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Intracellular degradation of misfolded proteins in polyglutamine neurodegenerative diseases

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Abstract

A number of neurodegenerative diseases, including Alzheimer's, Parkinson's, and polyglutamine diseases, are characterized by the age-dependent formation of intracellular protein aggregates and neurodegeneration. Although there is some debate surrounding the role of these aggregates in neurotoxicity, the formation of aggregates is known to reflect the accumulation of misfolded and toxic proteins. The degradation of misfolded proteins occurs mainly via the ubiquitin–proteasome and autophagy pathways. In neuronal cells, polyglutamine protein inclusions are present predominantly in the nucleus, which is not accessible to autophagy. It remains unclear how the ubiquitin–proteasomal and autophagy pathways remove misfolded proteins in the different subcellular regions of neurons, where disease proteins become misfolded and aggregated in an age-dependent manner. Here we discuss the key findings to date about the roles of the ubiquitin–proteasome system and autophagy in polyglutamine diseases. Understanding how these two pathways function to clear mutant polyglutamine proteins will further the development of effective treatments for polyglutamine and other neurodegenerative diseases.

Keywords

Ubiquitin; Proteasome; Autophagy; Polyglutamine; Huntingtin; Neurodegeneration

1. Protein degradation pathways

Eukaryotic cells have two main routes for clearing misfolded or toxic proteins: the ubiquitin–proteasome and autophagy–lysosome pathways. The ubiquitin–proteasome system (UPS) clears most soluble proteins in the cytoplasm and nucleus and plays a key role in degrading short-lived and misfolded proteins. Protein clearance by the UPS involves two sequential reactions, a tagging reaction and a subsequent degradation of the tagged proteins in the proteasome (Ciechanover, 2005). The tagging reaction requires ubiquitin, a small and highly conserved peptide that is universally distributed among eukaryotes and conjugated to proteins for their degradation by the proteasome. This reaction consists of three steps. In the first, a ubiquitin monomer is activated by forming an intermolecular thiol ester with the ubiquitin-activating enzyme (E1) in an ATP-requiring reaction. Next, activated ubiquitin is transferred to a Cys residue in the active site of a ubiquitin-conjugating enzyme (E2). Ultimately, ubiquitin is linked by its C-terminus through an amide isopeptide linkage to the e-amino group of a Lys residue in the substrate protein, which is catalyzed by a ubiquitin–protein ligase (E3) that confers specificity to the process by selectively binding to a protein target. Activated ubiquitin

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molecules are sequentially added to the first ubiquitin proteins, forming a polyubiquitin chain. Proteins tagged with chains of four or more ubiquitins are then recognized by the 26S proteasome for degradation (Chau et al., 1989; Deveraux et al., 1994). Ubiquitin monomers are released after proteasome degradation or are actively removed by the ubiquitin carboxyl-terminal hydrolases (Mayer and Wilkinson, 1989).

The 26S proteasome is known as an energy-dependent multicatalytic protease localized both in the nucleus and the cytoplasm that degrades polyubiquitylated proteins. It is composed of three major subunits: one 20S catalytic core and two 19S regulatory caps. In the inner part of the 20S complex, there are three types of catalytic subunits that execute the corresponding catalytic activities of the proteasome (trypsin-like, chymotrypsin-like, and peptidylglutamyl-peptide hydrolyzing (PGPH) activity) (Layfield et al., 2003). In addition to recognizing the substrates for the 20S proteasome, the 19S regulatory caps facilitate access of the target proteins to the 20S proteasome by unfolding the substrates and opening the catalytic channel (Hershko and Ciechanover, 1998). Upon recognition of the polyubiquitylated substrates, 19S complexes release polyubiquitin chains. Deubiquitylating enzymes (DUBs) then disassemble polyubiquitin chains into ubiquitin monomers that can be reused (Kawakami et al., 1999).

