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Assays to monitor aggrephagy

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ABSTRACT

The presence of ubiquitinated protein inclusions is a hallmark of most adult onset neurodegenerative disorders. Results from several neurodegenerative model systems indicate that elimination of the disease-associated inclusions can lead to symptomatic reversal, and a better understanding of the mechanisms involved in accumulation and turnover of aggregation-prone proteins is therefore important. Autophagy has been found to contribute to protein aggregate clearance, and the term aggrephagy is used to describe the selective degradation of aggregation-prone proteins by autophagy. Here, we provide an overview of different disease-related model systems and assays that can be used to distinguish non-aggregated from aggregation-prone proteins, and how these assays can be used to determine turnover of protein aggregates by autophagy.

1. Introduction

Most neurodegenerative disorders, including tauopathies, synucleinopathies, TDP-43 proteinopathies and polyglutamine disorders, are characterized by the presence of ubiquitinated protein inclusions in affected cells. Whether such protein inclusions are produced to protect the host or are themselves toxic has been a matter of debate for a long time, but are most likely equally true. Abnormal protein folding is a major threat to cell function and viability, and can be caused by e.g., gene mutations, incomplete translation, misassembled protein complexes or post-translational damage, which again can be caused by various forms of cellular stress [1]. Several mechanisms have developed to prevent accumulation of toxic misfolded proteins, including upregulation of molecular chaperones to assist protein refolding and protein degradation.

Misfolded proteins are prone to expose hydrophobic or polar residues on their surface, which would normally be buried inside their structure, leading to aberrant interactions and triggering protein aggregation [2]. Aggregation of misfolded proteins leads to the formation of oligomeric intermediates, which again can develop into small protein aggregates. Such small protein aggregates can continue to grow and multimerize into larger aggregates or inclusions, and if such aggregates are not removed by the cellular degradation systems they can be transported to the microtubule-organizing center (MTOC) to form an aggresome, a structure encapsulated by a characteristic cage of intermediate filaments, known to sequester large amounts of aberrant proteins and believed to protect against their toxicity (Fig. 1) [1]. Transport to the aggresome requires the ubiquitin-binding microtubule deacetylase HDAC6 (histone deacetylase 6), which functions to link dynein motor proteins and poly-ubiquitinated proteins [1]. Indeed, cells lacking HDAC6 fail to clear protein aggregates but also to form proper aggresomes, making them sensitive to accumulation of misfolded proteins [1].

Interestingly, several studies have found that elimination of the aggregation-prone proteins causes symptomatic reversal in different neurodegenerative models [3]. It is therefore important to understand not only how such protein aggregates form, but also how they are potentially removed. Misfolded proteins can be degraded by the ubiquitin–proteasome system (UPS), through chaperone-mediated autophagy (CMA) or by macroautophagy (hereafter referred to as autophagy) (Fig. 2A). 80–90% of all proteins are degraded by the UPS, including short-lived, abnormal, denatured or damaged proteins [4]. However, proteins must unfold to fit into the proteolytic chamber of the proteasome, and this is not possible for highly aggregated proteins. Moreover, it is found that proteasome function is susceptible to disruption or blockage by several aggregation-prone proteins [1]. A decrease in proteasomal turnover is often compensated by an increase in autophagic turnover and importantly autophagy is able to degrade protein aggregates [5]. The importance of autophagy in protein homeostasis is further indicated by conditional knockout of core autophagy

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genes in mice, leading to accumulation of ubiquitinated aggregates and neuronal degeneration [1].

It is now evident that ubiquitinated protein aggregates can be selectively recognized and targeted for degradation by autophagy, in a process named aggrephagy (Fig. 1) [6]. During aggrephagy, ubiquitin-binding autophagy receptors like p62/SQSTM1, neighbor of BRCA1 gene 1 (NBR1), Nuclear dot protein 52 (NDP52, also called CALCOCO2), optineurin (OPTN) and Tollip recognize aberrant ubiquitinated proteins and facilitate their recruitment into autophagosomes by binding to mammalian Atg8 homolog proteins of the microtubule-associated protein 1 light chain 3 (MAP1LC3, hereafter called LC3) and GABA(A) receptor-associated protein (GABARAP) protein subfamilies [6–8]. These autophagy receptors bind to LC3 or GABARAP proteins in the autophagosomal membrane through an LC3-interaction region (LIR) [8,9]. Additionally, p62 is able to polymerize via its PB1 domain, increasing the total avidity towards the autophagic membrane and enabling formation of larger tightly packed inclusions of misfolded protein [10]. Indeed, experiments show decreased formation of larger ubiquitin-positive aggregates in p62 KO mice, while smaller aggregates can still be formed in the absence of p62 [1]. Ubiquitin-binding autophagy receptors are often utilized as markers for inclusion bodies found in cells of patients with various forms of proteinopathies. p62 is for example found in Mallory bodies in alcoholic liver disease and protein aggregates in neurodegenerative diseases [11]. Another key player in aggrephagy is the large autophagic adaptor protein ALFY. ALFY is recruited to protein aggregates through an interaction with p62 and is required for their efficient clearance by autophagy [12]. ALFY also interacts with phosphatidylinositol 3-phosphate (PI3P), GABARAP and ATG5 [12,13], indicating that ALFY functions in recruitment of the autophagic membrane and the machinery required for sequestration and elimination of ubiquitinated protein aggregates.

