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Disruption of Protein Quality Control in Parkinson's Disease

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Parkinson's disease (PD), like a number of neurodegenerative diseases associated with aging, is characterized by the abnormal accumulation of protein in a specific subset of neurons. Although researchers have recently elucidated the genetic causes of PD, much remains unknown about what causes increased protein deposition in the disease. Given that increased protein aggregation may result not only from an increase in production, but also from decreased protein clearance, it is imperative to investigate both possibilities as potential PD culprits. This article provides a review of the systems that regulate protein clearance, including the ubiquitin proteasome system (UPS) and the autophagy-lysosomal pathway. Literature implicating failure of these mechanisms—such as UPS dysfunction resulting from environmental toxins and mutations in α-synuclein and parkin, as well as macroautophagic pathway failure because of oxidative stress and aging—in the pathogenesis of PD is also discussed.

Parkinson's disease (PD) is one of the most frequent neurodegenerative disorders, yet the cause of sporadic PD, which occurs in the absence of genetic linkage and accounts for more than 90% of all diagnosed cases, is still unknown. The primary neuropathological hallmark of PD is the degeneration of the nigrostriatal dopaminergic pathway (Dauer and Przedborski 2003). Simply put, PD symptoms result from a loss of dopamine, a neurotransmitter that normally sends signals in the brain to control body movement. The emergence of abnormal motor symptoms, including resting tremor, rigidity, slowness of voluntary movement, and postural instability, are all evidence of nigrostriatal dopaminergic pathway degeneration (Dauer and Przedborski 2003). This loss of neuromelanin-containing dopaminergic neurons serves as the basis of PD diagnoses, which can only definitively be made at autopsy, because more than forty different neurological diseases can show signs of parkinsonism (i.e., clinical features of PD). Such diagnoses are customarily based on the presence of intraneuronal, eosinophilic inclusions called Lewy bodies (LBs). These inclusions, or protein clumps, have been found throughout the diseased brain of PD patients.

Two distinct and not mutually exclusive pathological events believed to underlie the demise of the nigrostriatal dopaminergic neurons in sporadic PD are mitochondrial impairment

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and oxidative stress (Dauer and Przedborski 2003). However, the identification of PD-causing genetic mutations in α-synuclein (Polymeropoulos et al. 1997; Kruger et al. 1998; Zarranz et al. 2004), parkin (Kitada et al. 1998), DJ-1 (Bonifati et al. 2003), PINK1 (Valente et al. 2004), ATP13A2 (Williams et al. 2005; Ramirez et al. 2006), and leucine-rich repeat kinase-2 (LRRK2) (Paisan-Ruiz et al. 2004; Zimprich et al. 2004) have triggered a dramatic paradigm shift in the way researchers consider the question of PD pathogenesis. Indeed, the continued study of the cellular functions of each of the PD-related genes indicates that protein misfolding, as well as dysfunction in the protein degradation systems, may play a pivotal role in the cascade of deleterious events implicated in the neurodegenerative process of PD. These novel directions have also reinvigorated interest among researchers in LBs and other types of proteinaceous deposits found in PD brains, not just as neuropathological hallmarks of disease, but rather as putative effectors of PD pathogenesis.

THE PD CULPRIT: INCREASED PROTEIN MISFOLDING AND AGGREGATION OR DECREASED PROTEIN CLEARANCE?

By now, it is well recognized that protein aggregates in brain tissue is a feature shared by a number of prominent, age-related neurodegenerative diseases, including PD (Ross and Poirier 2004). Strict quality control mechanisms that act to coordinate the rates of protein synthesis with degradation normally prevent such intracellular aggregates from forming (Balch et al. 2008; Powers et al. 2009). However, prolonged exposure to various stressors places an incredible burden on these mechanisms. When these mechanisms fail, aggregation-prone proteins abnormally accumulate, as observed in neurodegenerative diseases such as PD (Ross and Poirier 2004). Although the composition and localization of characteristic protein aggregates differs from disease to disease, their presence suggests that protein deposition per se, or some related event, might be toxic to neurons.

Determining a pathogenic mechanism, which can account for the increased levels of misfolded and aggregated proteins in dopaminergic neurons in PD could dramatically alter therapeutic strategies to lessen the severity and detrimental consequences of the disease. Misfolded proteins, either soluble or insoluble and contained within aggregates, could be neurotoxic through a variety of mechanisms. Damage caused by protein aggregates, perhaps by a crowding effect, may lead to cell deformations or interfere with trafficking systems. It might be expected that the frequency of aggregates would correlate with the magnitude of neurodegeneration. This important relationship has not yet been convincingly shown in postmortem tissue samples from sporadic PD patients. Instead, the formation of aggregates may reflect a state of cellular distress (Lee et al. 2002; Petrucelli et al. 2002). In addition, it is hypothesized that inclusion formation could be the end result of an active process meant to sequester harmful, soluble misfolded proteins from the cellular milieu (Kopito 2000). Thus, aggregate formation, although possibly indicative of a "cell under attack," may simply be a defensive measure aimed at alleviating toxicity through the removal of soluble misfolded proteins (Cummings et al. 1999, 2001; Warrick et al. 1999; Auluck et al. 2002). The ability of chaperone proteins (e.g., Hsp70) to protect against neurodegeneration provoked by disease-related proteins, including α-synuclein-mediated dopaminergic neuronal loss, is consistent with the view that soluble misfolded proteins are the neurotoxic species, and not protein aggregates (Auluck et al. 2002; Muchowski 2002).

