Focus

Ubiquitin-Mediated Degradation of Cellular Proteins: Why Destruction is Essential for Construction, and How it Got from the Test Tube to the Patient's Bed

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Abstract

Between the 1960s and 1980s, the main focus of biological research was nucleic acids and the translation of the coded information into proteins. Protein degradation was a neglected area and regarded by many as a scavenger, non-specific and end process. While it was known that proteins are turning over, the large extent and high specificity of the process – where distinct proteins have half-lives that range from a few minutes to several days – have not been appreciated. The discovery of the lysosome by Dr. Christian de Duve did not change this view significantly, as this organelle is involved mostly in the degradation of extra- and not intracellular proteins, and it was clear that lysosomal proteases, similar to those of the gastrointestinal tract, cannot be substrate specific. The discovery of the complex cascade of the ubiquitin pathway has changed this view dramatically. It is now clear that degradation of cellular proteins is a highly complex, temporally controlled, and tightly regulated process that plays major roles in a broad array of basic pathways during cell life and death. With the multitude of substrates targeted and processes involved, it is not surprising that aberrations in the pathway have been recently implicated in the pathogenesis of many diseases, certain malignancies and neurodegeneration among them. Degradation of a protein via the ubiquitin pathway involves two successive steps: a) conjugation of multiple ubiquitin moieties to the substrate, and b) degradation of the tagged protein by the downstream 26S proteasome complex with release of free and re-utilizable ubiquitin. Despite intensive research, the unknown still exceeds what we currently know on intracellular protein degradation and major key problems remain unsolved. Among these are the modes of specific and timed recognition of the myriad substrates of the system and the nature of the mechanisms that underlie aberrations in the system and pathogenesis of diseases.

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Ubiquitin-mediated proteolysis

Like other macromolecular components of the organism, proteins are in a dynamic state of synthesis and degradation. During proteolysis, the peptide bond that links amino acids to one another is hydrolyzed and free amino acids are released. The process is carried out by a diverse group of enzymes called proteases. During proteolysis, the energy invested in the generation of the peptide bond is released. Distinct proteolytic mechanisms serve different physiological purposes and allow the body to accommodate to changing environmental and pathophysiological conditions. One can distinguish between destruction of "foreign" and "self" proteins. "Foreign" proteins that are contained in our diet are degraded "outside" the body, in the lumen of the digestive tract. To avoid immune response, the epithelial lining of the digestive tract prevents penetration of these proteins into the body, and they must be decomposed to yield non-antigenic amino acids that enter the body and serve as building blocks for synthesis of its own proteins. "Self" proteins can be divided into extracellular and intracellular, and these two groups of proteins are degraded via two distinct and independent mechanisms. Extracellular proteins such as the blood coagulation factors, immunoglobulins, albumin, cargo carrier proteins (such as the core protein of the low density lipoprotein, or transferrin), and peptide hormones (such as insulin), are taken up via pinocytosis or specific receptor-mediated endocytosis. They are then carried via a series of vesicles (endosomes) that fuse with primary lysosomes where they are degraded. During this process, the extracellular proteins are never exposed to the intracellular environment (the cytosol), and remain "extracellular." Degradation of proteins in lysosomes is not specific, and all engulfed proteins exposed to the lysosomal proteases are degraded at the same rate. Several observations led to the prediction that degradation of intracellular proteins must be carried out by completely different mechanisms. The process is highly specific and different proteins have half-life times that range from a few minutes (like the tumor suppressor protein p53) to several days (like the

muscle proteins actin and myosin). Paradoxically, from a thermodynamic point of view, degradation of intracellular proteins requires metabolic energy. It was assumed that energy is required to endow the system with a high degree of specificity and control, as it is inconceivable that trypsin- or chymotrypsin-like proteases will act freely in the cytosol. Also, inhibitors of lysosomal degradation do not affect the process, a finding that suggested that the process occurs in the cytosol. The assumption that the proteolytic enzymes and their substrates reside in the same cellular compartment predicted the existence of a tightly regulated machinery that must utilize metabolic energy for control. The discovery of the ubiquitin-proteasome proteolytic pathway resolved most of these enigmas.