Autophagy refers to a cellular degradative pathway that involves the delivery of cytoplasmic cargos to the lysosome (Levine and Kroemer, 2008; Mizushima et al., 2008). At least three forms of this pathway have been identified: macroautophagy, microautophagy, and chaperone-mediated autophagy. These forms differ in their physiological functions and in the cargos they deliver to the lysosome. Macroautophagy (often referred to simply as autophagy) is a main pathway for eukaryotic cells to degrade long-lived proteins and organelles. Macroautophagy is characterized by the formation of double membrane-bound autophagic vacuoles, called autophagosomes, which then fuse with lysosomes to form autophagolysosomes, in which lysosome enzymes degrade its content and inner membrane. During microautophagy, there is no autophagosome formation; rather, material enters the lysosome directly for degradation. The third form, chaperone-mediated autophagy, is usually activated under conditions of stress and can only degrade some soluble cytosolic proteins containing a targeting motif biochemically related to the pentapeptide Lys-Phe-Glu-Arg-Gln (KFERQ) (Massey et al., 2004). As little is known about the involvement of chaperone-mediated autophagy and microautophagy in polyQ diseases, this review focuses on the role of macroautophagy or autophagy in polyQ diseases.

There are over 20 genes in yeast (known as the ATG genes) that encode evolutionarily conserved proteins, which are essential for the execution of autophagy (Mizushima et al., 2008). Tissue-specific disruption of ATG genes has revealed a critical role for basal autophagy in protein quality control in mice. For example, depletion of atg5 and atg7 in neurons results in the accumulation of ubiquitin-positive protein aggregates and cellular degeneration (Hara et al., 2006; Komatsu et al., 2006). These findings suggest that the impairment of autophagy reduces the turnover of cytoplasmic proteins, leading to the accumulation of misfolded or damaged proteins that are subsequently aggregated and ubiquitinated.

Both the UPS and autophagy are critical for cell survival and a variety of cellular functions. They are involved in cellular differentiation, growth, and apoptosis. Altered UPS or autophagy function can lead to a wide range of disturbances, such as abnormal animal development and cell degeneration (Ciechanover, 2005; Rubinsztein, 2007; Levine and Kroemer, 2008; Mizushima et al., 2008). However, there are fundamental differences between the UPS and autophagy. The UPS normally clears soluble and short-lived proteins, whereas autophagy degrades long-lived proteins and degenerated organelles. The activity of the UPS often remains at a high level, whereas basal autophagy constitutively occurs at low levels in cells for the performance of homeostatic functions and is rapidly upregulated by starvation, growth factor

withdrawal, or high bioenergic demands (Levine and Kroemer, 2008). Furthermore, the UPS is ubiquitously presented in various cellular compartments, including the nucleus (Bader et al., 2007), whereas autophagy is restricted to the cytoplasm. Such important distinctions merit a discussion of the roles the UPS and autophagy in removing disease proteins that accumulate differentially in various subcellular regions in cells.

2. Misfolded and aggregated proteins in polyglutamine diseases

In this review, we examine the roles of the UPS and autophagy in polyglutamine (polyQ) diseases, which present a clear case that misfolded proteins cause neurodegeneration. Although we will focus on Huntington's disease (HD) in particular, all polyQ diseases are caused by the expansion of a CAG repeat in various disease genes; the CAG repeat is translated to a polyQ tract in the associated disease proteins. At least nine neurodegenerative disorders, including HD, several types of spinocerebellar ataxia (SCA), and spinobulbar muscle atrophy (SBMA), are caused by the expansion of a polyQ stretch in specific target proteins (Orr and Zoghbi, 2007). This expansion of the polyglutamine tract leads to abnormal protein conformations, such as a β-sheet structure (Perutz et al., 1994). As a result, mutant polyQ proteins can form insoluble aggregates in cells. Although there is considerable debate over whether aggregates have a toxic versus protective effect, the aggregates apparently result from the accumulation of misfolded proteins and are therefore an indicator of the intracellular capacity to clear or remove misfolded proteins.

HD represents a typical polyglutamine neurodegenerative disorder. It is autosomal dominant, affecting men and women equally. The disease is characterized by motor dysfunction, cognitive decline, and psychological dysfunction, with selective neurodegeneration that occurs preferentially in the brain striatum (Gusella et al., 1993; Vonsattel and DiFiglia 1998). The expansion of a CAG repeat (>37 CAGs) encodes a polyQ stretch in the N-terminal region of mutant huntingtin (htt), a large-sized protein (348 kDa) that is ubiquitously expressed in various types of cells and interacts with a number of proteins (Li and Li, 2004).