In PD patients with either α-synuclein point mutations (Polymeropoulos et al. 1997; Kruger et al. 1998; Zarranz et al. 2004) or gene multiplication (Singleton et al. 2003), dopaminergic neurodegeneration is thought to be caused by mutant or excess levels of normal α-synuclein adopting toxic conformations (Bussell and Eliezer 2001) or interfering with the cellular handling of misfolded proteins. In sporadic PD, there is a similar focus on both protein-damaging modifications and dysfunction of protein degradation systems (see below). For example, it has been proposed that oxidative stress may trigger impaired protein homeostasis in sporadic PD. Giasson and collaborators have shown

that LBs in PD contain oxidatively modified α-synuclein, which shows a greater propensity to aggregate compared to nonoxidized α-synuclein (Giasson et al. 2000). In addition, several environmental toxins can also induce misfolding or aggregation of α-synuclein (Uversky et al. 2001; Manning-Bog et al. 2002). However, irrefutable evidence that misfolded proteins and related aggregates are toxic to dopaminergic neurons in PD is still lacking.

Regardless, given that increased protein aggregation may result not only from an increase in production, but also from decreased clearance caused by a dysfunction in either the ubiquitin-proteasome and/or autophagic/lysosomal pathways, it is imperative that both possibilities be taken into consideration. We will now review the two major protein degradation systems, as well as the research, which suggests that breakdowns in these two systems are involved in the pathogenesis of PD.

THE UBIQUITIN PROTEASOME SYSTEM

The ubiquitin-proteasome system (UPS) is one of two protein degradation systems believed to go awry in Parkinson's disease. The UPS degrades proteins, especially short-lived proteins, in eukaryotic cells, or cells that contain a variety of organelles enclosed within internal membranes (Hershko and Ciechanover 1998; Ciechanover et al. 2000; Ciechanover and Brundin 2003). The UPS regulates the degradation of key regulatory proteins that control signal transduction, cell cycle progression, apoptosis, as well as cellular differentiation (Hershko and Ciechanover 1998). In addition to involvement in these processes, the UPS also degrades proteins that are misfolded and/or damaged, possibly caused by mutation, environmental stress, or intrinsic folding inefficiency (Hershko and Ciechanover 1998; Ciechanover et al. 2000; Ciechanover and Brundin 2003). Thus, UPS dysfunction has been implicated in a wide range of conditions, including neurodegenerative diseases, cancer, inflammation, and autoimmunity (Hershko and Ciechanover 1998; Schwartz and Ciechanover 1999; Ciechanover et al. 2000; Ciechanover and Brundin 2003).

How It Works

UPS-mediated protein degradation is a complex, multistep process initiated when a protein is "tagged" or covalently bound to ubiquitin (Fig. 1C). The process is ultimately completed when the 26S proteasome complex degrades the tagged protein (Fig. 1E). The UPS uses a series of enzymes to covalently link ubiquitin polypeptide chains to proteins, marking those proteins as substrates for the proteasome and allowing for targeted and selective degradation. Ubiquitin is a highly conserved, 76 residue polypeptide, which as a monomer, is attached sequentially to proteins through the cascade of E1 ubiquitin-activating, E2 ubiquitin-conjugating, and E3 ubiquitin ligase enzymes (Fig. 1A–C). The ubiquitin-activating enzyme E1 catalyzes the formation of a thiol ester bond between the E1 cysteine residues and the carboxy-terminal glycine in ubiquitin via an ATP-dependent reaction (Fig. 1A). Then, one of several ubiquitin-carrier proteins or ubiquitin-conjugating E2 enzymes transfer ubiquitin from the E1 (Fig. 1B) to the protein substrate that is specifically bound to a ubiquitin-protein ligase E3 enzyme (Fig. 1C). During this latter reaction, ubiquitin is ligated to the lysine residue of the protein substrate. Once the first ubiquitin molecule has been conjugated to the protein substrate, additional ubiquitin molecules can be added to the internal lysine residues of ubiquitin to form polyubiquitin chains, which is an essential event, because a minimum of four ubiquitin molecules is required for efficient targeting to the proteasome (Fig. 1D). As both E1 and E2 enzymes nonspecifically tag proteins with ubiquitin, E3 enzymes confer target specificity to UPS proteolysis by binding specific proteins. Thus, protein ubiquitination, while epitomizing a generic cellular mechanism is, in fact, quite a selective process, with only specific proteins being degraded by this pathway at a precise moment in response to precise cellular events.

Degradation of polyubiquitinated proteins is then performed by a large protease complex called the 26S proteasome (Fig. 1E), which contains a common proteolytic core known as the