Degradation of a protein via the ubiquitin-proteasome pathway involves two discrete and successive steps: a) tagging of the substrate by covalent attachment of multiple ubiquitin molecules, and b) degradation of the tagged protein by the 26S proteasome complex. Conjugation of ubiquitin, a highly conserved 76 amino acid residue polypeptide, to the protein substrate proceeds via a three-step cascade mechanism. Initially, the ubiquitin-activating enzyme, E1, activates, in an ATP-requiring reaction, the C-terminal carboxyl group of ubiquitin (Gly residue) to generate a high energy intermediate with the enzyme. One of several E2 enzymes (ubiquitin-carrier proteins or ubiquitin-conjugating enzymes) transfers the activated ubiquitin, via an E2~ubiquitin intermediate, to the substrate that is specifically bound to a member of the ubiquitin-protein ligase, E3, family. E3s catalyze the last step in the conjugation process – covalent attachment of ubiquitin to the substrate. The first moiety is transferred to an ε-NH_2 group of an internal Lys residue of the protein substrate to generate an isopeptide bond. In successive reactions, a polyubiquitin chain is synthesized by processive transfer of additional activated ubiquitin moieties to an internal Lys residue of the previously conjugated ubiquitin molecule. This multi-cycle process leads to the synthesis of a substrate-anchored polyubiquitin chain that is recognized by the downstream 26S proteasome complex. E3s play a key role in the ubiquitin-mediated proteolytic cascade, as they bind the target substrates and serve as the specific recognition elements of the system. The proteasome is a large, 26S, multicatalytic protease that degrades polyubiquitinated proteins into small peptides. It is composed of two sub-complexes: a 20S particle that carries the catalytic activity, and a regulatory 19S particle. The 20S particle is a barrel-shaped structure composed of four stacked rings, two identical outer α rings and two identical inner β rings. The α and β rings are composed each of seven distinct subunits, giving the 20S complex the general structure of $\alpha_{1\text{-}7}\beta_{1\text{-}7\beta}1\text{-}7\alpha_{1\text{-}7}$. The catalytic sites are localized to some of the β subunits. Electron microscopy shows that each extremity of the 20S barrel is capped by a 19S particle. One function of the 19S complex is presumably to recognize ubiquitinated proteins and other potential substrates of the proteasome. A ubiquitin-binding subunit of the 19S particle has indeed been identified, however its mode of action has not yet been discerned. A second function of the 19S complex, and most probably of its ATPase subunits, is to open an orifice in the α ring that will allow entry of the substrate into the proteolytic chamber. Finally, since a folded protein would not be able to fit into the proteasomal channel, it is assumed that the 19S particle unfolds the polypeptide chain and inserts it into the 20S particle. Both the channel-opening function and the unfolding of the substrate require metabolic energy. Following degradation of the substrate, short peptides derived from the substrate are released along with free and re-utilizable ubiquitin that is cleaved via the activity of ubiquitin recycling enzymes (ubiquitin C-terminal hydrolases, isopeptidases) [For a general review and a monograph on the ubiquitin system, see refs. 1,2]. For a general scheme of the ubiquitin pathway, see Figure 1.

A major, yet unresolved, problem involves the mechanisms that underlie the high specificity and selectivity of the system. Why are certain proteins extremely stable while others are short-lived? Why are certain proteins degraded at a particular time point along the cell cycle or only following specific extracellular stimuli, while they are stable under most other conditions? It appears that specificity is determined by two distinct and unrelated groups of proteins. Within the ubiquitin system, substrates must be specifically recognized and bind, prior to their ubiquitination, to the appropriate E3 enzyme. In most cases however, substrates are not recognized directly and in a constitutive manner. In a few cases, such as that of APC (anaphase promoting complex, the E3 that ubiquitinates mitotic regulators), the E3 undergoes post-translational modification in order to be active and bind the substrate. In most other cases it is the substrate that undergoes a certain change(s) that renders it susceptible for conjugation. The stability of some proteins depends on their state of oligomerization, while many others must undergo post-translational modification, such as phosphorylation or association with ancillary proteins such as molecular chaperones that act as recognition elements in *trans*, in order to be recognized by the appropriate ligase. Others, such as certain transcription factors, have to dissociate from the specific DNA sequence to which they bind in order to be recognized by the system. Thus, in addition to E3s, the modifying enzymes (kinases, for example), the ancillary proteins and the DNA sequences to which substrate proteins bind, also play an important role in the recognition process.

Ubiquitin-mediated proteolysis of a variety of cellular proteins plays an important role in many basic cellular processes. Among these are regulation of cell cycle and division, differentiation and development, involvement in the cellular response to stress and extracellular effectors, morphogenesis of neuronal networks, modulation of cell surface receptors, ion channels and the secretory pathway, DNA repair, regulation of the immune and inflammatory responses, and biogenesis of organelles. The list of cellular proteins that are targeted by ubiquitin is growing rapidly. Among them are cell cycle regulators such as G1 and mitotic cyclins, cyclin-dependent kinase inhibitors, such as $p27^{Kip1}$, and proteins involved in sister chromatid separation, such as Cut2. Tumor suppressors such as

p53, transcriptional activators and their inhibitors, including E2F-1, fos, myc, NF-κB and IκBα, cell surface receptors such as the growth hormone receptor and the T cell receptor, and endoplasmic reticulum proteins such as the cystic fibrosis transmembrane conductance regulator and HMG-CoA reductase, the rate-limiting enzyme in cholesterol biosynthesis that is the target of statins, are also degraded by the ubiquitin system. Cellular quality control mechanisms remove selectively via targeting to the ubiquitin system, mutated and denatured/misfolded proteins that are harmful to the cell. The system degrades them selectively while retaining their undamaged, functional counterparts.

