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FORUM REVIEW ARTICLE

Protein Misfolding and Aggregation in Parkinson's Disease

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Abstract

Protein aggregation as a result of misfolding is a common theme underlying neurodegenerative diseases. In Parkinson's disease (PD), research on protein misfolding and aggregation has taken center stage following the association of α-synuclein gene mutations with familial forms of the disease, and importantly, the identification of the protein as a major component of Lewy bodies, a pathological hallmark of PD. Fueling this excitement is the subsequent identification of another PD-linked gene, parkin, as a ubiquitin ligase associated with the proteasome, a major intracellular protein degradation machinery that destroys unwanted, albeit mainly soluble, proteins. Notably, a role for parkin in the clearance of insoluble protein aggregates via macroautophagy has also been implicated by more recent studies. Paradoxically, like α-synuclein, parkin is also prone to misfolding, especially in the presence of age-related stress. Similarly, protein misfolding can also affect the function of other key PD-linked genes such as DJ-1, PINK1, and perhaps also LRRK2. Here, we discuss the role of protein misfolding and aggregation in PD, and how impairments of the various cellular protein quality systems could precipitate these events and lead to neuronal demise. Towards the end of our discussion, we also revisited the role of Lewy body formation in PD. *Antioxid. Redox Signal.* 11, 2119–2134.

Protein Misfolding—An Imperfection of Life

PROTEIN MISFOLDING is a perfect example of life's imperfection. Despite the fidelity of gene transcription, it has been estimated that as many as one-third of all newly synthesized proteins in eukaryotes may not achieve native conformations (126). Even when natively folded, proteins have a low margin of stability and are constantly exposed to damaging conditions including temperature elevation and various post-synthetic modifications (*e.g.*, oxidation, glycation, and nitrosylation) that could promote their denaturation (39). Because of the constant threat of protein misfolding, and the potential danger the accumulation of misfolded proteins would pose to cellular function and survivability, the cell has evolved several complex surveillance machineries to detect and repair faulty proteins, and also to destroy those that are beyond repair rapidly in order to maintain exquisite intracellular protein homeostasis. Amongst these, the chaperone system plays a major role in supporting the correct folding and refolding of proteins (75). Molecular chaperones, which include members of the heat-shock protein (Hsp) family, also sense misfolded proteins and direct them for proteolysis when a native folding state could not be attained (Fig. 1). In this case, targeted proteins are usually tagged with ubiquitin, the polymerization of which on a substrate would typically act as a signal for degradation by the 26S proteasome, a large protease complex consisting of a narrow, barrel-shaped 20S proteolytic core in association with two 19S regulatory caps, one on each side of the barrel's openings (Fig. 2) (115). Three enzymes (*i.e.*, ubiquitin-activating (E1), -conjugating (E2), and -ligating (E3) enzymes) participate in the generation of a polyubiquitin chain on a substrate, whereby successive isopeptide linkages are formed between the terminal residue (G76) of one ubiquitin molecule and a lysine (K) residue (most commonly K48) within another. Although the G76–K48 chain linkage is the most common form of polyubiquitin, ubiquitin self-assembly can occur at any lysine residues within the molecule (at positions 6, 11, 27, 29, 33, 48, and 63) (111, 114). In addition, proteins can also be monoubiquitinated (111, 114). Notably, both K63-linked polyubiquitination and monoubiquitination of proteins are not typically associated with their degradation.

In recent years, it has become clear that polyubiquitinated proteins can also be removed via the autophagy–lysosomal pathway, especially under conditions of cellular stress. Indeed, increasing evidence suggests that autophagy acts as

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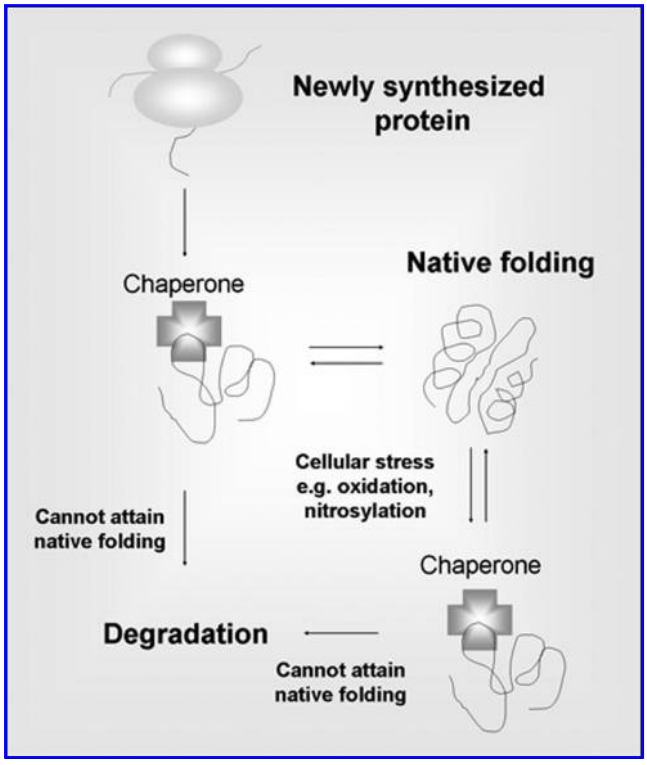

FIG. 1. The chaperone system. Molecular chaperones assist in both initial protein folding and subsequent maintenance of protein conformation under changing environmental conditions. Many factors (*e.g.*, oxidation, nitrosylation) can lead to protein unfolding or misfolding. Chaperones can function in an ATP-dependent manner to catalyze the refolding of partially denatured or denatured proteins into their native states. However, if the attempt to refold the damaged protein is unsuccessful, chaperones can collaborate with the cell's degradation system to rid the unwanted protein.

a compensatory degradation system when the ubiquitin–proteasome system (UPS) is impaired (55, 106). A major form of autophagy that is responsible for the bulk degradation of cytoplasmic proteins or organelles is known as macroautophagy (Fig. 3) (91). This process involves the sequestration of substrates by a phagophore that expands into a double-membrane structure called autophagosome or autophagic vacuole (AV) that engulfs the substrate. The autophagosome then fuses with a lysosome to form autolysosomes, within which the inner membrane of the autophagosome is broken down and the cargo degraded by acidic lysosomal hydrolases. Another form of autophagy is chaperone-mediated autophagy (CMA), which involves the direct translocation of unfolded substrate proteins across the lysosomal membrane through the actions of a cytosolic chaperone hsc70, and an integral lysosomal membrane receptor LAMP2A (lysosome-associated membrane protein type 2A) (91) (Fig. 3). Whereas CMA specifically degrades soluble proteins, macroautophagy has the capacity to degrade large structures, including aggregated proteins that have failed to pass through the narrow pore of the proteasome. Emerging evidence supports a model of macroautophagy-mediated aggregate clearance in which nondegradable protein aggregates are trafficked along microtubules to the microtubule organizing center (MTOC) juxtaposed to the nucleus to facilitate their capture by lysosomes (Fig. 4). The term "aggresomes", coined by Johnston and Kopito a decade ago (59), is popularly used to describe these juxtanuclear structures that co-localize with the MTOC marker, γ-tubulin. Aggresomes are often also enriched in ubiquitin, and their formation appears to be a general response of cells towards proteasome impairment or overloading (*e.g.*, when the capacity of the proteasome is exceeded by the production of aggregation-prone misfolded proteins). Consistent with this, aggregation-prone proteins often generate aggresome-like structures when ectopically expressed in cultured cells in the presence of proteasome inhibition (165). Importantly, we and others have demonstrated that macroautophagy induction promotes the clearance of aggresomes, whereas the reverse is true when the bulk degradation system is inhibited (32, 56, 105, 165), although work from Rubinstzein's laboratory suggests that autophagy is clearing the monomeric and oligomeric precursors of aggregates rather than aggresomes (161). Be it oligomers or aggregates, macroautophagy appears to represent a final line of defense against the build-up of toxic misfolded proteins in the cell. The importance of macroautophagy to neuronal homeostasis is elegantly illustrated by two recent studies showing