Identification of the genetic mutation underlying polyQ diseases enables the generation of a variety of cellular and animal models for studying polyQ disease pathogenesis. Studies of the HD models have provided clear evidence that the extent of protein misfolding and aggregation is correlated with the length of polyQ tracts. The earlier onset of neurological symptoms in HD patients is positively associated with the longer polyQ tract (Andrew et al., 1993; Snell et al., 1993). Multiple N-terminal htt fragments containing the polyQ tract are present in the brains of HD patients and mice (Zhou et al., 2003), and various cleavage sites in the N-terminal region of htt were identified (Li and Li, 2006). Smaller N-terminal htt fragments containing an expanded polyQ tract are more prone to misfolding and aggregation and appear to be more toxic than full-length mutant htt. For example, the N-terminal htt fragment encoded by the htt exon 1 only possesses 67 amino acids plus the polyQ tract. Expansion of this polyQ tract can cause exon 1 htt to form aggregates in various mouse and cell models and leads to the death of transgenic mice (Davies et al., 1997). Cellular models that express different htt fragments also show that smaller htt fragments form more aggregates and are more toxic than full-length mutant htt (Hackam et al., 1998; Gutekunst et al., 1999). Comparing different HD animal models demonstrates that mice with more severe neurological phenotypes often have an earlier accumulation of N-terminal mutant htt fragments in their brains, which leads to the formation of prominent aggregates in neuronal nuclei and processes (Wang et al. 2008a). N-terminal htt fragments containing expanded polyQ tracts seem to cause cytotoxicity via multiple pathological pathways, which can be classified as nuclear toxicity that affects gene transcription and cytoplasmic toxicity that increases oxidative stress, affects mitochondrial function, and reduces intracellular trafficking (Borrell-Pagès et al., 2006; Li and Li, 2006).

Thus, a key step towards neurodegeneration is the accumulation of misfolded and toxic proteins in cells.

3. UPS activity in polyglutamine diseases

In various cellular and animal models of polyQ diseases, as well as in the post-mortem brains of polyQ disease patients, nuclear polyQ inclusions are positively labeled by antibodies against ubiquitin and proteasome subunits (DiFiglia et al., 1997; Cummings et al., 1998). This colocalization raises the possibility that the sequestration and altered subcellular localization of UPS components by polyQ inclusions could impair UPS function. Several approaches have been used to assess UPS activity. The small flurogenic substrates that are specific for each of the proteolytic activities, such as chymotrypsin-like, trypsin-like, and peptidylglutamyl-peptide hydrolyzing (PGPH) activity, are widely used to measure UPS activity in cell lysates. Furthermore, there is an indirect assay of UPS function based on the expression of degron-reporter proteins in which a UPS degradation signal is fused to a green fluorescent protein (Bence et al., 2001; Lindsten et al., 2003). The modified protein (such as GFPu) has an extremely short half-life and will accumulate only if the UPS is not working efficiently, providing a sensitive tool to measure the UPS in living cells. Using these assays and specific inhibitors of the proteasome, more polyQ aggregates have been found in cellular models upon pharmacological inhibition of proteasome activity (Bence et al., 2001; Waelter et al., 2001; Jana et al., 2001).

To determine whether the proteasome is capable of digesting a polyQ peptide, Venkatraman et al. performed experiments with peptides containing 10–30 residues of glutamine, flanked by 2 Lys residues to enhance their solubility, and incubated them with purified proteasomes (Venkatraman et al., 2004). They found that the proteasome could not digest expanded polyglutamine sequence efficiently, but only cut the flanking basic residues. Their findings raise the important possibility that expanded polyQ tracts or aggregation-prone peptides cannot be digested by and then released from the UPS, resulting in an impairment of the function of the UPS. This idea is supported by two key findings: polyQ protein-mediated impairment of UPS function occurs in the absence of detectable aggregates, and there is a lack of interference of protein aggregates on 26S proteasome function in vitro (Bennett et al., 2005). In the presence of the proteasome activator PA28gamma(K188E), however, human red cell proteasomes are capable of cleaving Q–Q bonds multiple times within a polyQ tract of 10 contiguous glutamines (Pratt and Rechsteiner, 2008). It seems that reaction components such as PA28gamma might influence the ability of the proteasome to cleave polyQ peptides.