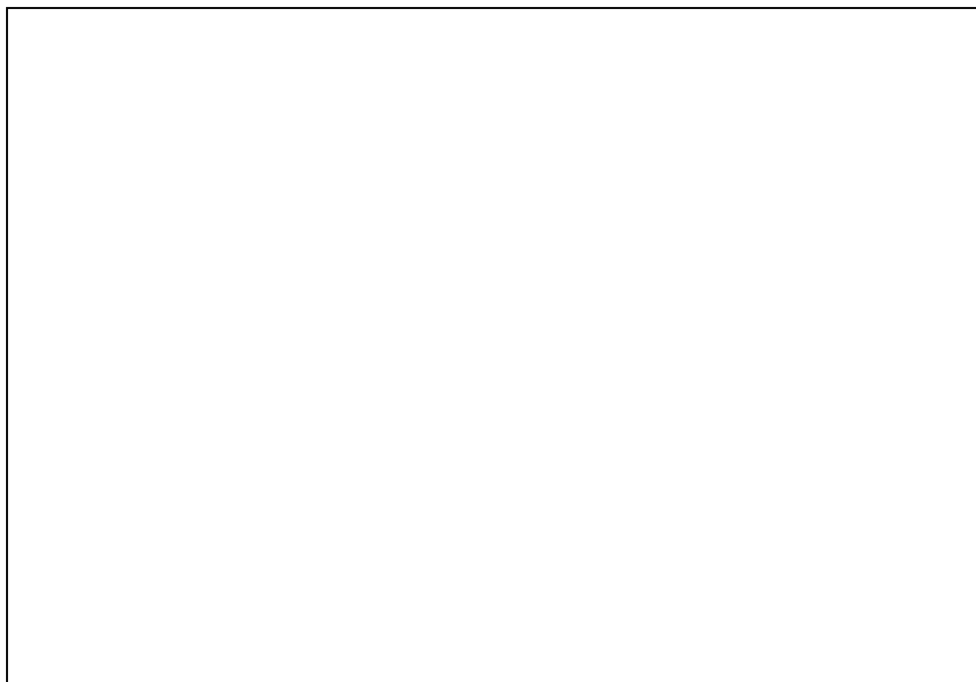

Figure 1. The ubiquitin proteolytic pathway. (1) activation of ubiquitin by the ubiquitin-activating enzyme, E1, a ubiquitin-carrier protein, E2 (known also as ubiquitin-conjugating enzyme), and ATP. (2) binding of the protein substrate, via a defined recognition motif, to a specific ubiquitin-protein ligase, E3. (3) multiple cycles of conjugation of ubiquitin to the target substrate, initially to an ε-NH_2 group of an internal Lys residue of the substrate followed by successive cycles of conjugation to the ε-NH_2 group of Lys48 of the previously conjugated ubiquitin moiety to generate a polyubiquitin chain. (4) degradation of the ubiquitin-tagged substrate by the 26S proteasome complex with release of short peptides. In the case of cellular antigens, such as viral proteins, these peptides are presented on MHC class I molecules to cytotoxic T cells. (5) ubiquitin is recycled via the activity of ubiquitin-C-terminal hydrolases (UCHs; isopeptidases).

With the numerous substrate proteins targeted and the multitude of processes involved, it is not surprising that aberrations in the ubiquitin system have been implicated in the pathogenesis of many inherited and acquired human pathologies. In some cases, the linkage between the proteolytic system and the resulting pathology is direct, while in others it is less obvious.

The ubiquitin system in the pathogenesis of human diseases

With the many processes and substrates targeted by the ubiquitin system, it was not surprising to find that aberrations in the system underlie the pathogenesis of many known and probably some yet unknown diseases. For some diseases the linkage between the pathology and aberrations in the system is obvious and clear, while for others it is indirect and at times presumptive. While inactivation of a major enzyme such as E1 is obviously lethal, mutations in enzymes or in recognition motifs in substrates that do not affect vital pathways, or that affect the involved process only partially, result in a clear phenotype. Likewise, acquired changes in the activity of the system will also evolve into clear pathologies. The pathological states associated with the ubiquitin system can be divided into two groups:

- those that result from loss of function – mutation in a ubiquitin system enzyme or target substrate that results in stabilization of certain proteins
- those that result from gain of function – abnormal or accelerated degradation of the protein target.