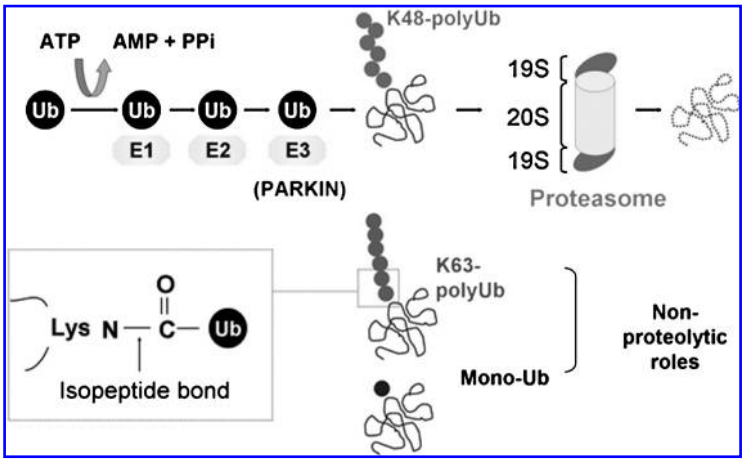

FIG. 2. The ubiquitin–proteasome system. Under normal conditions, proteins destined for proteasomal degradation are tagged with a chain of K48-linked ubiquitin (Ub) via multiple rounds of a linear reaction catalyzed by ubiquitin-activating (E1), -conjugating (E2) and -ligating enzymes (E3). An example of an E3 is parkin. The 26S proteasome is a large protease complex consisting of a barrel-shaped 20S proteolytic core in association with two 19S regulatory caps, one on each side of the barrel's openings. The 20S catalytic core is characterized by three distinct proteolytic activities: chymotrypsin-like, trypsin-like, and peptidyl glutamylpeptide hydrolytic. The components of the 19S cap play vital roles in the initial steps of substrate proteolysis, including the recognition, unfolding, and translocation of substrate proteins into the narrow lumen of the proteolytic core. Proteins can also be monoubiquitinated, or polyubiqutinated via alternative lysine linkages, such as K63. Both mono-Ub and K63-polyUb of proteins are not typically associated with proteolysis, but instead, are thought to sub-serve regulatory roles.

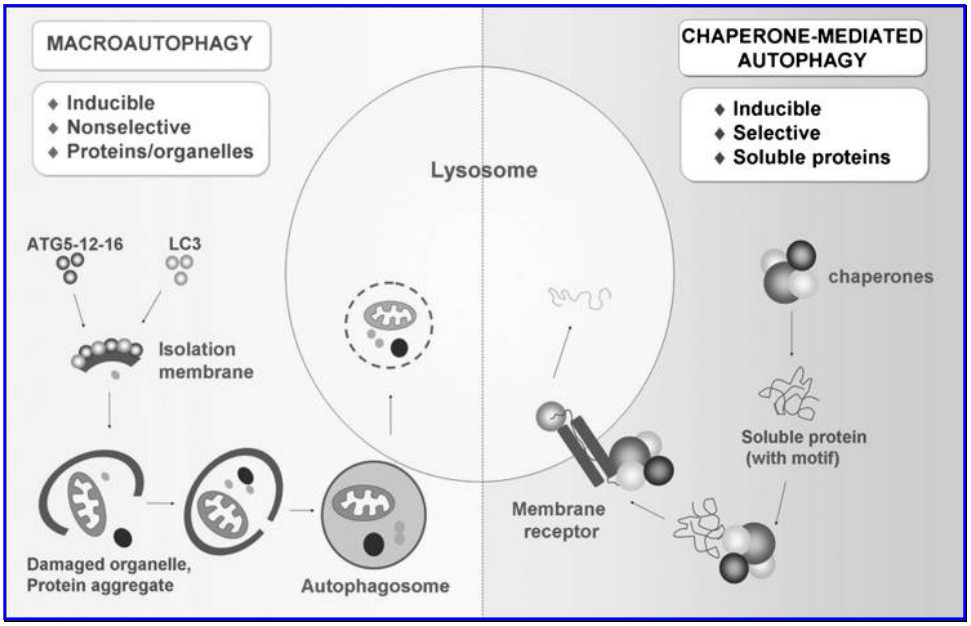

FIG. 3. The autophagy system. Two of the various types of autophagy in mammalian cells are shown here. In macroautophagy, intracellular components (including protein aggregates) are sequestered by an isolation membrane (phagophore) to form an autophagosome, a process involving the Atg5–12–16 complex formation and LC3 (Atg8). The autophagosome's cargo is then disposed off following its fusion with the lysosome. In chaperone-mediated autophagy, proteins are directly targeted to lysosomes by a chaperone complex. Upon binding to the membrane receptor, the cargo is translocated into the lysosomal lumen with the assistance from a luminal chaperone and is rapidly degraded.

that its ablation in neural cells of mice results in extensive neurodegeneration accompanied by widespread inclusion pathology (47, 69). Together, the chaperone, ubiquitin–proteasome, and autophagy systems thus function in synergism to effectively counterbalance the threat of protein misfolding and aggregation. Conceivably, in the event of system malfunction, or when the production of misfolded proteins exceeds the cellular capacity to remove them (*e.g.*, arising from genetic mutations), the resulting elevated levels of misfolded/aggregated proteins could precipitate cellular demise, as appears to be the case in neurodegenerative disorders such as Parkinson's disease (PD).

