-like and b-like proteins (Tanahashi *et al.*, 1993). There is sequence similarity, not only within the a- and b-subunit families, but also between the a- and b-sequences themselves (Zwickl *et al.*, 1992), suggesting that all subunits are derived from a single ancestral gene early in evolution.

The 20S proteasome is a 700 kDa protein complex with a sedimentation coefficient of 20S, composed of approximately 12-15 different polypeptide subunits in the molecular range 19 to 36 kDa (Peters, 1994). Some authors have reported the presence of RNA. This enzyme has been designated multicatalytic proteinase owing to its unusual ability to cleave peptide bonds carboxy-terminal to basic, hydrophobic and acidic amino acid residues (Wilk & Orlowski, 1983). These three activities, catalysed by three distinct active sites, are commonly referred to as trypsin-like, chymotrypsin-like and peptidyl-glutamyl hydrolysing activities, respectively. Evidence for the existence of even more peptidase activities has been reported. Degradation of both proteins and peptides is ATP-independent (Peters, 1994). Characterization of the catalytic properties of the protease has not yet allowed its classification as a serine, aspartate or thiol protease and sequence analysis of its polypeptides has not revealed similarities with any other known proteases (Zwickl *et al.*, 1992; Rivett, 1993). Therefore, the 20S proteasome may represent a novel class of proteolytic enzyme.

The cellular functions of the 20S proteasome remained largely unclear until it was shown that the 20S particle forms the proteolytic core of an even larger protein complex, the 26S protease, 26S proteasome or megapain, which is responsible for protein degradation in the Ub pathway. Indeed, in eukaryotic cells, the 20S proteasome is unable to degrade multi-ubiquitylated proteins (Fig. 3).

The 26S proteasome is assembled from a cylindrical proteolytic core complex (the 20S proteasome) and two cap-shaped polar 19S regulatory protein complexes. The resulting protease is a dumb-bell-shaped complex, 45 nm×19 nm in size (Fig. 4). The attached cap complexes appear roughly V-shaped and are bound to the 20S core particle in opposite orientations, giving the 26S proteasome a *trans* configuration.

The three particles (19S, 20S and 26S complexes) exist in a dynamic state of equilibrium and interconversion, with ATP favoring the 26S complex assembly:

$$2 \quad 19\text{ S complex} + 20\text{ S complex} \underset{-ATP}{\overset{+ATP}{\rightleftharpoons}} 26\text{ S complex}$$