Targeted inactivation of specific ubiquitin system genes in animals provide a glimpse into the broad spectrum of pathologies that can result from aberrations in ubiquitin-mediated proteolysis and predict that many more ubiquitin system-related diseases will be discovered.

Malignancies

It was noted that the level of the tumor suppressor protein p53 is extremely low in uterine cervical carcinomas caused by high risk strains of the human papillomavirus. Detailed studies both *in vitro* and *in vivo* have shown that the suppressor is targeted for ubiquitin-mediated degradation by the HPV oncoprotein E6 coded by high risk species of the virus (E6-16 or 18) [3]. Low risk strains that encode slightly different E6 proteins (such as E6-11) do not transform cells and do not target p53 for degradation. Degradation is mediated by the E3 enzyme E6-AP (E6-associated protein), where E6 serves as an ancillary protein

HPV = human papillomavirus

that allows recognition of p53 in *trans*. It associates with both the ligase and the target substrate and brings them, via the generation of a ternary complex, to the necessary proximity that is assumed to allow catalysis of conjugation to occur. Removal of the suppressor by the oncoprotein leads probably to malignant transformation in the absence of the cellular DNA damage sensing and control mechanism.

Aberrations in ubiquitin-mediated regulation of β-catenin, which normally plays an important role in signal transduction and differentiation of the colorectal epithelium, may play an important role in the multi-step development of colorectal tumors [4]. These tumors develop in 50% of the western world population by the age of 70, and in 10% of these individuals (5% of the population) the tumors progress to malignancy. Fifteen percent of these patients have a genetic predisposing defect leading to the development of the malignancy. In the absence of signaling, glycogen synthase kinase-3-β is active and promotes, following phosphorylation, degradation of β-catenin via the ubiquitin-proteasome pathway [5]. Stimulation promotes dephosphorylation, stabilization and subsequent activation of β-catenin via complex formation with otherwise inactive subunits of other transcription factors. β-catenin interacts with the 300 kDa tumor suppressor APC (adenomatous polyposis coli) that appears to regulate, in a yet unknown manner, its intracellular level. Aberrations in degradation of β-catenin lead to its stabilization, accumulation, and subsequent oncogenic activation, and can result from two distinct mechanisms: mutations in the protein itself and mutations in the targeting machinery. Mutations in the E3 recognition domain of β-catenin, in which the amino acid residues that are phosphorylated by GSK3β are substituted with amino acids that cannot be modified, have been described in several colorectal carcinomas as well as in malignant melanomas. These mutations result in a protein species that cannot be phosphorylated, and therefore cannot be recognized by the ubiquitin ligase. Colon cancer cells that do not express APC, or that harbor APC proteins that are mutated in one of the catenin-binding clusters, do not associate with β-catenin. Here too, the protein accumulates in the cytosol as an active transcriptional complex. Expression of full length APC in these cells leads to degradation of excess β-catenin and to abrogation of the *trans*-activation effect. It is possible that APC, while not a ubiquitin ligase itself, acts in the degradation of β-catenin as an ancillary targeting element in *trans*, similar to the action of E6 on p53 [6].

An interesting case is that of the cyclin-dependent kinase inhibitor $p27^{Kip1}$. The protein acts as a negative growth regulator/tumor suppressor and plays an important role in proliferation of mammalian cells. It binds and negatively regulates the activity of CDK2/cyclin E and CDK2/cyclin A complexes and thus inhibits cell cycle progression from G1 and entrance into the S phase. Its level is high in quiescent cells, but following mitogenic stimuli it is rapidly degraded by the ubiquitin system, allowing the CDK/cyclin complexes to drive the cell into the S phase [7]. The level of p27 is markedly reduced in several malignancies, including breast, colorectal and prostate carcinomas, and in many of these cases there is a strong correlation between the low level of p27 and the aggressiveness of the disease – namely tumor grading, clinical staging and poor prognosis [8]. Levels of p27 have become an important and novel prognostic factor for survival, recurrence and evaluation of therapy, where extremely low expression predicts poor prognosis. Dissection of the mechanism that underlies the decrease in p27 revealed that, unlike the case of other tumor suppressors such as p53 that are mutated and stabilized in many tumors, the rapidly degraded p27 is of the WT species. It is probably abnormal activation of the ubiquitin system that underlies the accelerated degradation of the suppressor.

Another interesting case involves pVHL, the von Hippel-Lindau protein. Mutations in one germ line copy of *VHL* predisposes individuals to a wide range of malignancies, including renal cell carcinoma, pheochromocytoma, cerebellar hemangioblasomas, and retinal angiomas. A hallmark of $VHL^{-/-}$ tumors is a high degree of vascularization that arises from constitutive expression of hypoxia-inducible genes including the master switch transcription factor hypoxia-inducible factor 1-α, the crucial vascular endothelial growth factor. It has recently been shown that pVHL is a ubiquitin ligase that is involved in targeting of HIF1-α for ubiquitin- and proteasome-mediated degradation. It is possible that the high, non-regulated activity of HIF1-α, and possibly other targets of pVHL, underlie the high frequency of malignant transformations observed in carriers of mutations in pVHL.