Misfolding and Aggregation of α-Synuclein in PD—A Killer Unfolds

PD is a prevalent neurodegenerative movement disorder characterized principally by the rather selective loss of dopaminergic neurons in the *substantia nigra pars compacta* (SNpc) of the midbrain, although other areas of the brain such as the dorsal motor nucleus of the vagus, locus ceruleus, and olfactory nuclei are frequently also affected (12, 57, 170). Most cases of PD occur in a sporadic manner. However, a subset of PD cases is inheritable and attributable to mutations in specific genes, which include *α-synuclein*, *parkin*, *DJ-1*, *PINK1*, and

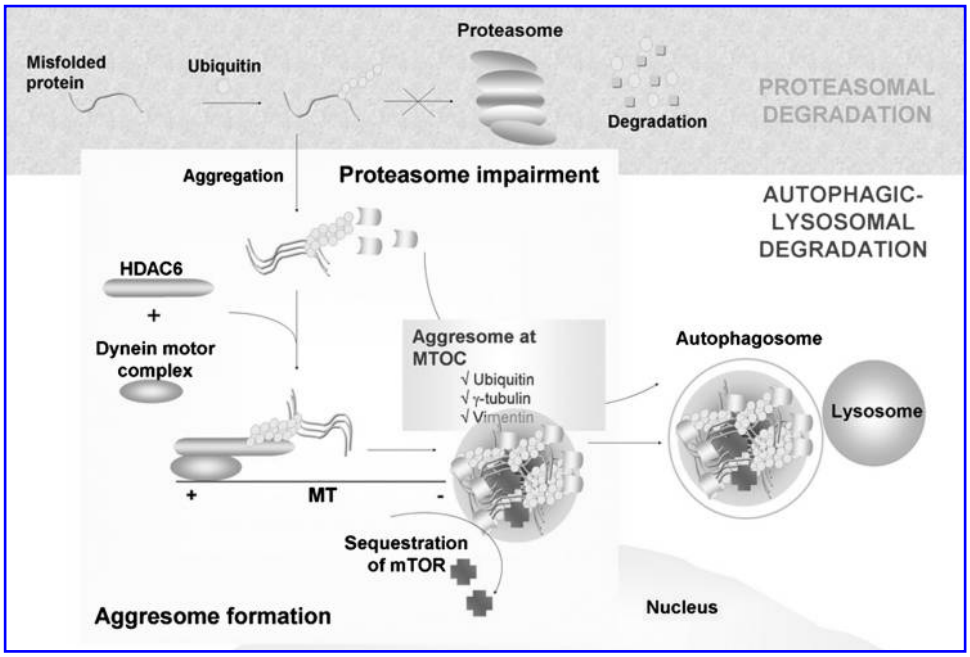

FIG. 4. Aggresome formation and clearance. Under normal cellular conditions, ubiquitinated proteins destined for degradation are targeted to the proteasome. Under conditions of proteasome impairment, the accumulated cargo proteins binds to HDAC6 and are retrogradely transported along the microtubule (MT) network to the MTOC by the dynein motor complex to form an aggresome. Aggresomes are thought to represent staging grounds for the disposal of protein aggregates *via* the macroautophagic route.

LRRK2 (124, 147). Notably, the functional characterization of these PD-linked genes has provided tremendous insights into the molecular pathogenesis of sporadic PD. A resulting area of intense focus is the role of aberrant protein homeostasis in disease pathogenesis. An initial hint pointing towards the involvement of protein misfolding and aggregation in PD comes from the observation that intraneuronal inclusions, known as Lewy bodies (LBs), occur in affected regions of the diseased brain in numbers that far exceed their occasional presence in the normal brain (12). Importantly, α-synuclein, a presynaptic terminal-enriched protein that is prone to misfolding and aggregation, was found to be a major component of LBs (136). This discovery was made shortly after the original association of α-synuclein gene mutation with dominantly-inherited forms of PD (117), which we now know could occur as substitutions (A53T, A30P, and E46K), duplication or triplication (71, 132, 171) Notably, disease-associated α-synuclein mutations have been shown by many groups to increase the accumulation of the protein and/or its propensity to aggregate (20, 37, 100, 150).

In its native state, α-synuclein is typically unfolded (or intrinsically disordered) (153, 162), but the protein is extremely sensitive to its environment and can be molded into an assortment of structurally unrelated conformations including a fibrillization-prone partially folded structure, as well as various α-helical and β-sheet species occurring in both monomeric and oligomeric states (150) (Fig. 5). Paradoxically, a protein's flexibility to fold also potentially increases its chances to misfold and thereby aggregate. In the case of α-synuclein, the protein has been demonstrated to form morphological diverse aggregates *in vitro*, including spherical or ring-like protofibrillar oligomeric intermediates, amorphous aggregates, and amyloid fibrils (40, 150). Kinetic studies have revealed that α-synuclein aggregation follows a crystallization-like seeding mechanism characterized by a slow lag phase in which an oligomeric nucleus is formed, which then initiates the rapid growth of larger aggregates (167). Consistent with this mechanism, macromolecular crowding is known to favor α-synuclein aggregation (130, 151). Several groups have also demonstrated in different experimental models that various exogenous neurotoxicants linked to PD, including pesticides, herbicides, and metal ions, accelerate the aggregation of α-synuclein (81, 127, 152), thereby supporting a gene–environment interaction in disease pathogenesis. Further, phosphorylated, nitrated and oxidized forms of α-synuclein have been identified in LB and other synuclei-

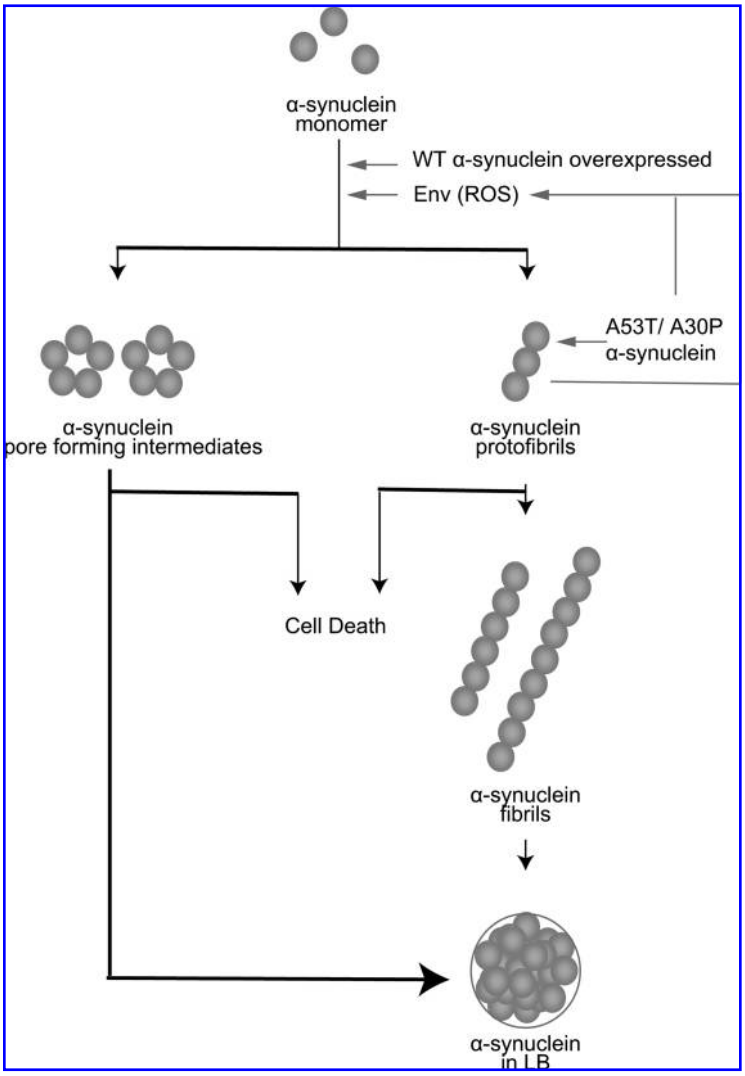

FIG. 5. α-Synuclein aggregation and toxicity. α-Synuclein is a natively unfolded protein that could adopt a variety of toxic conformations under different conditions. Protofibrillar α-synuclein intermediates could form pore-like structure capable of disrupting the integrity of membranes and vesicles, leading to cell death. Otherwise, α-synuclein could fibrillize and aggregate into LBs. Various factors, including disease-associated mutations as well as environmental (Env) stress could promote the formation of α-synuclein protofibrils and/or fibrils.

