Important Factors in the Formation and Clearance of Protein Aggregation

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Neurodegenerative diseases are major health challenges in the aged population and profoundly affect the life expectancy and quality of life of the elderly people. These diseases are caused by a build-up of aggregation-prone proteins (e.g. Amyloid β (Aβ) and tau in Alzheimer's disease, PolyQ proteins (Htt, Atx3n) in Huntington's disease, α-synuclein in Parkinson's disease, and TDP43 or SOD1 in Amyotrophic Lateral Sclerosis (ALS)). Build-up of these harmful proteins causes additional damages to the cells such as mitochondrial dysfunction, build-up of reactive oxygen species, and eventually causes cell death [1]. On the other hand, decreasing such aggregation-prone proteins has been shown to reverse neurodegeneration phenotypes, indicating the crucial importance of abnormal-protein build up as the crucial cause of neurodegenerative diseases. The build-up of these proteins are associated with a decline of protein degradation capacity of the cells, and consequently therapeutic strategies have been developed, and more are under development to enhance the clearance of these abnormal proteins by two major cellular protein degradation systems: the Ubiquitin-Proteasome System (UPS) [2] and the autophagy-lysosomal system [3]. In this editorial I would like to briefly review our current knowledge about the role of protein degradation systems in the development and therapy of neurodegenerative diseases. I will particularly focus on the cellular process of protein aggregation via key factors such as p97/VCP, p62, and HDAC6.

Cellular Protein Degradation Systems in the Development of Neurodegenerative Diseases

There are two major protein degradation systems in the cells. The UPS carries out degradation of mostly cellular proteins, particularly proteins marked by K48-linked poly-ubiquitination [2]. Degradation via the UPS is highly selective due to the ubiquitination of selected substrates by over 600 ubiquitin ligases [4]. The autophagy-lysosomal system, on the other hand, degrades cellular components by enclosing them in double-membrane autophagosomes, followed by subsequent fusion with lysosomes. Although initially regarded as a non-selective process that is activated during starvation to consume cellular components in order to provide energy and amino acids for protein synthesis [3], it is now accepted that autophagy can also select for particular substrates due to the presence of substrate adapter proteins such as p62 (aggrephagy [5]), Nix (mitophagy [6]), etc. The capacity of both the UPS [7,8] and autophagy [9] declines with ageing thus corresponds well with the association of neurodegenerative diseases with the elderly population. Therefore, it has been speculated that the decline of cellular protein degradation capacity may drive aggregate formation and promote neurodegeneration [9,10]. In 1987, ubiquitin was first identified in tau aggregates in Alzheimer's disease, first linking UPS with aggregate formation [11], and now it is evident that ubiquitin is present in almost every type of such protein aggregates. However, there lacks solid evidence for the hypothesis that proteasome dysfunction causes neurodegenerative diseases. In 2004, it was reported that in a mouse model, chronic exposure to proteasome inhibitors causes the build-up of α-synuclein in neurons and the emergence of Parkinson's disease symptoms [12]. Unfortunately, other groups cannot reproduce their results [13]. On the other hand, the build-up of aggregates may block proteasome functioning. It is proposed that the accumulation of ubiquitinated species in Alzheimer's disease is a result

of the inhibition of proteasome activity caused by Aβ peptides [14], and excessive levels of oxidized proteins inhibit proteasomal degradation probably by clogging the proteasome [15]. In fact, the emergence of aggregation-prone proteins are frequently associated with their point mutations (e.g. TDP-43 (M337V and Q331K) [16], α-synuclein (A53T, A30P) [17]), and poly-Q expansion (Huntingtin [18], ataxin-3 [19]) that makes them prone to aggregation but becomes poor substrates of the proteasome. Therefore, it appears unlikely that proteasome dysfunction is a major driving force for neurodegenerative diseases. By contrast, the importance of autophagy defects in the development of neurodegenerative diseases appears more prominent. Separate groups have demonstrated that inactivation of autophagy by neuron-specific knock-out key autophagy factors Atg5 [20] and Atg7 [21] promoted neurodegeneration in mouse models. The autophagic poly-ubiquitin binding protein p62 is found in most aggregates [22], and acetylation of PolyQ Htt enhances its degradation by autophagy [23].