In another case it was shown that c-Jun, but not its transforming counterpart v-Jun, can be multiply ubiquitinated and rapidly degraded in cells [9]. Detailed mechanistic analysis of the differential sensitivity to the ubiquitin system revealed that the δ domain of c-Jun, an amino acid sequence that spans residues 31-57 and is missing in the retrovirus-derived molecule, confers instability upon the normal, cellular protein. Deletion of the domain stabilizes c-Jun. Interestingly, the δ domain does not serve as a ubiquitination target. It may serve however as an anchoring site to the specific Jun ligase. The lack of the δ domain from v-Jun, a protein that is otherwise highly homologous to c-Jun, provides a mechanistic explanation for the stability and the possible resulting transforming activity of v-Jun. The loss of the δ domain during the retroviral transduction is yet another example of the "sophisticated" diverse mechanisms developed by viruses to ensure replication and continuity of infection.

GSK3β = glycogen synthase kinase-3-β
APC = adenomatous polyposis coli
CDK = cyclin-dependent kinase

HIF1-α = hypoxia-inducible factor-α
pVHL = von Hippel-Lindau protein

Liddle's syndrome

An interesting syndrome involves aberration in the regulation of kidney epithelial Na^+ channel. The channel, which is composed of three homologous subunits α, β and γ, is a short-lived protein that is degraded by the ubiquitin system and is recognized via a proline-rich (PY) motif by Nedd4, a member of the E6-AP family of E3 enzymes. Binding of Nedd4 is mediated via a WW domain in the ligase [10]. A mutation in the PY motif leads to stabilization of the complex, accumulation of pump subunits, excessive reabsorption of Na^+ and H_2O, and consequently, a severe form of hypertension.

Angelman syndrome

The ubiquitin system is probably involved in human brain development. A defect in the gene coding for the E3 enzyme E6-AP has been implicated directly as the cause of Angelman syndrome, which is characterized by mental retardation, seizures, out of context frequent smiling and laughter, and abnormal gait [11,12]. The target brain protein(s) that are most probably stabilized because of the mutation has not been identified. However, unlike E6-mediated targeting of p53, degradation of this putative protein is E6 independent and it is probably one of the native substrates of the ligase. The HPV, as part of its strategy, adopted the enzyme for targeting p53 in an E6-dependent mode. However, the physiological role of the enzyme involves targeting to a specific brain protein(s) that, when accumulated, leads to toxicity of the developing brain. Interestingly, the disease displays genomic imprinting, whereby a subset of mammalian genes can be expressed monoallelically in a parent-of-origin manner (uniparental disomy). The imprinting process epigenetically marks alleles according to their parental origin during gametogenesis. In the case of Angelman syndrome, the imprinted gene (that codes for E6-AP) is localized to chromosome 15q11-q13 where the deletions in the affected children were identified. Confirmatory studies in mice with uniparental disomy of the mouse homologue (Ube3a) show marked reduction in expression within Purkinje cells and hypocampal neurons, thus strongly establishing imprinting of brain E6-AP in Angelman syndrome pathogenesis [13].

Neurodegenerative diseases

Accumulation of ubiquitin conjugates have been reported in pathologic lesions of many chronic neurodegenerative diseases, such as the neurofibrillary tangles of Alzheimer's disease, brainstem Lewy bodies – the neuropathological hallmark – in Parkinson's disease, and nuclear inclusions in CAG repeat expansion diseases such as Huntington's disease and spinocerebellar ataxias. However, in all these cases, the pathogenetic linkage to aberrations in the ubiquitin system is still indirect. One factor that complicates the establishment of such a linkage is the realization that many of these diseases, such as Alzheimer's and Parkinson's, are not defined clinical entities, but rather syndromes with many distinct etiologies. Accumulation of ubiquitin conjugates in Lewy inclusion bodies in many of these cases is probably secondary, and may reflect unsuccessful attempts by the cell to remove damaged or abnormal proteins. This aggregation of brain proteins into presumably toxic lesions is emerging as a common, but poorly understood, mechanistic theme in sporadic and hereditary neurodegenerative disorders. It should also be noted that in most of these cases the target proteins have not been defined either.

