Orthogonal Cross-Seeding: An Approach To Explore Protein Aggregates In Living Cells[†]

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ABSTRACT: Protein aggregation is associated with the pathology of many diseases, especially neurodegenerative diseases. A variety of structurally polymorphic aggregates or preaggregates including amyloid fibrils is accessible to any aggregating protein. Preaggregates are now believed to be the toxic culprits in pathologies rather than mature aggregates. Although clearly valuable, understanding the mechanism of formation and the structural characteristics of these prefibrillar species is currently lacking. We report here a simple new approach to map the nature of the aggregate core of transient aggregated species directly in the cell. The method is conceptually based on the highly discriminating ability of aggregates to recruit new monomeric species with equivalent molecular structure. Different soluble segments comprising parts of an amyloidogenic protein were transiently pulse-expressed in a tightly controlled, time-dependent manner along with the parent aggregating full-length protein, and their recruitment into the insoluble aggregate was monitored immunochemically. We used this approach to determine the nature of the aggregate core of the metastable aggregate species formed during the course of aggregation of a chimera containing a long polyglutamine repeat tract in a bacterial host. Strikingly, we found that different segments of the full-length protein dominated the aggregate core at different times during the course of aggregation. In its simplicity, the approach is also potentially amenable to screen also for compounds that can reshape the aggregate core and induce the formation of alternative nonamyloidogenic species.

Accumulation of macroscopically observable, abnormal protein deposits is the cytological feature of many age-related neurodegenerative diseases, including Alzheimer's, Parkinson's, and Huntington's diseases (*1–3*). Although the characteristic pathological aggregates are primarily made up of the fibrillar aggregated form of the disease-associated protein, multiple aggregate morphologies are accessible to each individual amyloidogenic protein. The heterogeneity of aggregate morphologies may arise from (1) variations in the environment and aggregate growth conditions (*4–8*), (2) different segments of the primary sequence that govern the formation of the aggregate core (*8–13*), or (3) complex pathways of formation, including alternate aggregation routes or multistep assembly pathway(s), leading to multiple metastable intermediates (*14–20*). Current models propose that the species formed early in the course of aggregation, e.g., oligomers and protofibrils, are likely culprits in cytotoxicity and cell dystrophy (*21–25*). Conformation-specific antibodies that recognize only the oligomers (*26*) or bind only to the mature fibril state (*27*), and derived from amyloidogenic proteins of unrelated sequences, point to unique conformational epitopes present only in these states. This suggests that different moieties from a protein's sequence can be involved in the aggregate core and that the differing nature of intermolecular interfaces might be a rationale for the structural diversity of aggregated species, in turn reflecting the disease phenotype and determining the specific pathological features. Although clearly valuable, details of the structure of transiently formed intermediate species are presently lacking, particularly in the context of a cell, in large measure because of the technical difficulties due to their transient existence.

We have developed an orthogonal cross-seeding assay that can be applied directly to proteins expressed in cells in order to identify sequences or domains involved in the core of aggregates. Amyloid formation generally follows a nucleated-polymerization mechanism. Characteristic of this mechanism is the observation that preformed aggregates or seeds of the same protein can bypass the initiating energetically unfavorable nucleation event (*28*). Conceptually, the key to our approach is based on the highly discriminating ability of these seeds to recruit new monomers with equivalent sequences (*10, 29, 30*). The degree of sequence identity determines the efficiency of the seeding process and has been suggested to be crucial for the establishment of the long-range interactions that stabilize the core of the amyloid structures (*29*). To experimentally probe the nature of the aggregates of an amyloidogenic protein, the model expression host is cotrans-