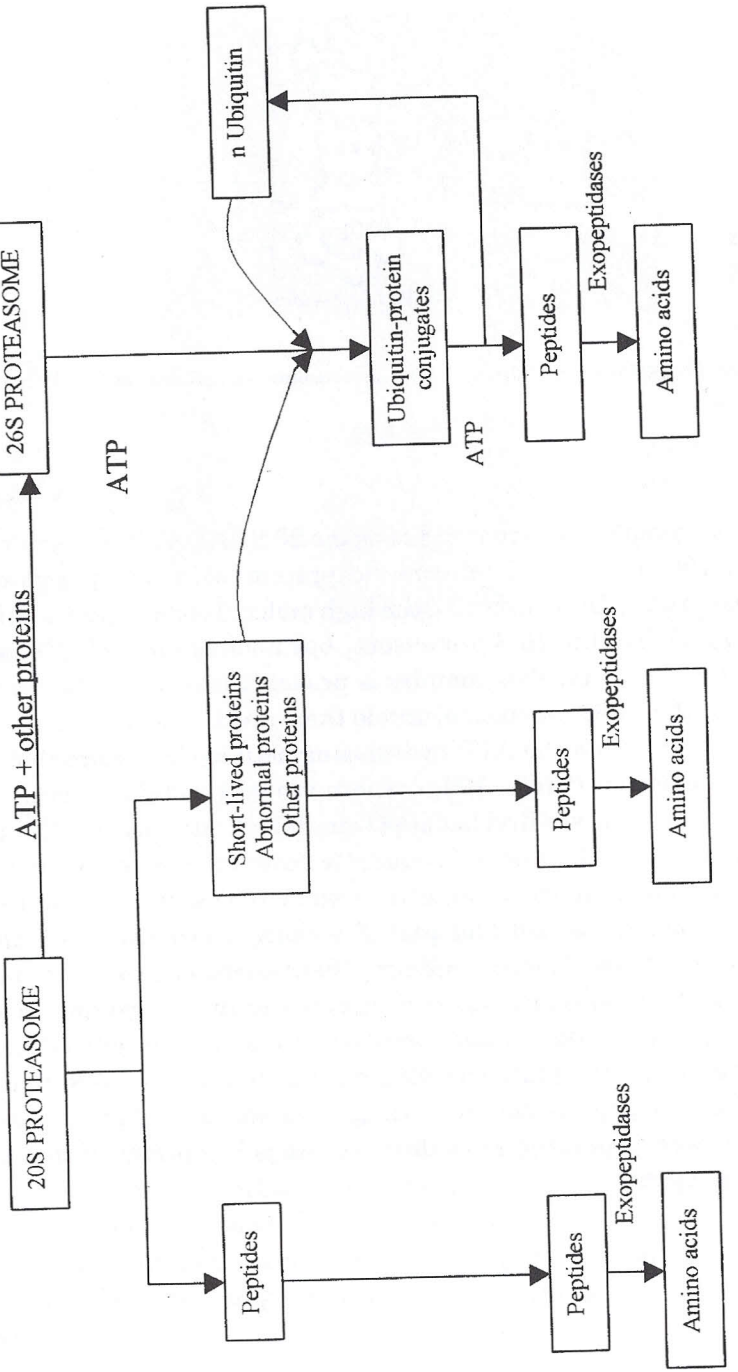

Fig. 3. Role of the 20S and 26S proteasomes in intracellular proteolysis.

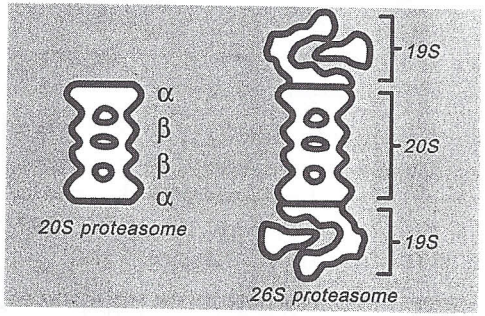

Fig. 4. Schematic presentation of 20S and 26S proteasome structures based on electron microscopy.

The 19 S cap complex, also referred to as the 20 S ball, ATPase complex, μ particle or PA700, is an heterooligomeric protein with an approximate molecular mass of 650 kDa, containing the high molecular mass polypeptides (35-100 kDa) specific of the 26 S proteasome but none of the 20 S protease. It exhibits ATPase activity. This complex is probably involved in binding to the substrates of the 26 S proteasome, unfold them and transport them to the proteolytic core (for which the ATP hydrolysing activity is required) of the cylindrical 20 S particle (Peters, 1994), where proteolysis takes place.

The 26 S proteasome was first isolated from rabbit reticulocytes (Hough, Pratt & Rechsteiner, 1987) and subsequently from other eukaryotic cells including animal and yeast. However, little is known concerning the protease in plants. It has been detected and partially characterized in *S. oleracea* (Fujinami *et al.*, 1994) and *L. minor* (Caeiro, 1996), being apparently similar to mammalian 26 S proteasomes. It has been detected in both cytoplasm and the nucleus. It is a very large protein complex, with an estimated molecular mass of 2 MDa and a sedimentation coefficient of 26 S. It is composed of more than 25 different polypeptide subunits, ranging in size from 22 to 110 kDa. Highly purified 26 S proteasomes contain all the polypeptides of the 20 S particle (Peters, 1994).