The case of PD highlights the complexity of the involvement of the ubiquitin system in the pathogenesis of neurodegeneration. Recent evidence has implicated mutations in α-synuclein, such as A53T, in the pathogenesis of neurodegenerative diseases. Initially, it was found in several cases of autosomal dominant familial PD. Later, the protein was shown to be a major component of LBs and Lewy neurites in sporadic PD, dementia with LBs, and the LB variant of AD. Also, studies of brains from patients with AD caused by genetic abnormalities demonstrated many α-synuclein-positive LBs [14]. The function of α-synuclein is not known. Furthermore, while it has been shown that the protein is targeted by the proteasome [15], it is not known whether the mutations affect its stability and lead to its accumulation and toxicity, and whether it is the conjugated form of synuclein that is accumulated in LBs. Recent findings in a German family with PD have revealed mutations in the gene coding for the ubiquitin carboxy terminal hydrolase isozyme UCH-L1. While the mutation did not lead to inactivation of the enzyme, the enzyme was clearly less active than its WT counterpart [16]. A third player in the pathogenesis of PD is parkin. Parkin is a 465 amino acids residues protein with moderate similarity to ubiquitin at the amino terminus and a RING-finger motif at the carboxy terminus. Mutations in the gene appear to be responsible for the pathogenesis of autosomal recessive juvenile parkinsonism, one of the most common familial forms of PD [17]. Interestingly, this disease is characterized by lack of LBs, the pathognomonic hallmark of sporadic forms of the disease. Later studies have identified parkin as a ubiquitin-protein ligase, E3, that acts along with the ubiquitin-conjugating enzyme UbcH7 and UbcH8, and that mutant parkins from AR-JP patients display loss of the ubiquitin ligase activity [18]. Parkin ubiquitinates and promotes degradation of CDCrel-1, a synaptic vesicle-enriched septin GTPase implicated in the inhibition of exocytosis through its interactions with syntaxin [19]. It is not known whether aberrations in the degradation of this substrate or of other, yet to be identified, substrates of parkin underlie the pathogenesis of AR-JP. Thus, three distinct pathologies, all linked strongly or weakly to activity of the ubiquitin system, have been associated with the pathogenesis of PD, though the underlying mechanisms have remained obscure.

AD is also characterized by accumulation of ubiquitin in neurofibrillary tangles and senile plaques, both characteristic of the neuronal abnormalities associated with the disease. They are

PD = Parkinson's disease
LBs = Lewy bodies
AD = Alzheimer's disease
AR-JP = autosomal recessive juvenile parkinsonism

also present in LBs characteristic to some forms of the diseases. Ubiquitin is found both in its free and conjugated forms. In these cases however, the target proteins and their role in the pathogenesis of the disease are still unknown. Also, it is not clear whether there is a defect in the ubiquitin system, or whether the presented substrates are misfolded/abnormal and cannot be digested by an otherwise normal system. It is also not clear whether accumulation of the proteins is toxic or whether the pathology is caused by upstream, yet unknown, factors. A more direct relationship between the ubiquitin system and pathogenesis of AD was established with the recent discovery of a frameshift mutation in the ubiquitin transcript, which leads to extension of the molecule with 20 amino acid residues [Ub(+1)], and which has been selectively observed in the brains of AD patients, including those with non-familial disease [20]. Ub(+1) is an efficient substrate for polyubiquitination, however the resulting polyubiquitin chains are refractory to disassembly by de-ubiquitinating enzymes [21] and potently inhibit the degradation of polyubiquitinated substrates by 26S proteasome. Thus, expression of Ub(+1) in the brain could result in dominant inhibition of the ubiquitin-proteasome system, leading to accumulation of toxic proteins with neuropathologic consequences. Additional and probably distinct players in the pathogenesis of AD are the presenilins 1 and 2 (PSs) that are involved in processing of the amyloid precursor protein APP. Numerous mutations causing early-onset AD have been identified in the *PS* genes, particularly in *PS1*. Both PS1 [22] and PS2 [23] are targeted by the ubiquitin system. However, degradation of the mutated proteins, which are still biologically active but can generate different processing products from APP, may differ from that of the WT proteins and contribute to the pathology of the disease.

Another group of genetic neurodegenerative disorders is caused by genomic instability that leads to an expanded 5'-CAG repeat in certain proteins, which is translated to a polyglutamine repeat. In the case of Huntington's disease, the affected protein is Huntingtin, a gene coding for a protein with unknown function. Similarly, in a series of diseases known as spinocerebellar ataxias 1-3, the affected proteins are Ataxins 1-3, respectively [24,25]. The polyglutamine-containing proteins aggregate and accumulate in intranuclear inclusion bodies, which are stained also for ubiquitin and part of it appears to be attached to the mutated proteins [24–26]. Huntingtin [27] and Ataxin 1 [28] are probably targeted by the ubiquitin system. Furthermore, at least for Ataxin 1, it has been shown that the *in vitro* degradation of the polyglutamine-containing and mutated protein is slower than that of the WT species [28], suggesting that the inability of the system to remove the mutated protein may underlie its accumulation and possible aggregation. However, it is not clear whether the aggregated protein and/or the inclusion bodies are toxic, and whether their generation underlies the pathogenesis of the disease. Initial experimental evidence suggests that the inclusion bodies may serve as an alternative scavenger mechanism to store proteins that cannot be removed by the system; and it is the "solubilization" of these proteins via expression of chaperones or removal of the conjugating enzyme [28] that aggravates the disease symptoms. According to these findings, the inclusion bodies play a protective rather than a toxic role.