Soluble expanded polyQ peptides are found to affect UPS function in vitro (Venkatraman et al., 2004) and in cells that express a fluorescent UPS reporter (Bennett et al., 2005). Michalik and Van Broeckhoven targeted polyQ proteins with N-terminal degradation signal for their digestion by the proteasome in transfected cells. Measuring the hydrolysis of fluorogenic proteasome substrates showed that soluble expanded polyQ proteins do not compromise proteasomal activity. A pulse-chase experiment demonstrated that soluble expanded polyQ proteins are completely and efficiently degraded by the proteasome (Michalik and Van Broeckhoven, 2004). In a mouse model that expresses a fluorescent reporter to reflect UPS function and a mutant polyQ protein (SCA7 protein), there was no correlation found between UPS impairment and polyQ-mediated retinal neuropathology (Bowman et al., 2005). Seo et al examined post-mortem brain tissues of HD patients and observed a decrease in proteasomal activity in the striatum and cortex (Seo et al., 2004). However, this decrease could be due to atrophy or degeneration already present in the HD patient brains. It seems more relevant to measure proteasomal activity directly in the brain tissues of polyQ disease animal models. Several studies that examined UPS activity in the whole cell homogenates of brain tissues detected no reduced UPS activity in HD mouse brains (Díaz-Hernández et al., 2003; Bett et

al., 2006; Wang et al., 2008b). However, quantifying polyubiquitin chains, an endogenous biomarker of UPS function, reveals that Lys 48-linked polyubiquitin chains accumulate early in HD mouse brains, suggesting that UPS dysfunction does occur in the HD brain (Bennett et al., 2007). It seems that different assays (in vitro vs. in cells and GFPu vs. ubiquitin or proteasome substrates) may have different sensitivities for detecting changes in the UPS in cells. As the use of whole cell lysates cannot address whether mutant polyQ proteins affect the UPS in a specific compartment in neurons, Wang et al. generated fluorescent reporters and targeted these reporters to the synapses in the mouse brain to measure synaptic UPS activity. They saw a decrease in UPS activity in the synapses of HD mice (Wang et al., 2008b). This decreased UPS activity is also confirmed by biochemical analysis of proteasome activity in isolated synaptosomal fractions from HD mouse brains. Although it remains unclear whether soluble or aggregated mutant htt can directly affect synaptic UPS, mutant htt was found to reduce mitochondrial trafficking in neuronal processes and to decrease the amount of mitochondria in the synaptosomal fraction. The smaller amount of functional mitochondria in nerve terminals results in a low level of ATP in the HD synapses, which could contribute to the reduced activity of synaptic UPS seen in HD mouse brains (Orr et al., 2008; Wang et al., 2008b). Thus, subcellular localization should also be taken into account when investigating potential changes in UPS activity in affected brains.

4. Autophagy in polyglutamine diseases

The idea that aggregation-prone or aggregated polyQ proteins are resistant to the UPS led to the investigation of polyQ protein clearance by autophagy. If autophagy clears polyQ proteins, then the activation of autophagy should reduce misfolded proteins and their associated cytotoxicity. In support of this idea, pharmacological activation of autophagy by rapamycin was found to lower the levels of mutant htt and other types of misfolded proteins, as well as their neurotoxicity in cellular and animal models (Ravikumar et al., 2004). Other proteins or molecules that can induce autophagic degradation were also found to reduce polyQ protein toxicity (Shibata et al., 2006; Zhang et al., 2007). ATG gene knockdown or knockout increases polyQ aggregate formation and the toxicity of polyQ-expanded proteins in *Caenorhabditis elegans* (Jia et al., 2007). Autophagy induced by overexpression of histone deacetylase 6, a microtubule-associated deacetylase, compensates for impairment in the UPS in a fly model of spinobulbar muscle atrophy (Pandey et al., 2007). Autophagic vacuole-like structures are reported in HD patient brains (Sapp et al., 1997). Moreover, overexpression of a variety of polyQ proteins leads to an increase in biochemical and morphological markers of autophagy-like structures in cultured cells (Kegel et al., 2000; Qin et al., 2003; Taylor et al., 2003; Ravikumar et al., 2004; Iwata A et al., 2005a; Yamamoto et al., 2006), as well as fly (Pandey et al., 2007) and mouse models (Petersen et al., 2001; Skinner et al., 2001) of polyQ diseases. In cultured neurons from transgenic HD (R6/2) mice, however, activation of the lysosome–autophagy system was only observed when the neurons are exposed to dopamine-mediated oxidative stress, suggesting that the combination of mutant htt and oxidative stress induces autophagy (Petersen et al., 2001).