nopathy lesions (33, 36), suggesting that unregulated post-translational modifications of α-synuclein may also contribute to the development of pathological inclusions. Other factors that catalyze α-synuclein misfolding and aggregation includes sequence truncations and interaction with membranes or proteins such as tau and FK506-binding proteins (150). Accumulated/aggregated α-synuclein that are not removed by the cell can physically disrupt cellular topography and/or interfere with normal cellular physiology, including provoking ER stress (133), mitochondrial dysfunction (52), and impairment in protein quality control systems (24, 145). Prolonged periods of such alterations would invariably lead to cell death. Various studies have indicated that protofibrillar, oligomeric forms of α-synuclein are also toxic and may actually be more potent neuronal killers than fibrillar, aggregated α-synuclein species. For example, the Lansbury's group has demonstrated that α-synuclein protofibrils form pore-like structures capable of permeabilizing membranes and vesicles, a physical disruption that could result in serious cellular injuries (72, 155). Remarkably, cytosolic DA can interact with α-synuclein to form adducts that stabilize α-synuclein protofibrils (21), which may explain why DA-producing neurons are especially susceptible to degeneration in PD.

Quality Control in the PD Brain—Systems Degeneration

How misfolded or aggregated α-synuclein manages to evade rapid degradation by the cell is an interesting question to explore, particularly when not one, but all the three quality control systems (*i.e.*, chaperone, UPS, and autophagy) appear to participate in the cellular management of α-synuclein. Chaperones are known to interact with α-synuclein and suppress its aggregation (6, 86). Chaperones also mediate the binding of α-synuclein to LAMP-2A on lysosomal membrane, which facilitates the protein clearance via CMA (24), although only α-synuclein monomers or dimers, but not oligomers, are degraded by this route. Apparently, α-synuclein degradation could also occur via the UPS or macroautophagy route (161), and the co-chaperone CHIP has been suggested to act as a molecular switch mediating α-synuclein degradation decisions between proteasomal and lysosomal pathways (129). Not surprisingly, the steady-state levels of α-synuclein are affected by inhibitors of proteasome, CMA, or macroautophagy. Indeed, simultaneous inhibition of these systems promotes a synergic formation of α-synuclein inclusions (121). Presumably, one or more systems involved in protein quality control are impaired in the PD brain. Notwithstanding this, it is remarkable to note that the clinical manifestation of PD typically requires several decades to surface in humans, even in individuals with overt α-synuclein or other PD-linked gene mutations. This implies that the various protein quality control systems must have functioned properly for a good length of time to keep toxic protein aggregation at bay, until age-related abnormalities set in that incapacitate the cell's ability to cope with the misfolded/aggregated protein load. The speed and extent of the damage would probably differ amongst individuals, being faster for those who harbor mutant genes or those who have a higher risk for the disease, perhaps by virtue of their genetic variations and/or their exposure to environmental neurotoxicants.

Alterations in the induction and/or function of chaperones have been associated with aging (95). Such alterations would decrease the robustness of the chaperone system and concomitantly increase the misfolded protein load for other quality control systems to handle. Notably, components of the Hsp70 system (Hsp70 and Hsp40) were found sequestered within LBs in postmortem PD brains (6), presumably reflecting a failed attempt by the chaperones to refold aggregated proteins. This irreversible immobilization of chaperones could conceivably contribute to their cellular depletion, thereby exacerbating the aggregation process. Indeed, the global balance of protein folding quality control in a *Caenorhabditis elegans* model of polyglutamine aggregation was found to be disrupted, which results in the loss of function of diverse metastable proteins that, in turn, enhance the aggregation of polyglutamine proteins (38). Consistent with this, overexpression of Hsp70 in a α-synuclein cell model reduces inclusions formation and concomitantly, its toxicity, and also the total levels of α-synuclein, suggesting that the chaperone might enhance refolding and/or promote degradation of α-synuclein (67, 86). Further, in a *Drosophila* α-synuclein model, Auluck *et al*. demonstrated that co-expression of Hsp70 with α-synuclein reduces α-synuclein aggregation and attenuates α-synuclein-mediated DA neuronal death in flies, a finding corroborated later by others in mice (6, 67). Moreover, transgenic flies fed with geldanamycin, an enhancer of Hsp70 expression and a modulator of stress response, similarly suppresses α-synuclein toxicity (5, 7). Collectively, these studies implicate a role for impaired chaperone function in precipitating α-synuclein aggregation and inclusion formation in PD.

Disruption of the UPS, which normally identifies and degrades intracellular proteins, is also thought to promote the toxic accumulation of proteins detrimental to neuronal survival, including α-synuclein. Support for this came from a broad range of studies, including genetics, gene-profiling, and postmortem analysis, as well as *in vitro* and *in vivo* modeling, although controversies surround these findings, especially the proteasome-inhibition rodent model generated by McNaught *et al.* where rats are subjected to subcutaneous injections of either naturally-occurring or synthetic proteasome inhibitor (88) [For a recent review, see reference (76)]. Nonetheless, several groups of investigators were able to demonstrate an association between UPS inhibition and the formation of α-synuclein-positive inclusion bodies (30, 31, 87, 88, 120). Notably, aggregated α-synuclein selectively interacts with the 19S cap and concomitantly inhibits the function of the 26S proteasome (135), thereby perpetuating a vicious cycle. More recently, Bedford *et al.* used a genetic approach to selectively deplete the level of 26S proteasome in the SN of mice and demonstrated in these mice an extensive degeneration in the nigrastriatal pathway that is accompanied by α-synuclein-positive inclusions resembling pale bodies (a structure commonly thought to be an early form of nigra LB) (8). This conditional model provides solid support for the role of proteasome dysfunction in PD pathogenesis and thus represents an important advance in the field. Although the precise basis of UPS dysfunction in sporadic PD remains unclear, the ATP-driven machinery is sensitive to age-related energy depletion and oxidative stress (13, 62, 119). Supporting this, proteasome activity in several brain regions of aged rodents, including the SN, was found to be significantly decreased

when compared to young animals (172). Further, we and others have shown that proteasome dysfunction could also be promoted by exposures to environmental toxins, particularly those that affect mitochondrial function or produce reactive oxygen species (10, 156, 160). Such age- and environment-related effects on proteasome function could result in the progressive accumulation of α-synuclein and other toxic proteins relevant to PD pathogenesis.

