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News and Commentary

Parkinsonism proteolysis and proteasomes

LM Schwartz*[,1], JR Nambu[1] and Z Wang[1]

[1] Department of Biology and Program in Molecular and Cellular Biology, University of Massachusetts, Amherst, MA 01003, USA
* Corresponding author: LM Schwartz, Department of Biology, University of Massachusetts, Amherst, MA 01003, USA.
Tel: (413)545-2435; Fax: (413)545-3243; E-mail: lms@bio.umass.e
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It is a general truism of science that the left hand side of an equation always attracts more attention than the right. The left hand side represents synthesis, while the right-hand side is dismissed as inactivation, a step-back from the can-do approach. Mitosis, transcription, translation and phosphorylation all attracted earlier and keener interest than did apoptosis, nucleases, proteases or phosphatases. With time however, further investigation always reveals that both sides play essential roles in the careful balance of regulation. The steady-state number of cells or molecules at any given time represents a balance between synthesis and degradation. Alter either and the balance shifts. Since most biological processes are controlled by the activity of specific proteins, the field of proteolysis has recently attracted considerable interest in the study of cellular regulation and molecular pathology. The ubiquitin–proteasome system (UPS), once dismissed as the cell's 'garbage disposal', is now appreciated as the complex, elegantly regulated pathway that is responsible for the selective degradation of specific proteins (reviewed in [1]). Subtle perturbations in this pathway lead to the inappropriate stabilization or degradation of key signaling molecules, which in turn can result in pathology.[2] For example, selective ubiquitination of the tumor suppressor protein p53 by papilloma virus E6 protein and the cellular E6-Associated Protein (E6-AP), leads to proteasome-dependent p53 degradation and enhanced rates of cervical cancer.[3] While a direct role for the UPS and disease has been well established for cancer it has been less clear if aberrations in this pathway are responsible for initiating neurodegenerative disorders. In this review, we describe the UPS and summarize recent findings directly linking defects in the UPS and the initiation of Parkinsonism.

Ubiquitin–protein conjugation is an ATP-dependent process that is mediated by three distinct classes of enzymes[1] (Figure 1). First in the cascade is the E1 ubiquitin-activating enzyme, which initially forms a thiolester bond with ubiquitin. Ubiquitin is then transferred to one of a number of E2 ubiquitin-conjugating enzymes (UBCs). Each species carry multiple independent E2 genes that serve unique cellular roles and the different E2 proteins cannot functionally substitute for one another.[4] The last enzyme in the cascade is the E3 ubiquitin–protein ligase, which recognizes specific target proteins and facilitates the transfer of ubiquitin from the E2 to the substrate. The lysine residue at position 48 on ubiquitin itself can serve as an acceptor for the covalent attachment of additional ubiquitin adducts. The subsequent appearance of poly-ubiquitin chains on a protein serves as a molecular tag to target the protein for degradation via the proteasome. The generation of these long polyubiquitin chains is facilitated by a novel class of E3s, referred to as U box or E4 proteins.[5,6] (Recent studies have demonstrated that formation of adducts on ubiquitin at position lysine 63 may serve a distinct, non-proteolytic signaling role.[7]).

The degradation of ubiquitinated target proteins is mediated by the 26S proteasome. Structurally, the proteasome is composed of a 20S core and regulatory caps of 11S (also known as PA28) and/or 19S (PA700).[8] The three dimensional structure of the 20S core particle is conserved across phylogeny from archeobacteria to mammals. It is a barrel composed of four stacked rings each of which contains seven subunits. The 19S caps serve to 'recognize' and unfold ubiquitinated proteins and deliver them into the catalytic chamber of the barrel, while the 11S caps appears to regulate the production and egress of short peptides from the proteasome.[9]

The UPS is so efficient that ubiquitinated proteins bound for degradation can be removed in a matter of seconds. Consequently, the accumulation of ubiquitinated substrates in cells often reflects underlying disease. For almost 15 years, pathologists have noted that many neurodegenerative disorders are characterized by the presence of nuclear or cytoplasmic inclusions rich in ubiquitin–protein conjugates,[10] (reviewed in [11]). In fact, these aggregates are often used as hallmarks in the differential diagnosis of neuropathological diseases. For example, the neurofibrillary tangles in Alzheimer's Disease contain aggregates of ubiquitinated neurofiliment proteins, most notably tau.[10] For many years the significance of these inclusions was unknown. Some suggested that they were the causative agents in cellular pathology and played an active role in subsequent demise of cells. Others felt that they were the irrelevant by-products of a deranged metabolism. Recently a third hypothesis has been put forward suggesting that such inclusions, termed aggresomes, represent a cellular defense to help protect the cell from effects of misfolded or aberrant proteins.[12,13] Aggresomes are cytoplasmic inclusions that coalesce in a dynein-dependent manner in the pericentriolar region of the cell just outside the nuclear envelope. Experimentally, aggresomes can be induced either by ectopic expression of aggregation-prone proteins or by pharmacological inhibition of the proteasome. In addition, Bence *et al*.[14] have demonstrated that individual cells that are forced to express aggregating proteins also display reduced UPS activity. This may establish a vicious