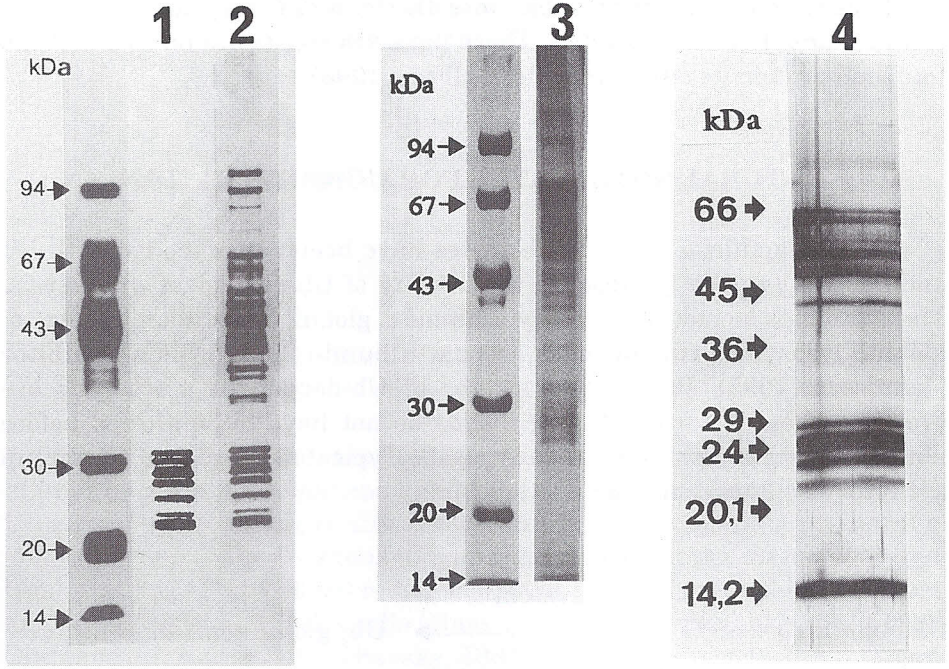

Fig. 5. Polypeptide patterns obtained by SDS-PAGE of the 20 S (lane 1) and 26 S (lane 2) proteasomes from human liver (Tanahashi *et al.*, 1993) and of the 26 S proteasomes from *S. oleracea* (lane 3; Fujinami *et al.*, 1994) and *L. minor* (lane 4; Caeiro, 1996). Molecular masses of standards are indicated in kDa.

The enzymatic properties of the 26 S proteasome are markedly distinct from the 20 S proteasome (Peters, 1994): ATP hydrolysis is required for proteolysis by the 26 S proteasome, in contrast to the energy independent activity of the 20 S proteasome; the 26 S complex exhibits isopeptidase activity, which removes Ub from bound substrates; the 26 S proteasome has a high affinity for multi-ubiquitylated proteins, although Ub-independent degradation by the 26 S complex has been demonstrated for the short-lived enzyme ornithine decarboxylase (Murakami *et al.*, 1992). Since ATPase activity, isopeptidase activity and substrate specificity are not attributed to the 20 S proteasome, they must be due to the attached cap complexes, either directly or by changing the properties of the 20 S core particle (Peters, 1994).

In eukaryotic cells, the proteasomes are the sites for degradation of most cell proteins (Rock *et al.*, 1994). These particles constitute up to 1% of the total cell protein (Coux, Tanaka & Golberg, 1996).

NATURAL SUBSTRATES FOR UBIQUITYLATION

Several artificial protein-substrates have been radiolabelled with ^{125}I and utilized *in vitro* to measure the activity of Ub-dependent proteolysis. These proteins include α-casein, cytochrome *c*, globin, α-lactalbumin, β-lactoglobin, lysozyme, ribonuclease, serum albumin and trypsin inhibitor (Jennissen, 1995). Primary substrates of Ub-dependent proteolysis are proteins whose N-terminal amino acids do not have to be altered before ubiquitylation and subsequent proteolysis. Typical substrates of this group are lysozyme, α-casein and β-lactoglobin.

$$\text{Globin} + n\text{Ub} + n\text{ATP} \xrightarrow{\text{Ub conjugating system}} \text{Ub}_n\text{-globin} + n\text{AMP} + n\text{PPi}$$

$$\text{Ub}_n\text{-globin} + m\text{ATP} \xrightarrow{\text{26S proteasome}} \text{Ub}_n\text{-peptides} + m\text{ADP} + m\text{Pi}$$

$$\text{Ub}_n\text{-peptides} \xrightarrow{\text{isopeptidases}} \text{peptides} + n\text{ Ub}$$

In contrast, secondary substrates are proteins whose N-terminal residues have to be altered before ubiquitylation and degradation (Jennissen, 1995). Ferber & Ciechanover (1987) showed that protein substrates with N-terminal acidic residues are not recognized by Ub-protein ligase and can only be degraded by the Ub system after being post-translationally modified by the addition of an arginine residue (necessity for arginyl-tRNA) to the N-terminus. The modification reaction is catalysed by arginyl-tRNA-protein transferase, which is specific for proteins possessing an N-terminal Asp, Glu or Cys. A prerequisite is that the N-terminus is not buried in the protein (Soffer, 1973). Bovine serum albumin (BSA), bovine α-lactalbumin and soybean trypsin inhibitor, which contain acidic N-terminal residues (Asp or Glu), are ubiquitylated and degraded after arginylation.