Targeting Cellular Protein Degradation Systems in Neurodegenerative Diseases Therapy

Targeting particular aggregation-prone proteins to increase their solubility or reduce their cellular levels has been a strategy for neurodegenerative disease therapy. For example, Homotaurine (Tramiprosat), which promotes the solubility of Aβ, and Tarenflurbil (R-flurbiprofen), which reduces the cellular level of Aβ, have entered clinical trials for the treatment of Alzheimer's diseases. Unfortunately, these agents did not reverse the cognitive decline of Alzheimer's disease patients in Phase III clinical trials [24]. This unsatisfactory outcome is related to the fact that neurodegenerative diseases are frequently caused by multiple types of aggregation-prone proteins. For example, both Aβ and tau are pathogens of Alzheimer's disease, and α-synuclein, which is best known for its role in forming Lewy bodies in Parkinson's disease, also contributes to 50% of Alzheimer's disease [25]. Consequently, targeting particular aggregation-prone protein may not be the most efficient therapeutic strategy. However, since UPS and autophagy clearly correlate with a build-up of aggregation-prone proteins, targeting these two protein degradation pathways is also become an important strategy to treat neurodegenerative diseases. Several activators of autophagy, such as CCI-779 (a rapamycin analog, [26]), Trehalose [27], Rilmenidine [28] have been shown in preclinical mouse studies to be able to alleviate neurodegeneration caused by PolyQ proteins. These strategies all target the initiating step of autophagy to promote the generation and assembly of autophagosomes to elevate the activity of general autophagy in the cells. Although these strategies have proven efficient in clearing aggregates and also

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damaged mitochondria (which generates reactive oxygen species and contributes to killing of neurons [1]), several undesired effects can also be caused by such a general elevation of autophagy. Hyper-activated autophagy by itself can cause autophagic cell death, and it has been shown that autophagy activation can promote the production of Aβ [29]. On the other hand, strategies to enhance proteasome activity to promote the degradation of aggregation –prone proteins are also under development. Finley et al. reported that inhibiting the proteasome-associated deubiquitinase Usp14 with a small molecule inhibitor IU-1 [30] can not only enhance ubiquitin-dependent protein degradation, but intriguingly, can also promote the degradation of aggregation-prone proteins such as tau or Atx3-PolyQ. Usp14 normally binds poly-ubiquitin chains of proteasome-associated substrates and cleaves from the distal end individual ubiquitins from the chain. Such shortening of ubiquitin chains may eventually cause the dissociation of ubiquitinated proteins from the proteasome to prevent their degradation. There are other strategies through which proteasome activities can be enhanced. For example, PKA and CaMKII-mediated phosphorylation of Rpt6 has been shown to promote proteasome activities in the cells [31,32]. Conversely, the phosphatase UBLCP1 dephosphorylates nuclear proteasomes and cause a decline of their activities [33]. Recently, it was found that tankyrase mediates ADP-ribosylation of PI31, a proteasome-associated inhibitory factor. This results in the dissociation of PI31 from 20S proteasome and an elevation proteasome activity in the cells [34]. These are promising strategies that have not yet reached clinical trials.

Important Factors Involved in Aggregation Formation and Clearance

Although raising the general levels of proteasome or autophagy activity are promising therapeutic strategy, such manipulations may profoundly affect many cellular functions since proteasome and autophagy controls almost every aspects of cellular functions. By contrast, it will be a more targeted approach to enhance the formation and clearance of aggregates, because these aggregates contain most harmful pathogenic proteins, and our understanding of key steps of aggregation formation and clearance has opened such possibilities to target these processes.

There is controversy concerning the location of these aggregates in the cells. Most studies report a single peri-nuclear aggresomes that contains not only ubiquitin but also the autophagy adaptor LC3 [35]. Kaganovich et al. [36] reported a new model in which protein aggregations form in two distinct compartments of the cells. One compartment (JUNQ, juxtanuclear quality control) is perinuclear, which contains proteasomes but not LC3, while the other compartment (IPOD, insoluble protein deposit) is located in the periphery of the cells, which contains no proteasomes but LC3 [36]. That study reports that proteins that cannot be easily folded are more likely to distribute to IPOD while JUNQ is a compartment in which cells attempt to refold or degrade the aggregated proteins. A subsequent report also demonstrated that different aggregation-prone proteins tend to distribute to different compartments. For example, while mutant SOD1 and poly-A containing proteins aggregate at JUNQ, insoluble proteins such as poly-Q containing proteins aggregate at IPOD instead [37]. Consequently, IPOD, but not JUNQ, resembles the aggresomes that are subsequently degraded by autophagy, but not proteasome. No studies have investigated the relationship between JUNQ, IPOD, and the perinuclear aggresome, but the difference between IPOD and aggresome may be due to whether or not the aggregate has been transported to the perinuclear region via HDAC6 (see below).