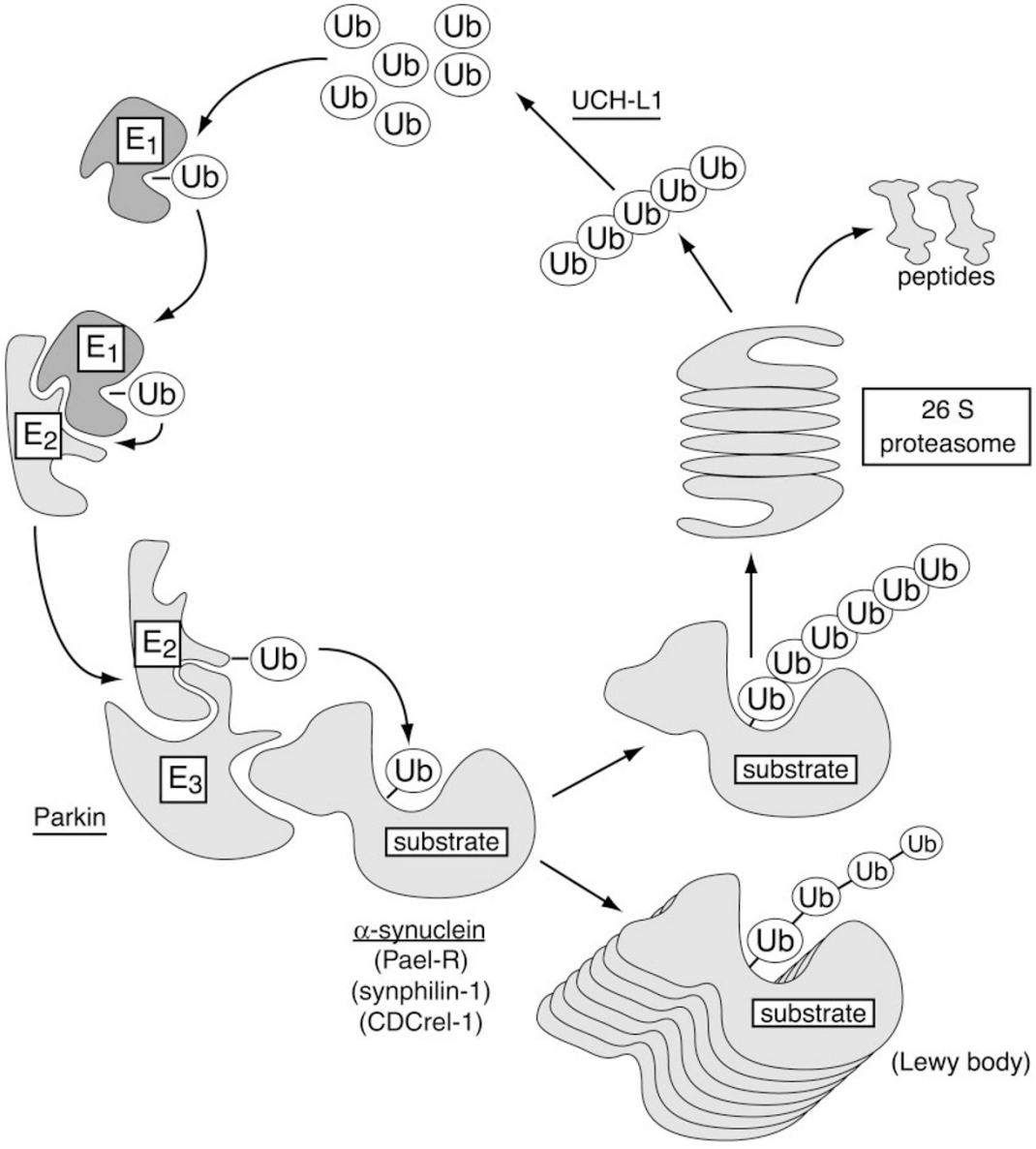

Figure 1 Parkinson's Disease components correlated with the ubiquitin–proteasome system. Ubiquitination of substrate proteins is mediated by a multi-enzyme cascade that facilitates the covalent addition of ubiquitin to cellular proteins. When multiple ubiquitin adducts are attached to proteins, they become targeted to the 26S proteasome for degradation. Mutations or post-translational modifications may reduce protein solubility and subsequent UPS-dependent degradation, resulting in the formation of aggregates. Mutations associated with familial forms of PD are underlined. Known substrates of the parkin ubiquitin E3 ligase are indicated by parentheses and are described in the text, except the synaptic versicle-associated protein CDCrel-1[31]

cycle whereby protein aggregation results in blockade of the UPS, thus allowing more misfolded proteins to accumulate. Cells that can form aggresomes may be in a better position to survive and degrade the backlog of misfolded proteins before irreversible pathological changes take place.