$$\text{Arginyl-tRNA} + \text{BSA} \xrightarrow{\text{arginyl-tRNA-protein transferase}} \text{arginyl-BSA} + \text{tRNA}$$

$$\text{Arginyl-BSA} + n\text{Ub} + n\text{ATP} \xrightarrow{\text{Ub conjugating system}} \text{Ub}_n\text{-arginyl-BSA} + n\text{AMP} + n\text{PPi}$$

$$\text{Ub}_n\text{-arginyl-BSA} + m\text{ATP} \xrightarrow{\text{26S proteasome}} \text{Ub}_n\text{-peptides} + m\text{ADP} + m\text{Pi}$$

$$\text{Ub}_n\text{-peptides} \xrightarrow{\text{isopeptidases}} \text{peptides} + n\text{Ub}$$

Besides artificial (foreign proteins in the sense of non-self, like extracellular or bacterial proteins) and denatured (unfolded, oxidised) proteins, the Ub proteolytic pathway is also involved in the breakdown and removal of abnormal (incomplete, proteolytically nicked, containing amino acid analogs) proteins. This has been demonstrated in rabbit reticulocytes, yeast, mouse ts85 cells and HeLa cells (Haas & Rose, 1982; Hershko *et al.*, 1982; Ciechanover, Finley & Varshavsky, 1984; Seufert & Jentsch, 1990). Therefore, the Ub system may be considered as a secondary system of defense which is essential for cell viability.

Despite the widely recognized physiological importance and the very large number of studies published on the Ub-mediated proteolytic pathway, the number of putative natural substrates remains surprisingly low. A complex linkage between ubiquitylation and degradation signals or second messengers possibly exists, making it difficult to unequivocally demonstrate the *in vitro* ubiquitylation of a particular target protein. Some proteins have been proposed in animal, plant and yeast cells as natural substrates for ubiquitylation. Non-catabolic ubiquitylations have been suggested for calmodulin, histones, arthrin, eye lens proteins, myosin light chain and several membrane receptors. Putative natural substrates for Ub-dependent proteolysis include phytochrome, cyclins A and B, p53 protein, MATα2 protein, MOS protein, several oncoproteins, calmodulin, hexokinase, regulatory subunits of protein kinase A and glyceraldehyde-3-phosphate dehydrogenase (Jennissen, 1995). However, no natural protein has yet been conclusively demonstrated to be degraded via the Ub-dependent proteolytic pathway. Only p53 protein and cyclin B are emerging as convincing candidates for a degradation by the Ub pathway (Jennissen, 1995).

The plant regulatory photoreceptor phytochrome was one of the first natural substrates reported to be degraded by the Ub pathway *in vivo* (Shanklin, Jabben & Vierstra, 1987). Red light, inducing the photoconversion $P_r \rightarrow P_{fr}$ is the signal necessary for ubiquitylation. The *in vivo* half-life of the P_{fr} form is approximately 100-fold lower than that of the P_r form. However, ubiquitylation of phytochrome appears to be a result of, or accompanied by, the aggregation of this protein (Jabben, Shanklin & Vierstra, 1989). Therefore, evidence is still lacking for final proof that P_{fr} is indeed degraded via the Ub pathway. Besides calmodulin and histones, tryptophan decarboxylase has also been reported to undergo ubiquitylation in plants (Fernandez & DeLuca, 1994).

It is widely accepted that the *in vivo* turnover of natural very-fast-turnover proteins is generally mediated by the Ub pathway (Ciechanover, Finley & Varshavsky, 1984; Finley, Ciechanover & Varshavsky, 1984). Indeed, several studies concluded that 90% of short-lived proteins in higher eukaryotic cells are generally degraded by the Ub-dependent proteolytic pathway. However, more recent work has shown that these conclusions were premature (Jennissen, 1995). The very-fast-turnover protein ornithine decarboxylase, for example, was recently shown to be degraded in a Ub-independent manner (Murakami *et al.*, 1992).