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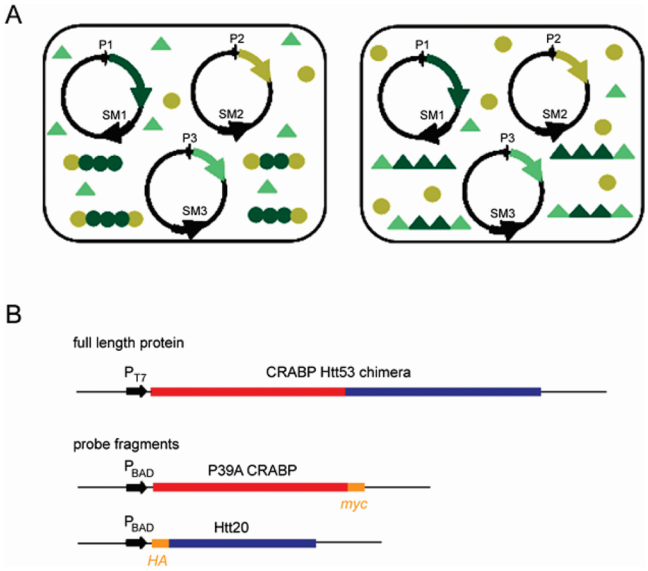

FIGURE 1: Principle of the *in vivo* cross-seeding approach. (A) The full-length aggregating protein (dark green) and segments of it (depicted in light green or dark yellow) are coexpressed on different plasmids under different promoters (marked P1, P2, and P3) and bear various selection markers (marked SM1, SM2, and SM3). Only one of the soluble probe fragments can be recruited to the various aggregates of the full-length amyloidogenic protein based on the adequate sequence similarity of the probe fragment to the aggregate core. (B) Schematic representation of the constructs used in this study. The expression of the amyloidogenic CRABP Htt53 chimera is under the control of the *T7* promoter. The two orthogonally expressed probe fragments P39A CRABP and Htt exon 1 with 20Q with a detection tag at either the N- or C-terminus represent the two domains of the full-length chimera, and their expression is under the control of the arabinose-inducible BAD-promoter. [All constructs with the CRABP domain contained a tetra-Cys motif for FlAsH binding, which we use as a read-out to follow the time-course of aggregation (*32, 33*); insertion of this motif does not alter properties of the CRABP.]

formed with multiple plasmids, one encoding the full-length amyloidogenic protein and others encoding peptide fragments comprising different parts of the full-length protein (Figure 1A). Provided there is tight control of the promoters, the synthesis of the probe peptides can be turned on and off in a tightly controlled, time-dependent manner. Recruitment of any of the probe fragments into the aggregates of the full-length parent protein will suggest that a portion of the full-length protein sequentially equivalent to the probe fragments comprises the aggregate core.

We used this approach to monitor directly in the cellular environment the structural evolution of the aggregate core of a chimera comprising a well-folded and stable β-sheet protein (cellular retinoic acid binding protein 1, CRABP[1]) fused N-terminally to exon 1 of huntingtin (Htt) containing a consecutive polyglutamine (polyQ) repeat in the pathological range (53Q), as implicated in the pathology of Huntington's disease (HD). At least nine slowly progressing hereditary neurodegenerative diseases, including HD, dentatorubral-pallidoluysian atrophy, and several spinocerebellar ataxias, are linked to an expanded unstable polyQ tract in otherwise unrelated disease proteins (*3*). The disease phenotype is manifest over a threshold value of ca. 35 glutamines, and the polyQ-dependent disease risk and tendency to aggregate are recapitulated both *in vitro* and in cell culture (*31*). Here, we show that the core of the aggregates of the CRABP Htt53 chimera evolves in the in-cell aggregation time course: early on, it is formed by the CRABP domain, and later, it is dominated by polyQ segments.

[1] Abbreviations: CRABP, cellular retinoic acid-binding protein I; HD, Huntington's disease; Htt, huntingtin; polyQ, polyglutamine; IPTG, isopropyl-β-thiogalactopyranoside.

EXPERIMENTAL PROCEDURES

Plasmid Constructs. DNA encoding mouse cellular retinoic acid-binding protein I (CRABP) with an N-terminal His-tag and an internal mutation that introduces a tetra-Cys-FlAsH dye binding site (previously described and used to directly monitor aggregation in cells (*32*)) was cloned under the control of the *T7* promoter in the pET16b (Am^R, Novagen) vector (*33*). The C-terminus was extended by the intact sequence of the full length Htt exon 1 with 53 CAG repeats (*32*). CRABP cloned in the pET16b plasmid was used as a template to introduce the amino acid substitution of Pro39 to Ala, and the resulting DNA encoding P39A CRABP was subcloned in-frame without a stop-codon into the pBAD/*myc* (Am^R, Invitrogen) plasmid. In a subsequent step, P39A CRABP with the C-terminal *myc*-tag (EQKLISEEDL) was subcloned into pBAD33 (Cm^R, ATCC 87402) vector under the control of arabinose-inducible promoter. The Htt exon 1 with 20 CAG repeats and with a 10-residue long, N-terminal *HA*-tag (YPYDVPDYA) was subcloned into the pBAD33 (Cm^R) behind the arabinose-inducible promoter. DNA encoding β_2m protein has been cloned under the *T7* promoter in the pET vector (a kind gift from Sheena Radford) (*4*). *E. coli* DH5α was used for all genetic manipulations, and all constructs were verified by DNA sequencing.