While the UPS has been implicated in neurodegenrative disorders, recent studies with Parkinson's Disease (PD) suggest a direct role in pathogenesis. PD is the second most common neurodegenerative disorder in the United States, affecting approximately 1% of people 65 or older.[15] The behavioral symptoms of PD are well characterized and include bradykinesis, tremors and rigidity. Neuroanatomically, the most obvious defects associated with PD are (1) the loss of dopaminergic neurons in the substantia nigra pars compacta and (2) the presence of Lewy bodies in surviving neurons. Lewy bodies are small cytoplasmic inclusions with a dense core and a protruding halo of filaments. While it has not been tested directly, it is assumed that Lewy bodies represent a neuronal version of aggresomes. Consistent with this hypothesis, Lewy bodies contain a variety of ubiquitinated proteins, most notably the protein α-synuclein,[16] a membrane-associated neuronal protein thought to be involved in synaptic vesicle

transport.[17,18] In fact, the presence of α-synuclein in cytoplasmic inclusions is considered diagnostic feature of Lewy bodies. (It should be noted that while Lewy bodies are one of the hallmarks of PD, they are also seen in other neurodegenerative disorders, including Dementia with Lewy Bodies and Multiple System Atrophy[19].)

PD is typically viewed as an idiopathic disorder, although it can also exhibit a significant genetic component. Mutations in three distinct genes, α-synuclein,[20] ubiquitin carboxy hydrolase L1 (UCH-L1),[21] and parkin[22] are associated with familial forms of PD. Mutations in the α-synuclein were the first to be associated with familial PD and result in the generation of a mutant protein that more readily aggregates and is less susceptible to proteasome-dependent degradation than the wild-type version.[20,21] Interestingly, the propensity for α-synuclein to aggregate is not restricted to mutated forms of the protein. Oxidative damage to wild-type α-synuclein can result in nitration of tyrosine residues leading to aggregation, Lewy body formation and Parkinsonism.[22] α-Synuclein is so prone to self-aggregation that merely over-expressing the wild-type protein can result in pathology. Targeted expression of normal human α-synuclein to the nervous system of mice or flies results in a PD-like disorder, complete with Lewy body formation, motor defects and loss of dopaminergic neurons.[23,24]

The two other genes identified in familial PD, UCH-L1 and parkin, are direct components of the UPS (Figure 1). UCH-L1, also known as PGP9.5, is a ubiquitin carboxy terminal hydrolase that is responsible for cleaving ubiquitin–ubiquitin bonds; it is one of the most abundant proteins in the central nervous system.[25] Following the proteasomal degradation of ubiquitinated proteins, UCH-L1 cleaves the polyubiquitin adducts into ubiquitin monomers in order to facilitate ubiquitin recycling. One German family with an autosomal dominant Parkinsonism, carries a UCH-L1 gene mutation that results in a Ile93Met amino acid substitution and reduced enzymatic activity of the corresponding protein.[21] Presumably this rare mutation results in reduced rates of both ubiquitin recycling and ubiquitin-dependent proteolysis.

The parkin gene is mutated in a form of familial PD known as Autosomal Recessive Juvenile Parkinsonism (AR-JP).[26,27] The parkin protein contains two RING domains, Cys-, His-rich metal-binding motifs that participates in protein/protein interactions. RING finger proteins have been shown to posses ubiquitin E3 ligase activity and it is now assumed that perhaps all RING proteins function in ubiquitination pathways.[28–30] In biochemical and cellular assays, parkin has been shown to be a ubiquitin E3 ligase that can auto-ubiquitinate and facilitate its own proteasome-dependent degradation.[31] Consistent with its role as an E3 ligase, parkin mutations identified from AR-JP patients disrupt the RING motifs and destroy E3 ligases activity.

One known parkin substrate that may contribute to AR-JP pathology is Pael-R (Parkin-Associated Endothelian Receptor-like Receptor), a putative G-coupled membrane protein.[32] In normal neurons, misfolding of Pael-R leads to rapid parkin-dependent ubiquitination and subsequent degradation. In the absence of functional parkin, Pael-R accumulates in the endoplasmic reticulum, where it appears to induce stress responses and cell death. Interestingly, dopaminergic neurons express high levels of Pael-R, which may help explain why they are preferentially compromised in AR-JP.