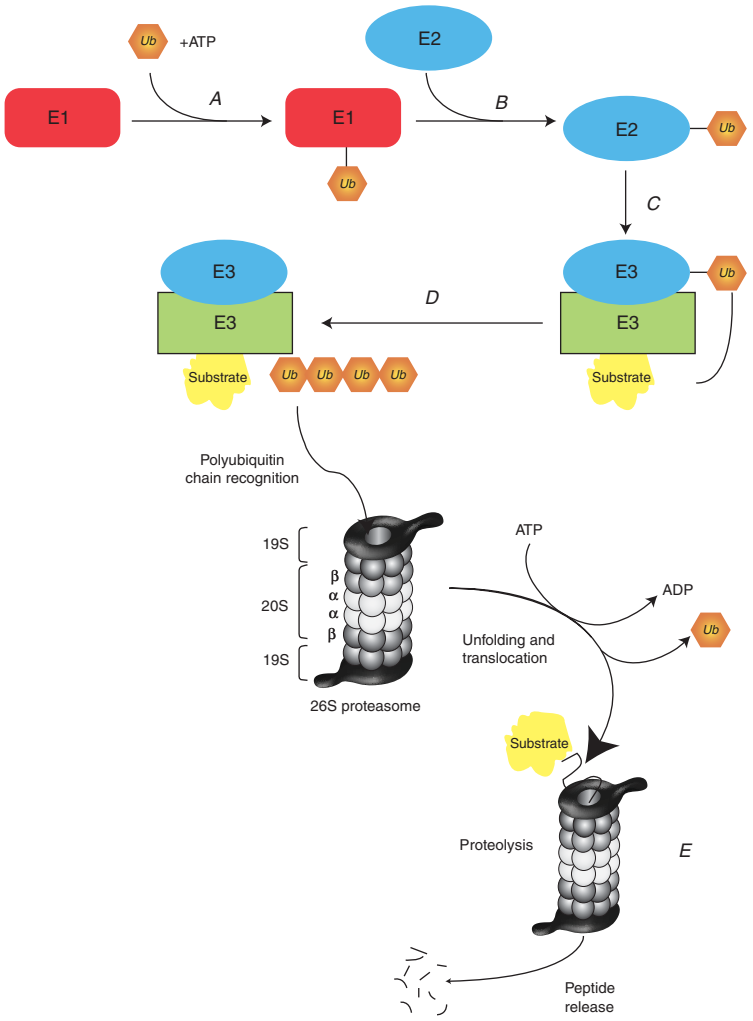

Figure 1. Representation of ubiquitin-proteasome proteolysis. Ubiquitin is first activated by the ubiquitin-activating enzyme E1 (*A*), an ubiquitin-carrier protein, E2, and ATP. The product of this reaction is a high-energy E2 ~ ubiquitin thiol ester intermediate (*B*). Protein substrates are then ubiquitinated by either binding of the substrate to a specific ubiquitin-protein ligase (E3), and then the E2-bound activated ubiquitin is transferred directly to the E3-bound protein substrate. Or alternatively, the activated ubiquitin can be transferred from the E2 to the E3, prior to its conjugation to the E3-bound substrate (*C*). Following conjugation of the first ubiquitin molecule to the protein substrate, additional ubiquitin molecules can be added to the internal lysine residues of ubiquitin to form a polyubiquitin chain on the substrate (*D*). The ubiquitinated substrate is then recognized and degraded by the 26S proteasome complex, leading to the release of short peptides (*E*). Ubiquitin is recycled via the activity of deubiquitinating enzymes.

20S proteasome (Hershko and Ciechanover 1998; Ciechanover et al. 2000; Ciechanover and Brundin 2003). The 20S proteasome is an open-ended, hollow structure that is composed of 28 subunits arranged in four, heptameric rings. The two outer rings are each composed of seven α-type subunits (α1–α7), whereas the two inner rings each contain seven β-type subunits (β1–β7) (Fig. 1). The proteolytic activity is contained within the inner rings, with only β1, β2, and β5 subunits respectively possessing caspase-, trypsin-, and chymotrypsin-like cleavage specificity

(Dick et al. 1998; Kisselev et al. 1999). These active sites allosterically regulate one another through substrate binding, leading to a proposed model in which single polypeptide chains are successively hydrolyzed by a structured and coordinated activation of these catalytic subunits (Voges et al. 1999; Navon and Goldberg 2001). However, Liu and colleagues (2003) present evidence that a disordered polypeptide loop, such as a β-hairpin structure, can also gain entry to the inner canal of the 20S proteasome, allowing for the endoproteolytic cleavage and partial degradation of unstructured proteins that is not dependent on ubiquitination. This describes a novel function of the proteasome, liberating active peptides from precursor proteins, as well as correcting folding defects in internal domains of large proteins.

UPS IMPAIRMENT IN PARKINSONIAN SYNDROMES

The Effects of α-Synuclein and Parkin Mutations on the UPS

Given that ubiquitinated proteins, heat shock proteins/chaperones, and components of the UPS have all been shown to accumulate within LBs, both protein misfolding and UPS dysfunction are implicated in neurodegenerative processes in sporadic PD (Lennox et al. 1989; Lowe et al. 1990; Ii et al. 1997; Auluck et al. 2002; McNaught et al. 2002; Schlossmacher et al. 2002). At this point, several studies have suggested that inhibition of proteasomal activity leads to accumulation of α-synuclein or the formation of ubiquitinated α-synuclein-containing aggregates (Rideout et al. 2001; Tofaris et al. 2001; Snyder et al. 2003). Because degradation, not ubiquitination, is rate-limiting for most UPS substrates (Pickart 2001), the presence of undegraded, stable ubiquitin conjugates in the brains of PD patients, as well as genetic and toxic models of PD, suggests that misfolded proteins in sporadic PD are properly recognized and ubiquitinated, but not efficiently degraded. Such inefficient degradation may be the result of intrinsic resistance to proteasomal degradation of α-synuclein-containing aggregates (Lee et al. 2004), or a reflection of the difficulty in unfolding stable protein aggregates by the 19S subunits of the proteasome complex (Voges et al. 1999). Moreover, the accumulation of α-synuclein and other aggregation-prone proteins has been shown to potently impair the activity of the UPS (Bence et al. 2001; Jana et al. 2001; Bennett et al. 2005; Emmanouilidou et al. 2010).