Compounding the age-associated defects in chaperone and UPS functions, the lysosomal pathway also registers signs of breaking down as cells age, which include changes in lysosomal volume, stability, and hydrolase activities, impaired AV formation and fusion, reduced rate of chaperone-mediated substrate translocation into lysosomes, as well as accumulation of indigestible materials or lipofuscin (82). Accordingly, both CMA and macroautophagy exhibit decline activity with age, resulting in inefficient turnover of intracellular components and the inability of cells to respond adequately to stress (82). Consistent with this, RNAi-mediated downregulation of LAMP2A expression reduces CMA activity and results in elevated intracellular levels of oxidized, unfolded and aggregated proteins (84). Since α-synuclein can be eliminated selectively in lysosomes by CMA, dysfunctional CMA could potentially also promote α-synuclein accumulation and aggregation. Notably, disease-associated α-synuclein mutants bind to the CMA lysosomal receptor with high affinity but are poorly translocated, resulting in the blockage of uptake and degradation of CMA substrates (24). The increase in cytosolic α-synuclein levels that ensued could favor its aggregation and concomitantly, amplify the burden of misfolded protein load for the cell. Interestingly, DA modification of α-synuclein impairs CMA-mediated degradation by a similar mechanism (83). After binding as monomers to the CMA translocation complex, membrane-bound DA-α-synuclein monomers appear to seed the formation of oligomeric complexes, which consequently placed the translocation complex under siege. Thus, DA-α-synuclein adduct formation in catecholaminergic cells not only promotes the stability of the toxic, pore-forming oligomeric intermediates, it also impairs CMA function. Consistent with this, CMA inhibition following L-DOPA treatment is more pronounced in ventral midbrain cultures containing dopaminergic neurons than in non-DA producing cortical neurons. Importantly, α-synuclein appears to be the principal mediator of DA-induced blockage of CMA, as ventral midbrain cultures derived from α-synuclein null mice are relatively spared from the inhibitory effects of DA on CMA (83). Although upregulation of macroautophagy in response to CMA inhibition is known to occur as a compensatory mechanism (83, 84), the bulk degradation system cannot replace CMA under stress conditions (84), where both pathways presumably needs to be maximally activated. Further, macroautophagy lacks the selectivity that characterizes CMA, and could result in nonspecific degradation of proteins that might be important for the stress response. Indeed, autophagy induction is a double-edge sword (*i.e.*, it could protect against or promote cell death under different circumstances) (15, 41, 174). Otherwise, a finely-tuned macroautophagy response could assist in the clearance of various α-synuclein species, including those sequestered within aggresome-like inclusions (105, 161, 165). Morphological evidence of AV accumulation is certainly present in PD as well as in several other neurodegenerative disorders (3). However, whether the phenomenon represents attempts by the neuron to clean up its cobwebs of aggregated proteins, or a prelude to cell death, or simply a failure in AV consumption remains poorly understood. What is more widely accepted currently is that in the final stages of neurodegeneration, a generalized failure of the different protein quality control systems probably contributes to the demise of affected neurons. In the case of PD, malfunction in these systems could be accelerated by aberrant forms of α-synuclein, although other PD-related proteins, as discussed below, could also conspire to hasten the process.

Parkin—Juggling a Stressful Triage Between UPS and Macroautophagy

Perhaps the most direct evidence supporting a relationship between proteolytic system disruption and neurodegeneration comes from the association of genetic mutations in the *parkin* gene with autosomal recessive parkinsonism (65), as subsequent studies by three independent groups demonstrated that parkin functions as a ubiquitin ligase associated with proteasomal degradation, and that disease-causing mutations of parkin compromise its normal role as an E3 enzyme (54, 128, 173). These findings collectively suggest that loss of parkin function could lead to a toxic accumulation of one or several of its substrates, thereby leading to neurodegeneration. Supporting this, numerous reported substrates of parkin, including CDCrel1 (16), CDCrel2a (16), Cyclin E (139), Pael-R (53), AIMP2/p38 (23, 156), and FBP1 (10), were found to accumulate in the brains of PD patients carrying parkin mutations, although the majority of these substrates do not exhibit elevated levels in parkin-null mice (112). Further, overexpression of CDCrel1 and AIMP2/p38 in cell culture or SN of mice result in marked neurotoxicity (25, 68). Similarly, mice overexpressing Pael-R in a parkin-null background display progressive and selective loss of catecholaminergic neurons (159). However, because none of the identified parkin substrates are exclusively expressed in DA neurons, it remains puzzling why this population of neurons are selectively vulnerable to parkin deficiency in parkin-related patients. Interestingly, parkin, as well as some of its substrates including Pael-R and AIMP2/p38, are localized to LB in sporadic PD (23, 99, 125). Parkin-related cases are, however, frequently devoid of classic LBs, as revealed by initial autopsy studies (48, 94, 141). Given parkin's role as an E3 enzyme and that LBs are usually enriched with ubiquitin, a logical hypothesis that ensued is that functional parkin may be required for, or otherwise facilitates, LB formation. Although we now know from more recent postmortem studies that parkin-linked patients are not necessarily devoid of LBs (28, 118, 123), the hypothesis remains attractive. Another interesting feature of parkin-related cases is that the clinical onset of symptoms tends to be earlier, typically before the age of 40 years, although some late-onset cases have also been described (11). It would appear that the lack of functional parkin markedly accelerates the degeneration process, suggesting that parkin is a key "neuroprotector." Indeed, parkin can afford cellular protection against a remarkably wide spectrum of cellular stress including those mediated by α-synuclein or DA, or arising from various exogenous sources (29, 92). Although the mechanism underlying parkin's multipurpose neuroprotective property is not fully elucidated, two major cellular pro-survival pathways, NF-kB and PI3K/Akt, have been implicated in parkin-

mediated protection (27, 49). Interestingly, the activation of both pathways by parkin is associated with its nonproteolytic ubiquitination activity. In recent years, it has become clear from the work by several groups, including ours, that parkin is a unique, multifunctional E3 ligase capable of mediating proteasome-associated K48-linked poyubiquitination as well as proteasome-independent monoubiquitination and K63-linked poyubiquitination (26, 27, 46, 49, 77, 85, 104). Further, we have proposed that nonproteolytic forms of ubiquitination, by virtue of their dissociation from the proteasome, could also participate in protein aggregation (78). Although this may appear counterintuitive for cellular survival, such a mode of ubiquitination that allows tagged proteins to evade the proteasome could conceivably become useful in times of proteasome overload or dysfunction, as discussed below.