2. Models and markers

Stress and other physiological demands on protein homeostasis can result in accumulation of misfolded ubiquitinated proteins into aggregates of various composition and size in any cell type. In neurodegenerative diseases, protein inclusions are often produced by the accumulation of a single protein, where the most common neuronal proteinopathies are caused by accumulation of mutant α-synuclein, superoxide dismutase 1 (SOD1), tau, transactive response DNA-binding protein-43 (TDP-43) or a mutated protein with extended polyglutamine repeats [6]. It should, however, be pointed out that a single disorder can be characterized by multiple aggregation prone proteins, and that certain aggregation-prone proteins can be implicated across multiple diseases (for example, alpha-synuclein and tau). Because of their clinical relevance these proteins are often used as models in autophagy research. However, in most such studies it has not been distinguished between selective and non-selective autophagy, and it is therefore not clear if degradation of all these aggregation-prone proteins is facilitated by autophagy receptors. Furthermore, many studies lack controls to distinguish whether aggregate clearance is mediated by macroautophagy or by other lysosomal pathways (e.g., CMA). Moreover, it is sometimes not clear whether it is prevention of aggregate formation or the clearance of preexisting aggregates that is being demonstrated. In future studies it will be important to address these distinctions. Here we will discuss how this can be achieved. We start by introducing some cell based model systems of protein aggregation and their physiological relevance, and finally we provide a discussion about the different methods that can be used to measure protein aggregate clearance.

2.1. Drug-induced ALIS/DALIS/p62 bodies and aggresomes

2.1.1. Aggresomes

As mentioned above, aggresomes are major repositories for misfolded protein aggregates and can be artificially generated by treatment with proteasomal inhibitors and/or overexpression of aggregation prone proteins [14]. Thus, a common way to study aggresomes is through treatment with proteasomal inhibitors such as MG132 or bortezomib, which cause accumulation of misfolded proteins and acceleration of the formation of perinuclear aggresomes [14–16]. Aggresome formation can be verified with co-localization of markers like HDAC6, γ-tubulin, ubiquitin and vimentin [14].

2.1.2. ALIS/DALIS/p62 bodies

Dendritic cell aggresome-like induced structures (DALISs) are ubiquitin-positive structures that are transiently formed in professional antigen-presenting cells, like dendritic cells and macrophages, and play an important role in MHC class I presentation [17,18]. DALISs are distinct from aggresomes, as they are more transient and not dependent on transport along microtubules, although also formation of DALIS is stress-induced [17,18]. Structures indistinguishable to DALIS are formed in other cell types in response to stressors like puromycin, oxidative stress, starvation, and transfection and are referred to as aggresome-like induced structures (ALIS) [19]. A major component of ALIS is p62, and such aggregates are therefore also referred to as p62 bodies [6]. Although p62 bodies may represent a broader class of protein aggregates, it is demonstrated that in many cases p62 bodies and ALIS are the same, and that p62 in fact is essential for ALIS formation [6,20]. The degradation of ALIS and DALIS can be performed by both the proteasome and by selective autophagy, and both p62 and ALFY are shown to facilitate their selective degradation through autophagy [6,20].

The antibiotic puromycin is a common tool used to generate ALIS/p62 bodies in cells. Puromycin is mistakenly inserted into the ribosome and prematurely stops translation during protein synthesis, resulting in defective ribosomal translation products (DRiPs) containing the drug at their C-terminal end [21]. This causes the rapid formation of Ub- and p62-positive ALIS. The clearance of such ALIS/p62 bodies can be analyzed at various time points after puromycin wash-out, using immunostaining with a p62 antibody or differential detergent extraction followed by p62 Western blotting (Fig. 2B) [20].

2.2. PolyQ expanded proteins

Several neurodegenerative diseases, like Huntington's disease (HD) and spinocerebellar ataxia (SCA), arise from the expansion of an unstable CAG triplet repeat within the coding region of a given gene. This results in the expansion of a stretch of glutamine residues (polyQ) in the protein, which renders the host protein toxic mainly through unknown gain-of-function mechanisms. A common hallmark of these diseases is the presence of protein inclusions.

2.2.1. Huntingtin

HD is caused by an autosomal dominant mutation that introduces a polyQ expansion (>35 repeats) N-terminally in the protein Huntingtin (Htt), promoting formation of toxic oligomers and aggregates of the mutant protein. Autophagy has been found to be important for degradation of mutant Htt protein and furthermore to reduce the associated toxicity both in cell culture and in mouse, fly, and zebra fish models of HD [6]. Although most HD studies do not discriminate between selective and nonselective autophagy, we and others have demonstrated that components of the