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Protein aggregation propensity is a crucial determinant of intracellular inclusion formation and quality control degradation

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ABSTRACT

Protein aggregation is linked to many pathological conditions, including several neurodegenerative diseases. The aggregation propensities of proteins are thought to be controlled to a large extent by the physicochemical properties encoded in the primary sequence. We have previously exploited a set of amyloid β peptide (Aβ42) variants exhibiting a continuous gradient of intrinsic aggregation propensities to demonstrate that this rule applies in vivo in bacteria. In the present work we have characterized the behavior of these Aβ42 mutants when expressed in yeast. In contrast to bacteria, the intrinsic aggregation propensity is gated by yeast, in such a way that this property correlates with the formation of intracellular inclusions only above a specific aggregation threshold. Proteins displaying solubility levels above this threshold escape the inclusion formation pathway. In addition, the most aggregation-prone variants are selectively cleared by the yeast quality control degradation machinery. Thus, both inclusion formation and proteolysis target the same aggregation-prone variants and cooperate to minimize the presence of these potentially dangerous species in the cytosol. The demonstration that sorting to these pathways in eukaryotes is strongly influenced by protein primary sequence should facilitate the development of rational approaches to predict and hopefully prevent in vivo protein deposition.

1. Introduction

Protein aggregation and the long term cellular persistence of aggregates are pathological hallmarks of a large set of human disorders, the so-called conformational diseases, such as Alzheimer's (AD), Huntington's (HD) and Parkinson's (PD) diseases, and diabetes type II or transmissible spongiform encephalopathies [1,2]. Moreover, protein deposition is also a common phenomenon during recombinant expression in simple organisms such as unicellular fungi or bacteria [3,4]. Interestingly, the aggregates formed in these microorganisms resemble those involved in the onset of the aforementioned disorders [5–7], indicating that protein self-assembly into β-sheet enriched amyloid-like structures is a generic process in direct competition with native protein folding [8–10], regardless of the considered host. Indeed, it has been shown that the large majority of polypeptides display at least one and often multiple aggregation-prone regions, usually involved in the formation of the hydrophobic core [11,12]. As it occurs with folding, the aggregation propensity of proteins represents an essential property of the behavior of polypeptides encoded in their primary sequences [13–15]. Different bioinformatic algorithms have exploited this feature to predict protein deposition and amyloid formation by identifying and quantifying aggregation-promoting regions within a given sequence [16–19]. Despite their success in predicting in vitro aggregation, the challenge is asserting if the predictive power of these programs also applies in a more complex biological context [16]. Addressing this question is not trivial, since quantitative evaluation of the aggregation propensities of polypeptides in vivo is technically complex. In a first step toward this direction, we exploited in previous works the competition between protein folding and aggregation in the bacterial cytosol to approximate intracellular aggregation rates. Essentially, we generated 19 variants of the Aβ peptide (Aβ42) in which the original Phe residue in position 19 was substituted by the rest of natural proteinogenic amino acids. This residue is located in the central hydrophobic cluster (CHC), a short stretch comprising residues from Leu17 to Ala21 that controls, to a large extent, the aggregation propensity of the entire sequence. The 20 Aβ42 mutants were fused to the green fluorescent protein (GFP), which acts as a reporter of the aggregation propensity of the Aβ moiety and were recombinantly expressed in *Escherichia coli*. The expression of the different peptide variants in *E. coli* resulted in the formation of cytoplasmic amyloid-like inclusion bodies whose

Abbreviations: Aβ42, 42 residue-length amyloid β peptide; AD, Alzheimer's disease; ATG1, autophagy-specific gene 1; cfu, colony-forming units; CHC, central hydrophobic cluster; DMSO, dimethyl sulfoxide; EtOH, ethanol; GFP, green fluorescent protein; HD, Huntington's disease; PD, Parkinson's disease; PMSF, phenylmethanesulfonyl fluoride; PQC, protein quality control machinery; SC-URA, synthetic complete medium deficient for uracil

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GFP activity reflected the aggregation rate of the corresponding mutant [20,21]. These data constitute the core of AGGRESCAN, a method implemented in our group to predict protein aggregation propensity [22,23]. We have shown recently that in this model system the cellular fitness cost induced by protein deposition is tightly regulated by the intrinsic properties of the polypeptide chain, linking thus phenotype and sequence [24,25].

We have applied here the same approach in yeast to provide a quantitative assessment of the aggregation properties of proteins and how they correlate with sequential features in a more complex eukaryotic background. The budding yeast *Saccharomyces cerevisiae* is an excellent model system for the study of human cell biology in health and disease, since the basic features of eukaryotic cell biology evolved before the split between yeast and metazoans. This organism shares with higher eukaryotes numerous fundamental cellular pathways involved in neurodegeneration, such as protein quality control, membrane trafficking, autophagy or oxidative stress [26,27]. On this regard, humanized yeast models for AD, HD and PD have been successfully developed, recapitulating some of the pathological features associated with these disorders [28–34]. Here we analyzed the aggregation properties of the above mentioned Aβ42 mutants when expressed in yeast. In contrast to bacteria, where degradative pathways did not seem to affect the fate of these polypeptides, in yeast Aβ42 variants are actively cleared from the cytosol by the yeast protein quality control machinery. The intrinsic properties of the peptides determine both their in vivo intracellular aggregation and degradation. This model system should allow to rationalize and predict the impact of sequential changes in the deposition and degradation of amyloidogenic polypeptides in eukaryotic environments.