Intriguingly, it has recently been reported that α-synuclein phosphorylated on Ser-129 (pS129) is degraded by the proteasome without undergoing ubiquitination, suggesting that proteasome impairment would specifically increase pS129-α-synuclein (Machiya et al. 2010). This is particularly important given the reports that ~4% of total α-synuclein has been shown to be phosphorylated at S129 in normal brain tissue (Fujiwara et al. 2002), though 90% of aggregated α-synuclein in LBs is phosphorylated at S129 (Fujiwara et al. 2002; Anderson et al. 2006). As phosphorylation of α-synuclein on the S129 residue has been shown to show a greater propensity to aggregate (Fujiwara et al. 2002; Chen et al. 2009), LB formation could result from the increased relative abundance of pS129-α-synuclein species under conditions of proteasomal impairment.

In addition, loss-of-function mutations in the parkin gene, which has been identified as an E3 ubiquitin ligase (Shimura et al. 2000; Zhang et al. 2000), cause a recessively inherited form of parkinsonism that typically emerges clinically before the age of 30. As many parkin mutations have been shown to abolish parkin's ubiquitin ligase activity, familial PD associated with mutations in parkin is believed to result from the compromised polyubiquitination and degradation of specific proteins (Kitada et al. 1998; Dauer and Przedborski 2003). Intriguingly, in human postmortem brain tissue, PD caused by mutations in parkin is associated with a loss of dopaminergic neurons, but not with the accumulation of LBs (Mizuno et al. 2001). Although familial PD cases associated with parkin mutations are very rare, this striking departure from the characteristic neuropathology associated with sporadic PD may indicate that parkin ligase activity is required for LB formation. Additionally, this finding could also provide support for the hypothesis that

aggregate formation is a cellular defense mechanism to temporarily alleviate toxicity of soluble, misfolded proteins through sequestration, in particular given the very early age of onset of familial PD associated with loss-of-function mutations in parkin.

In an attempt to model PD associated with impaired parkin function, investigators generated mice deficient in parkin expression, which show increase expression of putative parkin substrates, including aminoacyl-tRNA synthetase-interacting multifunctional protein type 2 and far upstream element-binding protein-1 (Corti et al. 2003; Ko et al. 2006). However, although several studies have stressed the multiplicity of parkin substrates (Ciechanover and Brundin 2003; Dauer and Przedborski 2003), exactly how their accumulation in the brain in response to a loss of parkin ligase activity might play a role in neurodegeneration remains unclear. Other investigations have suggested that a loss of parkin may trigger cell death by sensitizing neurons to various cytotoxic insults, including proteasome inhibition or mutant α-synuclein overexpression (Stefanis et al. 2001; Petrucelli et al. 2002), but here again the mechanisms that underlie this effect remain to be elucidated.

Environmental Toxins Associated with PD Are Detrimental to UPS

The relationship between UPS impairment and sporadic PD is evident because of a number of in vitro studies demonstrating decreased proteasomal activity on exposure to various pesticides and environmental toxins that have been implicated in PD, including rotenone, paraquat, and maneb (Betarbet et al. 2006; Wang et al. 2006). The in vivo administration of rotenone also led to a reduction in proteasome activity specifically in the ventral midbrain of rats (Betarbet et al. 2006). Intriguingly, use of osmotic minipumps to continually deliver the PD-linked toxin MPTP to mice for one month produced a PD-like phenotype, including depletion of striatal dopamine levels and neuronal loss in both the substantia nigra and locus coeruleus, which was accompanied by the formation of α-synuclein and ubiquitin-positive inclusions (Fornai et al. 2005). These mice also showed a decrease in proteolytic activity of the proteasome in striatal extracts, as well as a progressive decline in motor activity that was rescued by administration of dopamine agonists. Surprisingly, when experiments were replicated in mice lacking α-synuclein, neuronal loss, behavioral impairments, and the formation of ubiquitin-positive inclusions were alleviated (Fornai et al. 2005). This observation indicates that α-synuclein is required for the neurotoxic effects of MPTP. Perhaps most telling: impairments in proteolytic activity following MPTP administration were also alleviated in the absence of α-synuclein, suggesting that α-synuclein exacerbates the deleterious effects of PD-linked environmental toxins on UPS function. Furthermore, these findings imply that α-synuclein, and possibly UPS dysfunction, are critically involved in the manifestation of a PD phenotype. A potential feed-forward mechanism could be proposed in which the accumulation of aggregated α-synuclein in response to a toxic insult(s) (e.g., oxidative stress or an environmental toxin) would inhibit UPS function, leading to further accumulation of α-synuclein and other toxic, misfolded proteins. However, it should be stressed that despite the growing number of studies supporting a role for UPS dysfunction and protein misfolding in disease processes leading to PD, these observations imply a relationship between UPS dysfunction and PD, but do not conclusively show a causal role of UPS impairment in the development of PD.

AUTOPHAGY AS A CELLULAR CLEANING MECHANISM

Protein quality control is regulated not only by the UPS, but also by a process called authophagy. Autophagy, or the degradation of intracellular components inside lysosomes, literally means self-eating (Cuervo 2004; Levine and Klionsky 2004; Klionsky 2005; Yorimitsu and Klionsky 2005). Autophagy plays a major role in the maintenance of cellular homeostasis, the defense of cells against intracellular and extracellular aggressors, and in all processes requiring major

cellular remodeling, such as in embryogenesis and tissue differentiation (Mizushima 2005). Because autophagy contributes to the removal of proteinaceous aggregates seen in neurodegenerative diseases (Ravikumar et al. 2004; Iwata et al. 2005b), failure in the autophagic system may be responsible for the accumulation of misfolded proteins in some of these disorders, including PD.