For whatever reasons the proteasome becomes compromised in its function, it is difficult to imagine that the cell will continue to burden the machinery under such conditions with an endless stream of cargo proteins to be degraded. We developed a hypothesis ~2 years ago that nonproteolytic ubiquitination of proteins may help divert proteins originally destined for proteasomal degradation away from the system when it becomes overwhelmed under conditions of proteolytic stress (78). In our proposed model (78), the diverted ubiquitin-enriched proteins are then sequestered into inclusion bodies/aggresomes following their accumulation to be acted upon by the autophagy system. In this way, the cell could preserve its proteasome function over prolonged periods of proteolytic stress and recover thereafter. Supporting this, we have demonstrated that parkin-mediated polyubiquitination of synphilin-1 [a substrate of parkin (17)] normally occurs via K63-linked chains and that this nonproteolytic form of ubiquitin modification of synphilin-1 by parkin promotes its aggregation into inclusion bodies (77). Corroborating our findings, Olzmann *et al.* demonstrated that parkin-mediated K63 polyubiquitination of misfolded DJ-1 couples the protein to the dynein motor complex via the histone deacetylase 6 adaptor, thereby promoting its sequestration into aggresomes (104). As misfolded DJ-1 is usually rapidly degraded by the proteasome, their results also suggest that parkin-mediated DJ-1 aggresome formation takes place under conditions of proteasome impairment (93, 103). This capacity of parkin to divert misfolded proteins away from the proteasome may explain its extraordinary ability to preserve proteasome function under various conditions of proteolytic stress (113, 149, 156). Importantly, our recent work identified K63-linked polyubiquitin as a novel cargo selection signal for macroautophagy-mediated clearance of aggresomes (142, 143). We found that ubiquitin-positive, aggresome-like inclusions formed in cultured cells ectopically expressing either K63 ubiquitin mutant or the heterodimeric Ubc13/Uev1a E2 pair (which promote endogenous K63-linked ubiquitination) are rapidly cleared when autophagy is induced. In contrast, those generated in cells ectopically expressing either K63R ubiquitin mutant (which prevents K63-linked ubiquitin chain assembly), UbcH7 or UbcH8 resisted autophagy-mediated removal. Further, we also found that K63 ubiquitin-positive inclusions exhibit a predominant tendency (~80%) to colocalize with the lysosomal membrane protein LAMP-1, even in the absence of autophagy induction, whereas only a fraction (~20%) of K63R-ubiquitin-positive inclusions stains positively with LAMP-1 under the same conditions (143). This suggests an efficient recruitment of lysosomal structures to K63 polyubiquitinated proteins during aggresome formation, which concomitantly primes the structure for autophagic clearance. By being a multifunctional ubiquitin ligase capable of mediating both proteasome-associated K48-polyubiquitination and macroautophagy-associated K63-linked polyubiquitination, parkin may potentially act as an important link between the two major cellular degradation systems (Fig. 6). A role for parkin in the triage of misfolded proteins between proteasomal and lysosomal degradation thus seems appealing to us, and may explain why the loss

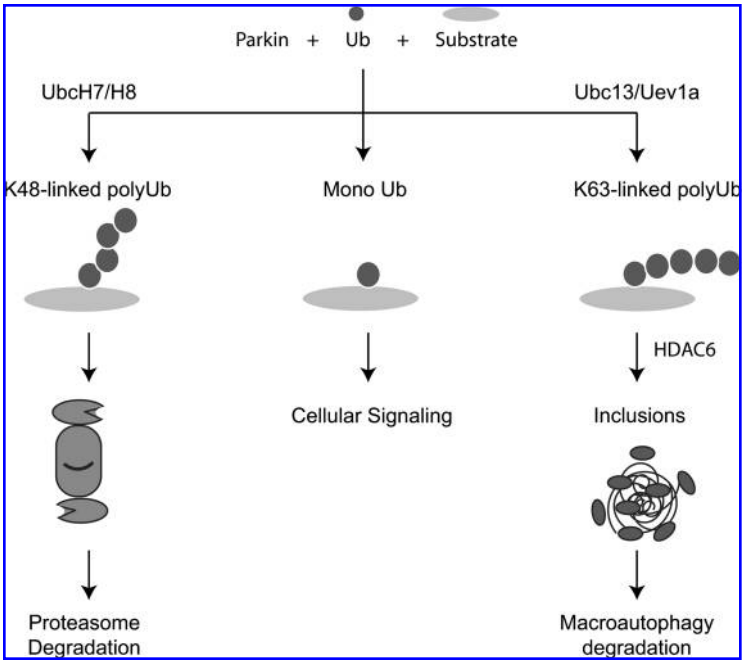

FIG. 6. Parkin is a multifunctional ubiquitin ligase. Parkin is a unique E3 enzyme capable of mediating various types of ubiquitin (Ub) modification on its substrates that would result in different outcomes. Parkin-mediated K48-linked polyubiquitination, which occurs in collaboration with degradation-associated E2s such as UbcH7 or H8, is responsible for substrate degradation via the proteasome. On the other hand, parkin-mediated monoubiquitination and K63-linked polyubiquitination of substrates are not coupled to the proteasome and may be involved in cellular signalling. Further, Ubc13/Uev1a-assisted, parkin-mediated K63-linked polyubiquitination could promote inclusions formation and may also be involved in Lewy body biogenesis.

of parkin function is detrimental to neuronal homeostasis in parkin-related patients, particularly for oxidation-prone DA-producing neurons.

Misfolding and Aggregation of Parkin in PD—The Paradox of a Quality Controller

Although disease-associated mutations of parkin were originally thought to result in the loss of its enzymatic activity, several groups have subsequently demonstrated that the majority of parkin missense mutants retain their catalytic competency (17, 46, 85, 138, 156). Instead, misfolding of parkin triggered by these mutations may be the major mechanism underlying parkin inactivation. Missense parkin mutations frequently alter the protein solubility and concomitantly promote its aggregation into aggresome-like inclusions (4, 22, 45, 46, 97, 138, 158). Interestingly, normal parkin in the brain also becomes progressively more detergent-insoluble with aging (110). As age represents an unequivocal risk factor for PD, the depletion of soluble parkin with age is unlikely to be a trivial association and may reflect the progressive modification of parkin by cellular stress. Consistent with this, we and others have found that a wide variety of PD-linked stressors, including those that produce oxidative and nitrosative stress, induce parkin solubility alterations and thereby its aggregation in a manner analogous to that brought about by several of its missense mutations (74, 156, 164). Remarkably, DA also modifies parkin in a similar fashion (74, 156). Furthermore, parkin appears to be uniquely susceptible to DA-induced modifications compared to several related E3 members such as HHARI, Cbl, and CHIP (73, 74, 166). Accordingly, detergent-insoluble parkin, but not HHARI, Cbl, and CHIP, accumulates in the PD brain (74, 156, 166). More recently, parkin solubility alterations has also been linked to α-synuclein accumulation/aggregation, although the exact mechanism underlying this α-synuclein-mediated phenomenon is not completely understood (61). As functional parkin exhibits a broad neuroprotective capacity, the alteration of parkin solubility induced by various extrinsic and intrinsic stressors provides a mechanism for parkin dysfunction that is relevant to the pathogenesis of sporadic PD (Fig. 7). This phenomenon is at the same time curiously paradoxical; as it appears that an important cellular manager of stress succumbs readily to stress instead.