APP = amyloid precursor protein

Cystic fibrosis

The CF gene encodes the CF transmembrane regulator, which is an 1480 amino acid residues chloride ion channel. Only a small fraction of the WT protein matures to the cell surface, whereas most of the protein is degraded from the ER by the ubiquitin system. Although more than 600 mutations have been described, the most frequent one (>70%) is ΔF508. The mutation leads to an autosomal recessive inherited multisystem disorder characterized by chronic obstruction of airways and severe maldigestion due to exocrine pancreatic dysfunction. Despite normal ion channel function, CFTRΔF508 does not reach the cell surface at all, and is retained in the ER from which it is degraded. It is possible that the rapid and efficient degradation results in complete lack of cell surface expression of the ΔF508 protein, and therefore contributes to the pathogenesis of the disease [29].

Immune and inflammatory response

Peptide epitopes presented to cytotoxic T cells on class I major histocompatibility complex molecules are generated in the cytosol from limited processing of antigenic proteins. It is now generally accepted that processing of most known MHC class I antigens is mediated by the ubiquitin-proteasome pathway [30]. An interesting finding is that the cytokine γ-interferon that stimulates antigen presentation leads also to induction and exchange of three proteasomal subunits in human cells, which in turn result in an alteration in the cleavage site preferences of the proteasome. The changes in activities probably result in peptides that terminate mostly with basic and hydrophobic residues, similar to the vast majority of known peptides presented on MHC class I molecules. They probably display a higher affinity to their interacting partners along the presenting route. The ubiquitin system degrades in a non-discriminatory manner both intracellular – "self" – proteins, as well as foreign – "non-self" – proteins such as viral proteins. Peptides from both populations are presented to CTLs, but those that are derived from "self" proteins do not elicit a T cell response. It is conceivable that aberrations in processing of these proteins, which can result in different cleavages, may lead to presentation of "self" peptides as "non-self." Such a mechanism can serve as an etiological factor in the pathogenesis

CF = cystic fibrosis
ER = endoplasmic reticulum
CFTR = cystic fibrosis transmembrane regulator
MHC = major histocompatibility complex
CTLs = cytotoxic C cells

of autoimmune diseases, but the antigens that elicit them have not been identified.

A wide array of immune and inflammatory disorders can also be caused by untoward activation of the immune system major transcription factor NF-κB. Activation of the factor stimulates transcription of many cytokines, adhesion molecules, inflammatory response and stress proteins, and immune system receptors. The factor is activated by the ubiquitin system via a two-step proteolytic mechanism: the first is the limited processing of the precursor protein p105 to yield the active subunit p50, and the second is signal-induced phosphorylation and subsequent degradation of the inhibitor IκBα that enables translocation of the factor into the nucleus where it exerts its transcriptional activity [31].

As described above, the HPV evolved a mechanism for proteolytic removal of p53 that enables continuous replication and propagation of the virus under conditions of continuous DNA damage that normally would have terminated with p53-induced apoptosis. Two other viruses evolved mechanisms that also exploit the ubiquitin system to evade immune surveillance. In one case, the Epstein-Barr virus nuclear antigen 1 persists in healthy virus carriers for life and is the only viral protein regularly detected in all EBV-associated malignancies, such as Burkitt's lymphoma. Unlike EBNAs 2-4 that are strong immunogens, EBNA1 is not processed and cannot elicit a CTL response. The persistence of EBNA1 contributes, most probably, to some of the pathologies caused by the virus. An interesting structural feature common to all EBNA1 proteins derived from different EBV strains is a relatively long and unusual Gly-Ala repeat at the C-terminal domain of the molecule. Transfer of a strong antigenic epitope from EBNA4 to EBNA1 prevented its presentation, while its insertion in an EBNA1 mutant that lacks the Gly-Ala repeat resulted in its presentation to the appropriate CTL. Similarly, insertion of the Gly-Ala repeat to EBNA4 inhibited CTL recognition of EBNA4. Thus, the Gly-Ala repeat constitutes a *cis*-acting element that inhibits antigen processing and subsequent presentation of potential antigenic epitopes [32]. It has been shown that while EBNA4 is degraded in an ATP-, ubiquitin-, and proteasome-dependent manner, EBNA1 is resistant to proteolysis. EBNA1 is degraded however by the system, following deletion of the Gly-Ala repeat [33]. An additional interesting observation involves the pathobiology of the human cytomegalovirus. The virus genome encodes two ER resident proteins, US2 and US11, that down-regulate the expression of MHC class I heavy chain molecules. The MHC molecules are synthesized, transported to the ER where they are glycosylated, but shortly thereafter, in cells expressing US2 or US11, are transported back to the cytosol, deglycosylated, and degraded by the proteasome following ubiquitination [34]. It appears that the viral products bind to the MHC molecules and escort/dislocate them to the translocation machinery where they are transported back into the cytosol. The detailed mechanism of action of the viral proteins is not known. They may diffuse laterally in the membrane and interact with the emerging nascent MHC chain, an interaction that does not allow insertion of the stop-transfer signal and proper anchoring of the molecule in the membrane. Alternatively, they may compete with the binding of the ER chaperone Bip/kar2, which may be necessary for proper folding of the MHC molecule. In any event, the removal of the MHC molecules does not allow presentation of viral antigenic peptides, thus enabling the virus to evade the immune system.