Three Types of Autophagy: Macro, Micro, and Chaperone-Mediated

There are different mechanisms by which substrates can be delivered to the lysosome, giving rise to three different types of autophagy in mammalian cells: macroautophagy, microautophagy, and chaperone-mediated autophagy (CMA).

Macroautophagy, the best-characterized form of autophagy, involves the sequestration of a complete region of the cytosol, including whole organelles, by a double-membrane vesicle known as the autophagosome (Fig. 2). The latter acquires the enzymes required for the degradation of its content after fusion with a lysosome. Macroautophagy is activated in response to nutrient deprivation, where degradation of intracellular components by this pathway provides cells with the amino acids no longer obtained by the diet, but required for the synthesis of proteins essential for survival (Mizushima et al. 2004). Physical and chemical stressors, as well as infectious agents, have also been shown to activate macroautophagy (Levine and Klionsky 2004). Activation of autophagy under these conditions is aimed at the removal of the aggressor itself (i.e., invading

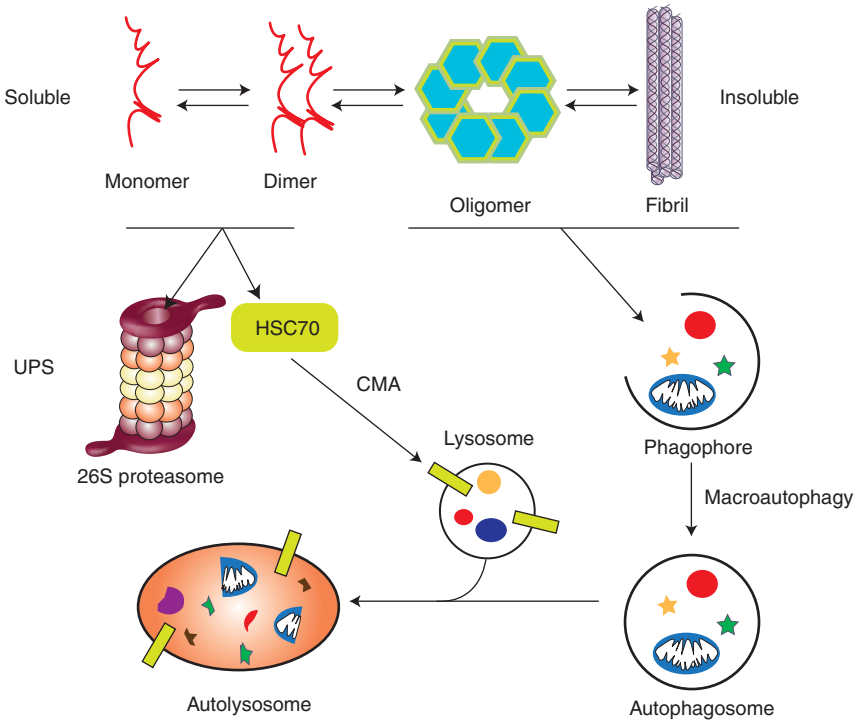

Figure 2. Impact of aggregation on mechanisms of protein degradation. Soluble misfolded monomers and dimers can be recognized by both the UPS or CMA-related chaperones, and subsequently degraded by either of these two pathways. In the case of CMA, cytosolic proteins (i.e., α-synuclein) are recognized by a chaperone (i.e., Hsc70), which delivers the target protein to the lysosome via a receptor protein present in the lysosomal membrane. However, on more complex assembly (oligomer or fibril formation) of the target protein, macroautophagy is the only mechanism available to clear the more insoluble and highly ordered aggregates.

microorganisms) or of the intracellular components that were damaged during exposure to stress. Furthermore, studies in genetically altered mice that lack one of the essential genes for macroautophagy have revealed that some level of macroautophagy is almost always present in tissues, and this basal activity plays an important role in the continuous turnover of organelles and clearance of misfolded and damaged proteins (Komatsu et al. 2005, 2006; Hara et al. 2006).

Complete regions of the cytosol can also be delivered to lysosomes for degradation via microautophagy. In this case, the lysosomal membrane invaginates or generates protrusions and tubulations to sequester the cytosolic content "in bulk." Microautophagy is a constitutively active pathway that contributes to the continuous, slow turnover of cytosolic proteins (Mortimore et al. 1988).

In contrast to "in bulk" degradation, cytosolic proteins can also be transported one-by-one into lysosomes for degradation by CMA. The intrinsic selectivity associated with this pathway permits the removal of damaged proteins without affecting normal proteins in the vicinity. The interaction of a chaperone with the substrate protein brings it to the lysosomal membrane, where it then binds to a lysosomal receptor protein (Fig. 2). After unfolding, the substrate protein is transported across the lysosomal membrane with the assistance of a second chaperone protein that is present in the lysosomal lumen (Cuervo and Dice 1996). As with all types of autophagy, once the substrate protein reaches the inside of the lysosome, it is rapidly degraded by potent hydrolases. Although some level of basal CMA activity is detectable in almost all cell types, this pathway is maximally activated under conditions of stress. Stressors known to activate this pathway include prolonged starvation, mild oxidative stress, and exposure to toxic compounds that alter the conformation of particular proteins (Massey et al. 2004).