Another feature of AR-JP is that while these patients have early-onset Parkinsonism, they do not develop Lewy bodies.[33] This suggests that functional parkin is required for Lewy body formation and perhaps the trapping of specific substrates. To test this hypothesis, the Selkoe laboratory purified Lewy bodies from patients with classical PD (as opposed to AR-JP) and found that they contain parkin, the UBC-H7 E2 enzyme and intriguingly, a modified form of α-synuclein.[34] Thus, while the wild-type α-synuclein protein migrates at 16 kDa, the form isolated from Lewy bodies migrates at 22 kDa. Biochemical analysis suggested that this novel form is generated as a post-translational modification by O-linked glycosylation. This modified α-synuclein is specifically recognized by parkin and becomes heavily ubiquitinated.

Dawson and his collaborators have demonstrated that the α-synuclein binding protein synphilin-1, is another parkin substrate that accumulates in Lewy bodies.[35] Co-expression of α-synuclein with synphilin-1 is sufficient to induce the formation of Lewy body-like cytoplasmic inclusions in cultured HEK293 cells.[36] The synphilin-1 protein in these inclusions is only modestly ubiquitinated unless wild-type parkin is also ectopically expressed in these same cells. The mutant forms of parkin found in AR-JP patients fails to support synphilin-1 ubiquitination.

Taken together, the data presented in this review suggest the following general model for familial and perhaps idiopathic PD. Defects in ubiquitin-dependent proteolysis lead to the accumulation of misfolded and/or denatured proteins, which in turn form aggregates and endanger the cell. In many cases, this protein is α-synuclein, which aggregates due to either over-expression or post-translational modification (Figure 2). Alternatively, the protein may be Pael-R or perhaps other members of the hard-to-fold class of proteins. In normal cells, parkin forms a complex with its preferred E2 (UBC-H7), and selected substrates, such as synphilin-1 and Pael-R. This complex promotes substrate ubiquitination and proteasome-dependent degradation. In abnormal situations, these substrates accumulate to toxic levels and form nucleating seeds that facilitate aggregation within cytoplasmic inclusions, the Lewy bodies. The presence of these inclusions slows the rate of cellular damage and perhaps facilitates protein turnover. In patients with loss-of-function mutations in parkin, Lewy bodies do not form and cells die at an accelerated pace, hence the early onset of disease in AR-JP patients.

These data provide strong evidence linking various components of the UPS to the pathogenesis of Parkinsonism. It still remains to be determined if these insights can be extrapolated to all forms of Parkinsonism. If so, then they not only provide insight into the basic molecular mechanisms of disease, but also point to potential therapeutic targets. Interventions that reduce the insolubility of cellular proteins like Pael-R and α-synuclein may well

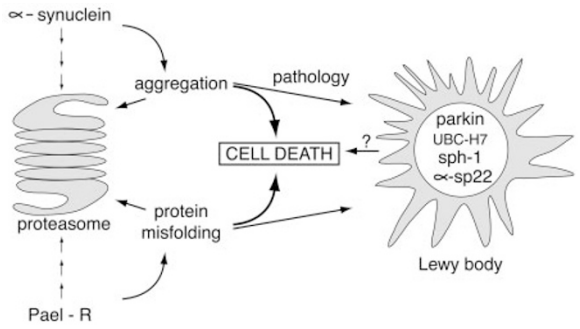

Figure 2 A generalized model of Parkinsonism. Some poorly soluble proteins such as α-synculein may be over-expressed mutated, or post-translationally modified in such as way that they are prone to aggregation. If they are not removed from the cytoplasm, they can ultimately induce cell death. In some cells, these denatured substrates can associate with the ubiquitin E3 ligase parkin and accumulate in Lewy bodies, which may reduce their destructive impact on cellular processes. In patients with loss-of-function mutations in parkin, Lewy bodies do not form, which leads to enhanced rates of cell death and an earlier onset of symptoms. Abbreviations: α-sp22=glycosylated α-synuclein; sph1=synphilin-1, Pael R=Parkin-Associated Endothelian Receptor-like Receptor, UBC-H7=ubiquitin-conjugating enzyme H7

slow the progression of the disease. In addition, pharmacological or molecular manipulations that selectively enhance the activity of parkin and related ubiquitin E3 ligases may also be of clinical value.

Note added in proof

CASK/LIN-2 has been shown to be an additional parkin substrate (Fallon *et al.* (2002) J. Biol. Chem. 277: 486–491). Expression of the molecular chaperone Hsp70 blocks dopaminergic neuron death in *Drosophilia* that express ectopic human α-synuclein (Auluck *et al.* (2002) Science 295: 865–868). Lewy body-like inclusions still form in these cells. These data support the hypotheses outlined in this review that suggest that misfolded proteins contribute to neuronal death and that Lewy bodies are not themselves toxic.

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