Because impairment of the UPS induces autophagy and knockout of autophagy in the mouse brain leads to neurode-generation with ubiquitin-positive pathology, it is likely that the two apparently distinct systems communicate with one another in the regulation of protein turnover. HDAC6 is found to be required for autophagy-dependent degradation of polyQ proteins (Iwata et al., 2005b; Pandey et al., 2007). These findings led to the idea that autophagy may act as a compensatory degradation system when the UPS is impaired, and that HDAC6 might be a mechanistic link in this compensatory interaction (Pandey et al., 2007).

5. Remaining questions

Studies of the UPS clearly show that inhibiting proteasomal function increases the levels of both soluble and aggregated forms of mutant htt (Jana et al., 2001; Zhou et al., 2003), suggesting that the UPS plays an important role in clearing misfolded polyQ proteins. Subsequent investigation of autophagy in polyQ diseases led to the hypothesis that the UPS may act as a primary route to clear soluble polyQ proteins, whereas autophagy functions to remove aggregation-prone or aggregated proteins (Rubinstein, 2007). Hence the discovery that the activation of autophagy can suppress polyQ toxicity is exciting, as it highlights a potentially therapeutic means of alleviating the neuropathology of polyQ diseases.

Nevertheless, there are several significant issues that remain unclear and require rigorous investigation. Unlike the UPS, autophagy is capable of digesting long-lived proteins and organelles. This unique property may allow autophagy to digest polyQ aggregates or inclusions in cells. If true, we should observe the presence of polyQ aggregates in lysosome and/or autophagosome, a membrane-bound structure, in the brain; however, there is still a lack of strong evidence to substantiate this. Several studies have shown that polyQ protein overexpression increases the incorporation of LC-3, an autophagosome marker, into autophagy-like puncta in transfected cells. Given that autophagy can be activated in response to various cellular stresses, it is likely that overexpression of misfolded proteins can induce autophagic degradation and autophagic structures in transfected cells. Also, LC3 can be incorporated into protein aggregates independent of autophagy (Kuma et al., 2007). An important issue is how autophagy functions when polyQ proteins are expressed at the endogenous level in the brain. HD repeat knock-in mouse models, which express mutant htt at the endogenous level, show the age-dependent accumulation of misfolded proteins in the brain (Li et al., 2000; Wheeler et al., 2000; Lin et al., 2001). It would be interesting to know whether altering autophagy function, such as treating the animals with rapamycin, can increase the clearance of mutant htt or other polyQ proteins in knock-in mouse models, and if so, which form (soluble or aggregated) of mutant polyQ proteins can be reduced. In addition, the half-life of different htt fragments and the influence of the polyQ domain on htt's half-life remain to be investigated, which will help understand how mutant htt turns over.

Interpretation of any results obtained with drugs that alter UPS and autophagy functions should take into account their potentially nonspecific effects. For example, rapamycin is known to activate autophagy by suppressing the mammalian target of rapamycin (mTOR), a protein kinase that can regulate multiple cellular functions independent of autophagy (Wullschleger et al., 2006). Indeed, rapamycin confers additional protection against apoptosis in polyQ protein-transfected cells (Ravikumar et al., 2006). Recent studies report that rapamycin decreases aggregation-prone polyQ proteins and aggregates in autophagy-deficient cells, suggesting that rapamycin reduces polyQ protein synthesis independent of autophagy (King et al., 2008). The use of Atg siRNA, which is more specific for inhibiting autophagy, in cultured cells has provided more compelling evidence that inhibition of autophagy can increase polyQ aggregates (Iwata et al., 2005a). Genetic mouse models that are deficient for important autophagy proteins, such as Atg (Komatsu et al., 2005), would allow investigation of the in vivo role of autophagy in polyQ diseases. However, given that autophagy is critical for a variety of cellular functions and that loss of autophagy leads to increased protein ubiquitination and neurodegeneration, we expect that deficient autophagy in polyQ diseases would exacerbate polyQ cytotoxicity. Also, the impairment of autophagy caused by mutant polyQ proteins can lead to defective turnover of organelles and other molecules. A key issue is distinguishing between primary consequences and those secondary to other impaired cellular functions as a result of inhibiting autophagy.