Macroautophagy, a Protective Mechanism against Protein Misfolding, Takes Aim at Insoluble Aggregates

The UPS plays a critical role in the removal of misfolded proteins that would otherwise form aggregates, whereas lysosomes selectively clear misfolded proteins via CMA. However, given that proteins within insoluble aggregates cannot unfold, which prevents their translocation into the barrel of the proteasome complex or across the lysosomal membrane, neither the UPS nor CMA are able to degrade insoluble aggregates (Fig. 2). Despite this, a number of studies have now shown that turning off expression of a toxic protein, even after the formation of protein aggregates, is sufficient to allow recovery from proteotoxic stress in various inducible models of degeneration (Yamamoto et al. 2000; Zu et al. 2004; Santacruz et al. 2005). Given the strict requirement that proteins must be capable of completely unfolding to be degraded by either the UPS or CMA, it is unlikely that either of these pathways contributes to aggregate removal and the subsequent recovery from proteotoxic stress. In contrast, macroautophagy can sequester and deliver entire regions of the cytosol to the lysosome for degradation, regardless of the state of solubility of the sequestered proteins. Therefore, it is most likely that macroautophagy mediates the clearance of these insoluble, protein aggregates.

In fact, pharmacological activation of macroautophagy has been shown to decrease the number of intracellular aggregates and protect against neurotoxicity induced by various aggregation-prone proteins, including mutant forms of huntingtin or α-synuclein (Ravikumar et al. 2004). In addition, conditions that promote intracellular aggregate formation have been reported to lead to an activation of macroautophagy (Kamimoto et al. 2006; Nixon 2006; Williams et al. 2006). In support of this, there is an increased abundance of autophagic vacuoles, indicative of an up-regulation in macroautophagy, in postmortem brain tissue from patients affected by different neurodegenerative disorders characterized by abnormal protein aggregation (Kegel et al. 2000; Larsen et al. 2002; Nixon et al. 2005; Nixon 2006). Given that dying neurons appear to contain a greater amount of autophagic vacuoles, it was originally hypothesized that activation of macroautophagy was a mechanism of cell death (reviewed by Nixon 2006). However, the beneficial effects of enhanced macroautophagy observed in differ-

ent experimental models of neurodegeneration is suggestive of a defensive, prosurvival role for macroautophagy in these disorders.

Despite this, the notion of cell death by autophagy has not been completely abandoned, as recent experimental evidence suggests that excessive up-regulation of macroautophagy dramatically reduces cell viability, and under these conditions, inhibition of macroautophagy is protective (Pattingre et al. 2005). Therefore, it is possible that under conditions of increased protein misfolding, macroautophagy is initially activated as a protective mechanism to promote clearance of damaged proteins, but if the harmful situation is not resolved, persistent macroautophagy activates apoptotic mechanisms that are used to completely eliminate the irreversibly damaged cell. In support of this hypothesis, the presence of aggregated proteins seems sufficient to up-regulate macroautophagy, perhaps by promoting an inhibition of the UPS (Iwata et al. 2005a). In addition, blockade of CMA, which has been shown to occur in the presence of some abnormally folded proteins, also leads to an induction of macroautophagic activity (Massey et al. 2006). Therefore, activation of macroautophagic "in bulk" degradation is a cellular mechanism to attempt to compensate for the inhibition of degradative pathways that under normal conditions would promote the removal of these soluble proteins.

Oxidative Stress and Aging Cause Macroautophagic Pathways to Fail

With macroautophagy left as the only remaining pathway for the turnover of intracellular components in the compensatory stage of neurodegenerative disorders, it can be inferred that small alterations in this system may greatly impact neuronal survival. Oxidative stress and aging are believed to be two of the main aggravating factors precipitating failure of macroautophagic pathways. Conditions that promote oxidative stress overload the macroautophagic system, and oxidized proteins and damaged organelles engulfed by autophagosomes can become a source of reactive oxygen species inside either autophagosomes or lysosomes. These reactive species can eventually damage lysosomal hydrolases and other components required for the lysosome/autophagosome fusion, resulting in the accumulation of undegraded products inside these cellular compartments. Free radicals can also induce nonspecific protein cross-linking, which increases the indigestibility of the substrates and promotes their accumulation inside lysosomes in the form of an autofluorescent pigment known as lipofuscin (Terman and Brunk 2004). Age-related changes in the lysosomal system also play an important role in macroautophagic failure and could explain the late onset of many of these neurodegenerative disorders, as both macroautophagy and CMA activity have been shown to decrease with age (Cuervo et al. 2005). The main defect in macroautophagy is the diminished ability of lysosomes to clear autophagic vacuoles, which form to trap damaged intracellular components that need to be removed. However, a combination of defective autophagosomal-lysosomal fusion with the impaired activity of lysosomal hydrolases with aging is believed to contribute to the accumulation of undigested materials inside old cells.

The critical role of macroautophagy in the removal of aggregated proteins is evidenced by brain-specific deletion of either Atg5 or Atg7 (essential autophagy genes), which in mice is associated with an abnormal phenotype that is characterized by ubiquitin pathology, the accumulation of insoluble, proteinaceous inclusions, and motor impairment (Iwata et al. 2005a; Komatsu et al. 2006, 2007). As the protein aggregates present in Atg5 and Atg7 KO mice are positive for ubiquitin, it is believed that in addition to targeting proteins for degradation by the UPS, ubiquitination could also target proteins for degradation by selective autophagy (Kirkin et al. 2009b). Although there are seven possible ubiquitin–ubiquitin linkages (including K6, K11, K27, K29, K33, K48, and K63), the functional role of each is unknown (Peng et al. 2003; Kim et al. 2007; Ikeda and Dikic 2008). It has been proposed that in addition to a role in endocytosis and DNA repair, K63-linked polyubiquitin chains also target proteins for degradation by autophagy (Seibenhener et al. 2007;

Olzmann and Chin 2008; Tan et al. 2008; Kirkin et al. 2009b). However, ubiquitin absolute quantification mass spectrometry revealed that all detectable poly-ubiquitin linkages accumulate in autophagy-deficient mice in approximately equal ratios, suggesting that it is unlikely that a specific poly-ubiquitin linkage acts as a signal for autophagic degradation (Riley et al. 2010). However, following the inactivation of Atg5, aggregation-prone proteins and not diffuse cytosolic proteins were shown to preferentially accumulate, which did not correlate with the increase in poly-ubiquitinated substrates (Riley et al. 2010).