It appears that evolution of certain viruses involved tight "collaboration" with the ubiquitin system, where the system is "exploited" and serves the viral replication and propagation machinery.

Muscle wasting

The ubiquitin system plays a major role in pathophysiological processes in the muscle. Muscle degeneration that follows long-term immobilization, denervation, and severe catabolic states such as occur in sepsis and cancer-induced cachexia, leads to activation of the ubiquitin pathway and induction of many of its enzymatic components. This in turn results in massive degradation of muscle proteins [35,36]. The nature of the signaling mechanisms is still obscure, and it appears that cytokines such as tumor necrosis factor-alpha and IL-6 are not involved, at least not directly.

Drug development for targeting aberrant activity of the ubiquitin system

Because of the central role the ubiquitin system plays in a broad array of basic cellular processes, the development of drugs that modulate the activity of the system can be difficult. Inhibition of enzymes common to the entire pathway, such as the proteasome, may affect many processes non-specifically, though a narrow window between beneficial effects and toxicity can be identified for a short-term treatment. One approach can be the development of small molecules that bind and inhibit specific E3s. For example, specific phosphopeptide derivatives that span the phosphorylation targeting domains in different substrates can serve as "bait" to the respective E3s [37]. This approach can turn however into a double-edged sword. In the case of p27 and IκBα, where phosphorylation destabilizes negative regulators, inhibition of the E3 can increase the level of the proteins and consequently control disregulated cell cycle and curb untoward activity of the immune system. Therefore, compounds that display such activity can be tested as potential drugs for treatment of certain forms of malignancies and uncontrolled inflammatory states, respectively. However, the similarity between the phosphorylation sites of IκBα and β-catenin may lead also to stabilization of catenin which is an activator,

EBV = Epstein-Barr virus
EBNA = Epstein-Barr nuclear antigen

IL = interleukin

whereby its excessive transcriptional activity can result in malignant transformation. A better approach could be the development of small molecules that are substrate specific and bind, preferably, to specific substrates or to their ancillary proteins rather than to an E3. When accelerated degradation of a tumor suppressor such as p53 results in exposure of cells to malignant transformation, selective inhibition of the recognition machinery can potentially reverse the malignant phenotype. Indeed, peptides that bind specifically to HPV-E6 and prevent its association with p53 interfere with p53 targeting. The peptides were able to induce p53 with subsequent reversal of certain malignant characteristics or induction of apoptosis in HPV-transformed cells [38]. Treatment directed at increasing the level of $p27^{Kip1}$ resulted in regression of the malignant phenotype of prostate carcinoma in experimental models. While it is not clear that they act via the ubiquitin system, IL-6 [39], and phenylacetate [40], for example, lead to G1 arrest by increasing the level of p27.

Concluding remarks

The versatility of protein ubiquitination as a cellular regulatory mechanism is now well established and appears to be comparable to that of phosphorylation, another well-studied post-translational protein modification. Indeed, as we have seen in many cases, protein phosphorylation and ubiquitination go hand in hand in the regulation of many cellular processes. However, the enzymology of ubiquitination appears to be more complex than that of protein phosphorylation, and the mechanism of polyubiquitin chain formation is currently not clear. What we can certainly expect in the near future is the identification of an ever-increasing number of substrates of the ubiquitin system and of their specific E2/E3 complexes. It remains to be seen whether all the new E3s will belong to the now known classes, or whether new types of E3s will be identified. Consequently, but also in parallel, we can expect to see the development of an exciting area – that of specific modulators and potential drugs that can interfere with specific substrate recognition in a variety of pathological states.

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