DEGRADATION SIGNALS OF THE UBIQUITIN SYSTEM

Degradation signals are features of proteins that confer metabolic instability. The number of distinct Ub-dependent degradation signals on the proteins destined for catabolism is unknown but is likely to exceed ten (Varshavsky, 1997). Internal sequence motifs, N-terminal alterations of the target protein and post-translational modifications that affect the conformation of the protein (e.g. phosphorylation, dephosphorylation, ligand-binding) may constitute requisites for ubiquitylation. Degradation signals on the substrate protein appear to comprise two essential elements: an amino acid sequence or a conformational determinant and one or several internal Lys residues, the sites of ubiquitylation. On the other hand, degradation signals can be active constitutively or conditionally. Conditional degradation signals are controlled through phosphorylation or interactions with other proteins, whose binding may sterically shield an otherwise constitutive degradation signal. Alternatively, the interaction with another protein may confer a short *in vivo* half-life to the substrate protein. A number of Ub-dependent degradation signals have recently been proposed, including the N-degron and the N-terminal Ub (degrons are motifs that target proteins for degradation by the Ub-dependent pathway or other systems).

The N-degron comprises two essential determinants: a destabilizing N-terminal residue and one or more internal Lys residues of a substrate. In fact, it was found that the *in vivo* half-life of a protein depends on the identity of its N-terminal residue – a relation termed the N-end rule (Bachmair, Finley & Varshavsky, 1986).

Bachmair, Finley & Varshavsky (1986) devised an elegant and ingenious experiment to study the effect of the N-terminal amino acid residue on the Ub-dependent rate of degradation. A chimeric gene was constructed encoding a Ub-Xaa-β-galactosidase fusion protein which could be expressed in *S. cerevisiae*. The Ub molecule is spontaneously cleaved off the nascent protein, exposing amino acid X (Xaa) as the N-terminal residue of the *Escherichia coli* β-galactosidase. In this way, 16 similar β-galactosidase proteins were constructed differing exclusively in their N-terminal amino acids. The authors concluded that the free N-terminus determined the half-life of such b-galactosidases in yeast – N-terminal Arg conferred a half-life of 2 min whereas Met one of 20h. The short-lived b-galactosidase molecules were multi-ubiquitylated implying their degradation by the Ub pathway. It followed, that in addition to the N-end signal, a second signal in the form of an acceptor Lys residue for multi-ubiquitylation was essential (Bachmair & Varshavsky, 1989).

It must be emphasized that, although very attractive, there is no clear evidence for a role of the N-end rule on Ub-mediated proteolysis. Over 80% of the cell soluble proteins are N-terminally blocked, mainly by acetylation, which excludes them from the N-end rule (Han & Martinage, 1992). The remaining 20% of unblocked proteins are probably also not eligible for this system because they generally possess N-terminal residues that protect them from degradation by the N-end rule (Hershko & Ciechanover, 1992). This may be due to the specificity of the initiator methionine aminopeptidase, which may only remove Met if the second amino acid residue is stabilizing according to the N-end rule (Varshavsky, 1992). The N-end rule dependence of protein degradation and the E3 enzyme apparently display exactly the same substrate specificity. The low biological significance of the N-end rule is therefore suggested by observing that the deletion of E3 enzyme in yeast is not lethal (Bartel, Wunning & Varshavsky, 1990).

Multi-Ub chains linked to a substrate protein function as secondary signals for degradation, because the initial conjugation of Ub with the

substrate is mediated by the primary degradation signal. However, it was found that linear Ub fusions (DNA encoded), bearing a non-removable N-terminal Ub moiety, are short-lived, its Ub functioning as a primary degradation signal. Eukaryotes contain a number of genes encoding proteins that bear domains highly similar to Ub.

Sequences rich in Pro, Glu, Ser and Thr, referred to as PEST, have been proposed to function as degradation signals (Rechsteiner & Rogers, 1996).

Most of the damaged or structurally abnormal proteins are recognized and destroyed by the Ub system, apparently due to the exposure of their normally buried degradation signals (Varshavsky, 1997). Therefore, normal and otherwise long-lived but nascent polypeptides, with their degradation signals still exposed before the acquisition of their native conformation, may be regarded by the Ub system as damaged proteins. Folding of newly formed proteins, in some cases with the aid of chaperonins, may be in kinetic competition with the pathways that target them for degradation.

CONCLUDING REMARKS

Over the last two decades much effort was put in trying to understand the ubiquitin system. Consequently, a huge amount of information has accumulated on ubiquitylation and de-ubiquitylation, particularly in what animal cells are concerned. On the other hand, there is little doubt about the great importance of ubiquitin metabolism in eukaryotic cells. Nevertheless, fundamental gaps still exist in our knowledge about ubiquitin, specially in determining the true biological significance of the ubiquitin system and the physiological relevance of the ubiquitin-mediated proteolytic pathway.

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