There are four autophagic receptors in human cells that bind poly-ubiquitinated proteins [3]. Among them, p62 is most extensively studied and its role in causing the aggregation of poly-ubiquitin conjugates has been well established [5]. p62 contains an Ubiquitin-Association (UBA) domain that binds poly-ubiquitin chains, and also an N-terminal PB1 domain to allow oligomerization. In this process, p62 promotes the aggregation of poly-ubiquitin conjugates. Furthermore, p62 also associates with key autophagy factors such as LC3 (Atg8 in yeast) that are bound on the membrane of growing autophagosomes [5], thus promoting the sorting of these aggregates into autophagosomes for degradation. These properties of p62 make it an attractive target to pharmaceutically activate in order to clear poly-ubiquitinated aggregates by autophagy, but not affecting autophagy generally. Regulating the phosphorylation of p62 appears to be an effective way. Phosphorylation of p62 at S403 by Casein Kinase 2 (CK2) [38] or TBK1 [39] promotes its ability to sort ubiquitin conjugates into aggresomes, while its phosphorylation at S351 by mTORC1 appears to promote an anti-oxidant transcriptional response by activating the transcription factor Nrf2 [40], which is also cytoprotective by inducing the expression of anti-oxidant enzymes and interestingly, also proteasome subunits [41] and p62 [42].

p97 is an AAA-ATPase with important function in ubiquitin-dependent degradation. It utilizes ATP to extract ubiquitinated proteins from the endoplasmic reticulum [43] or large protein structures such as myofibrils [44] and chromatin [45] to facilitate their degradation. Intriguingly, p97 was recently shown to be also important for the aggregation of poly-ubiquitinated proteins. In Inclusion Body Myopathy with Paget's disease and Fronto temporal Dementia (IBMPFD) model harboring dominant-negative p97 mutations that abolish its ATPase activity, cells fail to form a single perinuclear aggresome. Instead, PolyQ proteins were aggregated in several dispersed aggregates around the cells [46,47]. These aggregates also contain ubiquitin, LC3, and p62, but are defective in being delivered to autophagolysosomes for degradation [46,47]. Therefore, p97 also promotes the aggregation and degradation of abnormal proteins.

Another important factor is HDAC6, a deacetylase that plays important roles in various steps of aggregation formation and clearance. First, HDAC6 can interact with poly-ubiquitin conjugates (via its Znf-UBP domain) and microtubule dyneins simultaneously [48,49]. These interactions, together with the deacetylation of α-Tubulin by HDAC6 [50], promote the transport of poly-ubiquitin conjugates to the perinuclear aggresome. This process apparently confines the ubiquitin conjugates to a restricted cellular compartment and minimize its damage to the cells. Next, HDAC6 deacetylates cortactin to promote actin remodeling. This process facilitates the fusion of autophagosomes and lysosomes for degradation [51]. HDAC6 can also promote the protein folding by deacetylating Hsp90 to enhance its interaction with cochaperone p23 and its chaperone function [52], or to dissociate Hsp90 from HSF1 and cause the activation of the transcription factor HSF1 to induce the expression of additional chaperones [53]. Via these mechanisms, HDAC6 plays multifaceted roles in clearing abnormal and aggregation-prone proteins.

While HDAC6, p62, and p97 are all important for aggresome formation and clearance, the interaction between these molecules has not been thoroughly studied and clearly merits major research efforts. Binding of p62 to HDAC6 appears to enhance the deacetylase activity of HDAC6 [54]. p97 also interacts with HDAC6. On the one hand, p97 and HDAC6 appears to compete for binding to poly-ubiquitin conjugates to determine whether the ubiquitinated protein will be unfolded or aggregated [55]. On the other hand, expression of HDAC6 in cells harboring dominant-negative p97 mutation can facilitate

the sorting of aggregated Poly-Q proteins to autophagosomes for degradation [46,47], indicating that these factors act closely to promote the clearance of aggregated proteins by either the UPS or autophagy. In addition, p97 participates also in the dissociation of Hsp90-HSF1 complex by HDAC6 to promote HSF1-dependent induction of heat shock proteins [53]. Recently, Hao et al. reported an intriguing mechanism that at aggregated proteins, Rpn11 (a metalloprotease subunit that removes poly-ubiquitin chains from substrates en bloc [56]) of the proteasome produces unanchored ubiquitin chains [57]. Upon binding these unanchored ubiquitin chains, HDAC6 is activated, and subsequently deacetylate cortactin to promote the clearance of aggregated proteins. These studies provide intriguing example of the interaction between p62, HDAC6, and p97 in promoting the aggregation and clearance of poly-ubiquitinated proteins that ought to be investigated as a coherent system. Understanding of their synergistic action is still in its infancy and further knowledge would provide further guidance to therapeutically targeting these factors.

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