It is possible that mutant polyQ proteins can impair UPS function, which in turn causes more accumulation of misfolded proteins that further damage UPS function. However, because measuring proteasomal activity in cell homogenates of HD mouse brains did not reveal any significant decrease in proteasomal activity (Díaz-Hernández et al., 2003; Bett et al., 2006; Wang et al., 2008a,b), it remains unclear whether mutant polyQ proteins can directly affect the UPS in the brain. On the other hand, an age-dependent decrease in UPS activity is well correlated with the formation of polyQ aggregates in the mouse brain (Zhou et al., 2003; Wang et al., 2008a,b). The activities of both the UPS and autophagy decline during aging (Vernace et al., 2007; Martinez-Vicente et al., 2005), and this age-related decline is likely to contribute to the age-dependent accumulation of misfolded proteins and neuropathology in a variety of late-onset neurological disorders. Thus, a key issue is how the UPS and autophagy clear mutant polyQ proteins that are expressed at the endogenous level and accumulate progressively with age.

A striking feature of polyQ diseases is the presence of polyQ inclusions in the nucleus. Autophagy is known to be ineffective at clearing protein aggregates that are formed in the nucleus (Iwata et al., 2005a), and there is no evidence for the presence of autophagy in the nucleus. It has been postulated that autophagy does not clear aggregates directly, but clears aggregate precursors, thereby shifting the equilibrium away from aggregate formation (Rubinsztein, 2007). This may happen in the cytoplasm of transfected cells that overexpress polyQ proteins. In most polyQ disease mouse models, there are no prominent polyQ inclusions found in the cytoplasmic region of neuronal soma, or perikaryon. Because soluble polyQ proteins are likely (or at least more efficient than aggregated proteins) to enter the nucleus, a decrease in the clearance of soluble mutant polyQ proteins in the cytoplasm can cause more mutant proteins to move into the nucleus. The nucleus favors the accumulation of misfolded proteins because their nuclear export is impaired by expanded polyQ tracts (Cornett et al., 2005) and possibly because of an intrinsically lower capacity to remove misfolded proteins in the nucleus. Indeed, proteasomal activity in the neuronal nucleus is found to be lower than in the cytoplasm (Zhou et al., 2003; Wang et al., 2008a,b). In HD, mutant htt is also transported within neuronal processes (Colin et al., 2008) and accumulates in neuropil (axons and dendrites) and synapses, in which low UPS activity is also observed, which can promote the accumulation of misfolded htt (Wang et al., 2008a,b). Because the impairment of autophagy in neuronal processes can contribute to axonal dystrophy and neurodegeneration (Komatsu et al., 2007), subcellular localization also critically determines the extent to which misfolded proteins accumulate, as well as the cellular capacity to clear these misfolded proteins.

6. Conclusion

Although many issues remain to be clarified or tested, it is clear that the intracellular capacity to remove misfolded proteins is critical for reducing polyQ expansion-mediated neuropathology. Because this capacity diminishes with age, improving the cellular capacity to clear misfolded proteins should help slow or reduce age-dependent neuropathology. However, overactivation of the UPS and autophagy can also be harmful to important cellular functions. For example, UPS overactivation can lead to cancer (Miasari et al., 2008), and overactivation of autophagy can lead to cell degeneration (Kang et al., 2007). Thus, a better understanding of the processes and identification of the components involved in protein degradation by the UPS and autophagy will further the development of mechanism-based drugs that can specifically target only the disease proteins. Given that mutant polyQ proteins selectively accumulate in different subcellular regions in affected neurons, the various capacities of different cellular compartments to cope with misfolded proteins should also be considered. Understanding the mechanisms underlying the different activities of the UPS and autophagy during aging and in different subcellular regions could identify therapeutic targets.

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