2. Material and methods

2.1. Yeast strain and plasmids

Yeast strain BY4741 (*MAT* a *his*3Δ1 *leu*2Δ0 *met*15Δ0 *ura*3Δ0) was transformed with pESC(-Ura) plasmids (Stratagene), encoding for the Aβ42–GFP fusion protein and the 19 variants differing in the 19th residue of Aβ42, as previously described [3]. Standard lithium/polyethylene glycol protocol was used for the transformation and a glucose selective synthetic complete medium deficient for uracil (SC-URA) was employed for plasmid selection.

2.2. Protein expression

Yeast cells were grown overnight in glucose SC-URA medium at 30 °C and 100 μL was used to inoculate 5 mL of fresh medium. At an OD_{600} of 0.5, cells were changed to a fresh raffinose SC-URA medium. After 30 min, cells were changed again to a fresh SC-URA medium containing 2% of galactose as a source of carbon to induce the recombinant protein expression. After 15 h at 30 °C, cells were harvested, washed in sterile water and pellets were stored at −80 °C for further analysis.

2.3. Microscopy

Cells were washed three times with sterile PBS and 5 μL was placed on top of microscopy glass slides and covered with coverslips. Images were obtained at a 40-fold magnification using an emission filter for GFP under UV light excitation in a Leica fluorescence microscope (Leica DMBR, Heidelberg, Germany).

2.4. Fluorescence measurements

Cell pellets were resuspended in PBS to an OD_{600} of 1. The emission spectra of GFP were recorded on a Cary Eclipse Spectrofluorometer (Agilent Technologies, Santa Clara, CA, USA) in the range 500–600 nm with a data interval of 1 nm and using an excitation wavelength of 488 nm. The experiments were carried out in triplicates.

2.5. Immunoblotting analysis

Cell pellets were resuspended in PBS. 200 μL of each mutant was prepared to an OD_{600} of 20 and 5 μL was used for total fraction Western-blots. For soluble/insoluble fraction analysis, 100 μL was centrifuged and resuspended in the same volume of Y-PER protein extraction reagent from Thermo Scientific (supplemented with a protease inhibitor cocktail tablet) to induce cell lysis. After 20 min of incubation at room temperature under mild agitation, mixtures were centrifuged at maximum speed for 30 min. Insoluble fractions were resuspended in 100 μL of PBS containing a protease inhibitor cocktail tablet and used for Western-blot analysis, together with 100 μL of the soluble fraction. 5 μL of each sample was loaded in a 14% SDS-PAGE and blotted onto a PVDF membrane. Immunodetection was performed using β-amyloid antibody 6E10 from Covance and membranes were developed with the ECL method. Densitometries were performed using ImageJ software. The experiments were carried out in triplicates.

2.6. Flow cytometry analysis

Cells expressing Aβ42*wt*–GFP and Aβ42F19E–GFP were harvested and washed three times in 0.22 μm filtered PBS. Flow cytometry measurements were performed using a FACSCanto flow cytometer (BD Biosciences, San Jose, CA, USA) equipped with a 488 nm blue argon laser. Gated cells (by means of FSC and SSC parameters) were analyzed for green emission measured on a 530/30 nm BP filter. Obtained data were analyzed using BD FACSDiva 4.0 software.

2.7. Cell viability assays

Overnight cultures of Aβ42*wt*–GFP and Aβ42F19E–GFP grown in SC-URA medium supplied with glucose (or galactose, for colony-forming units assay) were washed with sterile water and diluted to an OD_{600} of 0.8. The experiments were carried out in triplicates.

For determining the colony-forming units per mL (cfu/mL), 100 μL of serial dilutions (10^{-3}–10^{-7}) was plated in medium supplied with glucose and galactose and incubated at 30 °C for 3 days. Only plates containing between 30 and 300 colonies were monitored.

For spotting assays, 8 μL of serial dilutions (10^{-1}–10^{-4}) was spotted in glucose and galactose plates, subsequently incubated at 30 °C for 2 days. This experiment was carried out with all Aβ42–GFP mutants.

For growth curves, overnight cultures diluted in water were used to inoculate 250 μL of fresh medium containing glucose and galactose to a final OD_{600} of 0.15 in 96 well plates. Culture growth was monitored overnight at 28 °C in a Victor 3 Plate Reader (Perkin-Elmer, Inc., Waltham, MA, USA) measuring the OD_{595} every 15 min.

2.8. Proteolytic degradation analysis

Yeast strains specifically used in these assays were: BJ5459 (*MAT* a *ura*3-52 *trp*1 *lys*2-801 *leu*2Δ1 *his*3Δ200 *pep*4Δ::*HIS*3 *prb*1Δ1.6R *can*1 GAL) [35], and Δ*erg6* and Δ*atg1*, both in BY4741 background.

In experiments with chemical compounds, the cell wall permeable yeast strain, Δ*erg6*, transformed with pESC(-Ura) plasmids, was incubated overnight with galactose SC-URA medium. Prior to drug application, protein expression was arrested by changing medium from galactose to glucose supplemented. Incubation with drugs was performed for 4 h at 30 °C. The chemical compounds and the final concentrations used in these assays were: phenylmethanesulfonyl fluoride (PMSF) dissolved in ethanol (EtOH) at 1 mM, rapamycin dissolved in dimethyl sulfoxide (DMSO) at 100 nM and MG-132 dissolved in DMSO at 50 μM; all of them from Sigma-Aldrich. EtOH and DMSO