This observation could be explained by taking into consideration a pathway for selective autophagy, which was initially characterized in yeast, and is known as the cytoplasm-to-vacuole targeting (Cvt) pathway. This pathway is dependent on oligomerization of substrate proteins, and not ubiquitination. The protein oligomers are then recognized and targeted by a receptor protein (Atg19) and subsequently sequestered within a Cvt vesicle, which eventually fuses with a lysosome (reviewed by Klionsky 2007). Interestingly, the formation of oligomers has been shown to be both necessary and sufficient for recognition by Atg19 in yeast (Kim et al. 1997). Therefore, to target proteins for degradation by selective autophagy, both ubiquitination and oligomerization have been proposed as potential signaling mechanisms (Riley et al. 2010). Similar to the yeast Cvt pathway, in which protein oligomerization drives selective autophagy, it has been hypothesized that substrate oligomerization may also serve as a signal for selective autophagy in mammalian cells (Riley et al. 2010). In support of this, inhibition of autophagy selectively led to the accumulation of an aggregation-prone fragment of the mutant huntingtin protein, rather than the monomeric, soluble protein (Riley et al. 2010). In addition, there was an increase in total ubiquitin and all major poly-ubiquitin linked chains in the insoluble fraction under conditions of autophagic impairment, and only a very small percentage of total ubiquitin was associated with the mutant huntingtin fragment. These findings indicate that the aggregation-prone protein is not targeted for selective autophagy by a specific ubiquitin conjugate, and additionally that the up-regulation in total ubiquitin levels is not the result of increased ubiquitination of a substrate protein (Riley et al. 2010).

IMPAIRED AUTOPHAGY AND PD

Some have proposed that activation of autophagic pathways could be used as a potential therapeutic approach to augment clearance of α-synuclein pathology in PD (Masliah et al. 2005). For example, under normal conditions, α-synuclein is degraded by both the UPS and through autophagy, being delivered to lysosomes through the CMA pathway for degradation (Webb et al. 2003; Cuervo et al. 2004). Pathogenic mutant forms of α-synuclein are still delivered to lysosomes by a cytosolic chaperone and bind to the lysosomal membrane receptor, but are unable to cross the lysosomal membrane and cannot be degraded in this compartment. α-synuclein mutants remain tightly bound to the lysosomal membrane, also interfering with the degradation of other CMA substrates (Cuervo et al. 2004). The similar ability to block CMA has been observed for wild-type α-synuclein when modified by dopamine (Martinez-Vicente et al. 2008). Cells respond to this blockage of CMA by up-regulating macroautophagy to guarantee normal rates of protein degradation (Massey et al. 2006). However, the absence of CMA leaves the cells more vulnerable to stress, and apoptotic cell death is readily activated in these cells after exposure to different stressors.

When α-synuclein aggregates can no longer be degraded by either the UPS or CMA, macroautophagy becomes the only proteolytic pathway able to remove these proteinaceous deposits from the neuronal cytosol. This view is consistent with the demonstration that aggregates of mutant α-synuclein can be cleared by macroautophagy (Webb et al. 2003), and activation of the latter under this condition is likely a result of the inhibition that mutant and modified α-synuclein exert on UPS and CMA activity (Iwata et al. 2005a; Massey et al. 2006). Although activation of macroautophagy during this compensatory stage prevents the intracellular accumulation

of aggregated α-synuclein, conditions with a negative impact on macroautophagic activity could precipitate failure of the clearance of toxic and aggregate forms of α-synuclein and eventually result in cell death.

α-Synuclein as a Disruptor of Normal Autophagic Degradation

The study of human postmortem brain tissue provides evidence for decreased CMA activity in PD, in which both LAMP2A and hsc70 levels were significantly decreased in the SN and amygdala from PD patients when compared to controls (Alvarez-Erviti et al. 2010). In contrast, LC3-II levels (a marker of autophagic activation) were increased in patients with diffuse Lewy Body Disease (DLB), and LC3 (marker for autophagic vacuoles) was colocalized with α-synuclein in most LBs and Lewy neurites, suggesting an increase in macroautophagy, as well as an attempt to clear α-synuclein pathology by up-regulating autophagic activation (Alvarez-Erviti et al. 2010). Mutant α-synuclein transgenic mice and human postmortem brain tissue from DLB patients also show an increase in beclin 1 and LC3-II levels, again suggesting that α-synuclein pathology is associated with an up-regulation in autophagic activity (Yu et al. 2009). In agreement with these findings, α-synuclein accumulation is linked to alterations in both CMA activity and lysosomal function (Cuervo et al. 2004; Martinez-Vicente et al. 2008; Xilouri et al. 2009).