Another pathogenic mechanism related to parkin misfolding and aggregation is less well understood at this moment, but may potentially explain why some heterozygous parkin carriers have increased risk for the disease. Although initially described as a recessive disorder, numerous reports suggest that single parkin mutations alone may confer increased susceptibility to developing the disease (19, 51, 63, 102). These include two case-control studies which demonstrated the presence of several heterozygous parkin mutations that are not found in control individuals (19, 102). Consistent with this, imaging studies revealed nigrostriatal dysfunction in heterozygous parkin carriers (51, 63). However, the genetic basis of this remains unclear, as most of the single parkin mutant allele does not appear to transmit in an autosomal dominant fashion as might be expected (66). Two parkin mutations that frequently occur in heterozygous state are R275W and G328E. Using *Drosophila* as a model, we demonstrated in R275W, but not G328E, mutant parkin-expressing flies an age-dependent, selective loss of DA neurons that is accompanied by progressive locomotion deficits (157). Similar observations were made by Sang *et al.* with transgenic flies overexpressing two other disease-associated parkin mutants (122). Together, our results suggest the interesting possibility that certain parkin mutations may directly exert neurotoxicity *in vivo*. Mechanistically, how select parkin mutants could mediate pathogenic outcomes *in vivo* remains to be elucidated. Nonetheless, it is tempting to propose that R275W parkin-mediated toxicity may be related to the protein's propensity to aggregate, as several groups, including ours, have previously demonstrated in cultured cells (4, 22, 45, 50, 158). Supporting this, *C. elegans* expressing an orthologous parkin mutant that is prone to misfolding are hypersensitive towards a variety of proteotoxic conditions, including ER stress and A53T alpha-synuclein-mediated aggregation (137). Collectively, these studies suggest that misfolded parkin may be a relevant pathogenic factor in at least some of the parkin-related PD cases (Fig. 7).

Misfolding and Aggregation of Other PD-Linked Genes

At least two other PD-linked gene products, PINK1 and LRRK2, have been reported to localize to LBs (1, 34, 98, 175). PINK1 has been identified to be a mitochondrial-related protein kinase whose mutations, like parkin, are associated with recessive parkinsonism (154). Accumulating evidence suggests that PINK1 and parkin function in a common pathway in the maintenance of mitochondrial homeostasis and function (18, 108, 169). Mutations of PINK1 can affect the protein stability and lead to its rapid degradation and thereby loss of function (9). On the other hand, pharmacological inhibition of proteasome function in cells overexpressing wild-

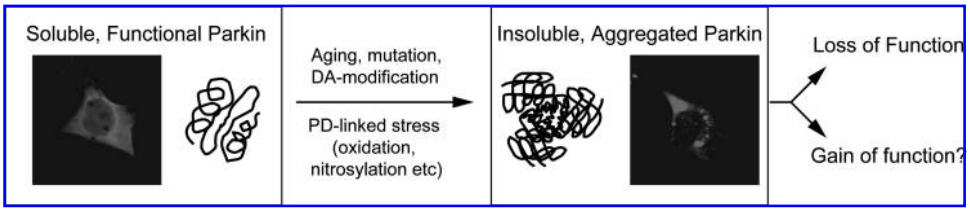

FIG. 7. Parkin misfolding. Parkin normally exists as a soluble, cytosolic protein. However, several disease-associated mutations, as well as normal aging and various forms of exogenous or endogenous PD-linked stress, could alter parkin's solubility and concomitantly promote its aggregation within the cell. The resulting immobilization of parkin within inclusion bodies would deplete the pool of soluble parkin in the cell, thereby compromising its protective functions. Certain species of misfolded parkin (*e.g.*, those triggered by selected parkin mutations like R275W) could exert direct neurotoxicity, although the mechanism underlying this phenomenon remains unclear.

type PINK1 promotes its sequestration into aggresome-like inclusions that stains positively with endogenous parkin as well as endogenous mitochondrial proteins (96). Notably, a recent report demonstrated the presence of mitochondria within intraneuronal inclusions formed in a genetic mouse model of proteasome impairment (8). The same report also showed the localization of mitochondria to nigral pale bodies in postmortem diseased brain samples, although classic LB appears to be devoid of the organelle. In contrast, PINK1 immunoreactivity is detected in a small subset (5–10%) of LBs predominantly in the halo (34, 98), but not in pale bodies (34), suggesting that the protein is recruited to the inclusion body at a rather late stage of its maturation and is probably not essential for its formation. Given that the mitochondrion is the purported residence of PINK1, it is unclear at this moment why the protein is not detected in pale bodies alongside with the organelle.

Like PINK1, LRRK2 contains a kinase domain that is capable of exhibiting a GTP-dependent phosphorylation activity (43, 134, 163). Although a dominant gain of function mechanism is suspected, the disease mechanism of LRRK2 mutations is not entirely clear at this moment. Notably, the inclusions pathology that accompanies SN degeneration in LRRK2 patients is pleiomorphic (*i.e.*, the presence of LB is not an obligate feature) (146). Nonetheless, a number of groups have reported the presence of LRRK2 in nigral LBs (1, 44, 175), although at least one group have disputed this observation (35). Apparently, differences in antibody specificity could be a confounding factor in these studies. Notwithstanding this, even when detected, LRRK2 appears to be present only in a small population of LBs (10–15%) and may not be a major contributor to the biogenesis of these inclusions. However, several LRRK2 mutants have been reported to aggregate when ectopically expressed in cultured cells (44). Whether this aggregation phenomenon reflects the tendency of LRRK2 mutants to become misfolded is debatable, as many of these aggregate-prone mutants do not lose their catalytic function but instead, exhibit elevated kinase activity, suggesting that the catalytic pocket at least is correctly folded (although it is possible that other regions of this large 2527 amino acid-containing protein are affected). The relevance of LRRK2 aggregation observed in cultured cells to DA neurodegeneration thus remains to be clarified. Interestingly, expression of LRRK2 mutants is associated with the shortening of neuritic processes (79), a phenomenon that is curiously promoted (rather than retarded) by autophagy (116). Indeed, mutant LRRK2-mediated neurite retraction is reversed when autophagy is inhibited via RNAi-mediated repression of key autophagy components (116). Together, these studies aptly illustrate the "Janus-like" role of autophagy as a promoter of cell survival or cell death under different circumstances.