Intriguingly, activation of autophagy by administration of rapamycin has been shown to decrease the deposition of α-synuclein (Sarkar et al. 2007). In addition, Spencer and colleagues show that α-synuclein overexpression in vitro and in vivo leads to the formation of inclusions in the cytosol and neuronal processes (Spencer et al. 2009). As these inclusions are positive for autophagic markers, including cathepsin D and LC3, as well as α-synuclein, it is believed these inclusions are actually abnormal autophagic vesicles, indicating that increased levels of α-synuclein disrupt normal autophagic processes. Based on the finding that overexpression of beclin 1 increases autophagy, as well as reduces cell death (Erlich et al. 2006; Hamacher-Brady et al. 2006; Pickford et al. 2008), Spencer and associates evaluated the consequences of beclin 1 overexpression on α-synuclein pathology. Following lentiviral-beclin 1 overexpression, there was a reduction in the deposition of α-synuclein, as well as a prevention of synuclein-mediated synaptic alterations, suggesting that an up-regulation in autophagy can both ameliorate synuclein pathology and restore neuronal function (Spencer et al. 2009).

Gaucher's and Niemann-Pick Disease, as Well as ATPase Gene Mutations, Increase Parkinsonism Risk

Interestingly, an increased susceptibility to develop parkinsonism and abnormal α-synuclein inclusion formation has been observed in patients with lysosomal storage disorders, including Gaucher's disease and Niemann-Pick disease (Tayebi et al. 2001; Varkonyi et al. 2003; Saito et al. 2004). In addition, mutations in a lysosomal protein encoded by the gene ATP13A2, which is believed to promote UPS and lysosomal dysfunction, are associated with a juvenile onset parkinsonian disorder known as Kufor-Rakeb syndrome (KRS) (Ramirez et al. 2006). KRS is a rare autosomal recessive form of PD that is characterized by extensive neurodegeneration, including dementia, and is associated with loss-of-function mutations in the neuronal P-type ATPase gene (ATP13A2) (Williams et al. 2005; Ramirez et al. 2006).

Evaluation of mRNA levels showed a relatively high expression of ATP13A2 in the brain, with the highest expression levels detected in the ventral midbrain, and the lowest expression observed in the cerebellum (Ramirez et al. 2006). Given that the midbrain is an affected area of the brain in PD, while the cerebellum is not, this pattern of expression is particularly significant to the pathogenesis of PD. In addition, Ramirez and associates localized the wild-type ATP13A2 to the lysosomal membrane, whereas expression of mutant ATP13A2 led to an abnormal accumulation in the ER, leading authors to propose that a loss of functional ATP13A2 promotes lysosomal dysfunction and impairs

autophagic pathways (Ramirez et al. 2006). Truncated mutants were also relatively unstable, with an enhanced stability of ATP13A2 mutants observed on proteasomal inhibition. This observation suggests that ATP13A2 mutants are increasingly more susceptible to proteasome-mediated degradation, and additionally that UPS dysfunction would lead to an increased expression of mutant, nonfunctional protein (Ramirez et al. 2006). Therefore, it is proposed that a loss of ATP13A2 function could simultaneously interfere with synuclein degradative pathways and prevent an adequate compensatory response to various cellular stressors, such as UPS dysfunction.

CONCLUSION

A number of the identified PD-related genes and risk factors have now been studied in some detail, and these investigations suggest that abnormal protein degradation and accumulation might be a critical factor mediating the degeneration of dopaminergic neurons in PD. As the oxidative environment in neurons is also believed to occupy a central role in neurodegeneration, of particular relevance to PD is the increased detection of dopamine quinones under conditions of oxidative stress (Bisaglia et al. 2007). Dopamine quinones have not only been shown to promote proteasomal and mitochondrial impairment (Berman and Hastings 1999; Fornai et al. 2003), but can also react with α-synuclein (Tessari et al. 2008). Dopamine-modified α-synuclein can then bind to CMA receptors and block entry of other CMA substrates as well (Martinez-Vicente et al. 2008). Inhibition of CMA under conditions of α-synuclein deposition have also been shown to prevent the CMA-mediated degradation of MEF2D, a transcription factor critical for neuronal survival, leading to the cytosolic accumulation of inactive MEF2D and thereby decreasing cytoprotective MEF2D activity (Yang et al. 2009).

In addition, although moderate oxidative stress has been shown to increase proteolytic activity in the cell (Grune et al. 1995, 1996, 1998; Kiffin et al. 2004; Bandyopadhyay and Cuervo 2007), severe oxidative stress can lead to protein misfolding and crosslinking, thereby preventing the ability of proteins to be unfolded and subsequently degraded by either the UPS or CMA (Friguet et al. 1994; Grune et al. 1995, 1998; Ullrich et al. 1999; Sitte et al. 2000a,b). As discussed above, oxidative stress can damage and impair the function of lysosomal constituents, and also promote the direct oxidation of catalytic subunits of the proteasome, leading to an impairment of proteolytic activity (Friguet et al. 1994; Okada et al. 1999; Keller et al. 2000a,b; Bulteau et al. 2001; Shamoto-Nagai et al. 2003). Thus, despite the active debate over the sole cause of PD, it is likely that oxidative and proteolytic stress, as well as mitochondrial dysfunction, are all intricately linked in a pathogenic feed-forward cycle responsible for the progression of PD. In support of this, reactive oxygen and nitrogen species influence proteasomal, lysosomal, and mitochondrial function, which in turn regulate the cellular response to oxidative damage. To disrupt this pathogenic cycle, it will be necessary to understand in greater detail the complex interactions between these different pathways.

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