In contrast to PINK1 and LRRK2, DJ-1 does not appear to be a component of LB (101). However, the familial PD-linked L166P DJ-1 mutant is prone to misfolding, which compromises its dimerization-dependent protective function (93, 103). As discussed earlier, depending on the cellular conditions, misfolded DJ-1 can either be rapidly degraded by the proteasome, or recruited to the aggresome via parkin-mediated K63-linked polyubiquitination. Several studies have suggested that DJ-1 operates as an antioxidant protein (14, 140), while a recent one suggests that DJ-1 acts as an atypical peroxiredoxin-like peroxidase that functions to scavenge mitochondrial H_20_2 (2). Obviously, these implicated roles of DJ-1 suggest that the loss of DJ-1 function may predispose DA neurons to stress-induced degeneration. Indeed, DJ-1-deficient mice and flies alike are hypersensitive to pharmacological inducers of oxidative stress (64, 80, 89, 90, 107, 168).

Revisiting the Role of LBs in PD

Although the aggregation of α-synuclein into LB is a defining pathological feature of PD, neither is the occurrence of LBs restricted to the SN in PD, nor is LB an obligate feature of SN degeneration (131). Moreover, LB also occurs in a number of other neurodegenerative diseases, including Alzheimer disease; dementia with LB; and pure autonomic failure, all of which stain positively for α-synuclein (131). The role of LBs in PD is thus not entirely clear, but their presence nonetheless serves as a useful signpost that light up the predilection sites of the disease. Using α-synuclein-positive LBs as a marker for PD progression, Braak *et al.* enumerated six neuropathological stages of the disease that, in essence, support the notion that PD is a multi-system disorder (12). Notably, the gastrointestinal and olfactory systems, and not the SN, are the first to be affected. The disease then advances to the brainstem

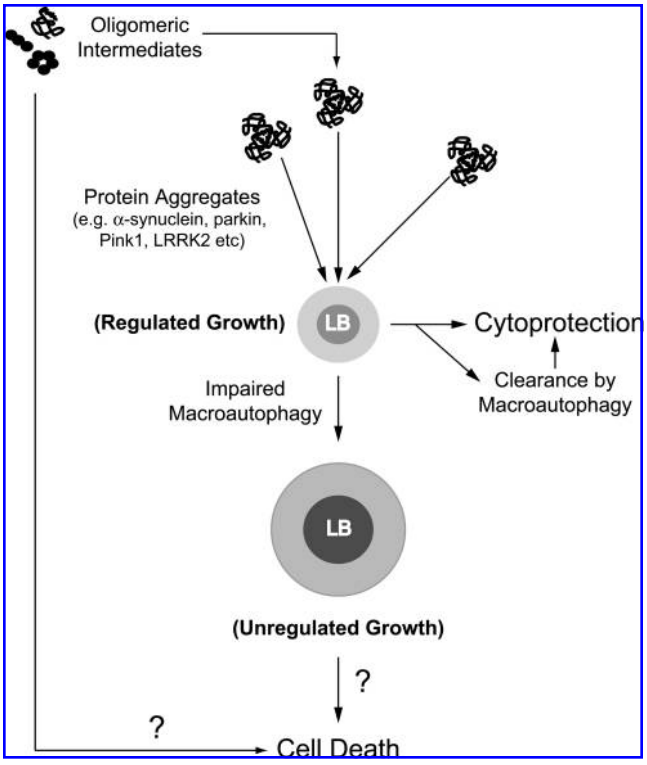

FIG. 8. Proposed model of LB biogenesis and clearance. The formation of LB helps to sequester protein aggregates arising from misfolded α-synuclein, parkin, Pink1, LRRK2, *etc*. that might otherwise be cytotoxic in their soluble forms. According to the aggresome theory, LB formation also facilitates the subsequent removal of these proteins via macroautophagy. The size of a growing LB is thus continuously being regulated. In the event of macroautophagy impairment, LB may grow to a size that becomes detrimental for cellular survival.

through the vagal nerve, and subsequently into the SN and mediotemporal structures before finally reaching the neocortex. Although the proposed pattern of disease progression appears largely valid for PD cases, variations do occur (58, 60). It should also be noted that a correlation between neuronal loss and LB burden is not taken into account in the article by Braak *et al*., and that emerging evidence suggests that LB biogenesis may be a "neuroprotective" response in that its formation may help to sequester soluble intermediates that are otherwise neurotoxic (109, 144, 148). The word "neuroprotective" here therefore means that cells with LBs survive better or longer than those that presumably contain diffuse components of the inclusion bodies, such as soluble α-synuclein oligomers. It does not imply that LBs are absolutely nonpathogenic. Since a growing LB potentially could affect cellular functions if its size were not regulated, it is conceivable that the inclusion body is continuously being cleared by macroautophagy so that its size is kept in check (Fig. 8). Given that neurons are capable of constitutive autophagy, it is tempting to speculate that the presence of matured LBs in neurons may indicate a failure by macroautophagy to remove the precursors of these structures. This could obviously arise from gross autophagy system dysfunction, or alternately, from an inability of certain types of LB to recruit the autophagy apparatus.

Relevant to this, we have recently demonstrated that the composition of an aggresome influences its clearance by macroautophagy (165). For example, whereas aggresomes generated in cells expressing α-synuclein are amenable to clearance by macroautophagy, those produced in cells expressing AIMP2/p38 (also a component of LB) are apparently resistant to autophagic clearance (165). Such impairments in the clearance of intracellular inclusions could initiate the neurodegeneration process, as evident in mice genetically ablated of components essential for macroautophagy (47, 69). Interestingly, a very recent study revealed that the population of nigral neurons in PD that contain LBs is rather constant, suggesting that LBs are continuously formed during the course of the disease and eliminated when the affected neurons die (42). Thus, while the aggregation of toxic diffuse proteins into LBs may confer a protective role, it would seem that the protective effect of LBs is dependent on the continuous clearance of these structures from the cell. This concept is consistent with the aggresome theory (70), where inclusions are thought to represent mere transit stations for protein cargoes destined for degradation to make their final exit from the cell.

Concluding Remarks

Over the last decade or so, protein misfolding and aggregation have emerged as an important theme in PD pathogenesis. Remarkably, the cell could tolerate these pathogenic events rather well for several decades, as seen in patients with aggregation-prone α-synuclein mutations. That disease-onset in these individuals takes a significant length of time to manifest reflects the amazing power of intracellular protein quality control systems to deal with misfolded/aggregated proteins. Preserving or harnessing the various protein homeostatic pathways may thus represent viable approaches in the treatment of PD as well as other neurodegenerative diseases.

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Abbreviations Used

AIMP2 = aminoacyl-tRNA synthetase-interacting multifunctional protein 2
AV = autophagic vacuole
CDCrel = cell division control related
CMA = chaperone-mediated autophagy
DA = dopamine
E1 = ubiquitin activating enzyme
E2 = ubiquitin conjugating enzyme
E3 = ubiquitin ligase
FBP = far upstream element-binding protein
Hsp = heat shock protein
LAMP = lysosome-associated membrane protein
LB = Lewy body
L-DOPA = 3,4-dihydroxy-L-phenylalanine
LRRK2 = leucine-rich repeat kinase 2
MTOC = microtubule organizing center
Pael-R = Parkin-associated endothelin receptor-like
PD = Parkinson's disease
PINK1 = PTEN-induced kinase
SNpc = substantia nigra pars compacta
UPS = ubiquitin-proteasome system

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