Growth Conditions and in Vivo Cross-Seeding. The full-length CRABP Htt53 (*T7*, IPTG-inducible promoter, Am^R) and either P39A CRABP (arabinose-inducible promoter, Cm^R) or Htt exon 1 with 20Q (arabinose-inducible promoter, Cm^R) were cotransformed in *E. coli* BL21(DE3) host, carrying the DE3 lysogen for high level expression of *T7* polymerase. For expression, only freshly transformed cells bearing the double antibiotic resistance were used. A single colony was used to inoculate LB medium containing 100 μg/mL ampicillin or 25 μg/mL chloramphenicol. Protein expression was induced either by adding isopropyl-β-thiogalactopyranoside (IPTG) (to 0.4 mM) for the *T7*-promoter based constructs or arabinose (to 0.2%) for the BAD promoter-controlled constructs. In the coexpression experiments, expression of the full-length CRABP Htt53 chimera or β_2m was induced by adding IPTG for 60, 120, 180, and 240 min after the culture reached $OD_{600} = 0.8$. IPTG was depleted by centrifuging the cells at 867*g* for 5 min and washing the cells twice with fresh medium. The cells were resuspended in the same volume of fresh medium supplemented with the corresponding antibiotics, and the expression of the short fragments was induced by adding arabinose (to 0.2%) for 30 min.

Slot-Blot Experiments and Immunodetection. Intact aggregates after the pulse-expression of the fragments were isolated from cells as described elsewhere (*34, 35*). The concentration of the aggregates was determined spectrophotometrically using the ϵ_{280} value of 21750 $M^{-1}cm^{-1}$ (*34*). Aggregates were diluted to 3 μM in 20 mM Tris·HCl at pH 7.5 containing 150 mM NaCl. Two-hundred microliters were

applied to a nitrocellulose membrane (Schleicher and Schuell) through a slot-blot manifold and washed three times with 200 μL of the same buffer. Immunodetection was accomplished using the *HA* (1:3000, Covance, Berkely CA) or *myc* antibodies (1:5000, Upstate Biotechnology). Visualization was accomplished via peroxidase-conjugated antirabbit or antimouse secondary antibody (Sigma Aldrich). The membranes were treated with 62.5 mM Tris·HCl at pH 6.7 containing 2% SDS and 100 mM β-mercaptoethanol for 30 min at 50 °C in a sealed container to strip the antibodies, rinsed three times with 10 mM Tris at pH 8.0 containing 150 mM NaCl and 0.05% Tween 20, and subsequently reprobed with polyQ antibody MW1 (1:1000 dilution) (Dev. Studies Hybridoma Bank, Iowa, USA), CRABP (1:1000, Abcam), or β_2m antibodies (1:5000 dilution) (Strategic Diagnostic, USA).

RESULTS

The Aggregate Core of CRABP Htt53 Evolves Structurally in the Time Course of Aggregation. In previous studies, we observed that the CRABP Htt53 chimera aggregates in a polyQ-length-dependent manner in a bacterial (*Escherichia coli*) *in vivo* model and that the aggregates evolved with time, from detergent-labile, small spherical aggregates into detergent-resistant fibrils (*32*). These morphologically varied aggregates initially could seed *ex vivo* the elongation of a slow folding variant of CRABP (*36*) and later the aggregation of Htt exon 1 with 20Q (*34*). Here, we sought direct in-cell evidence of how the involvement of different portions of the chimeric protein in the core of the aggregate correlated with the observed seeding properties *in vitro*. The full-length CRABP Htt53 protein was coexpressed in *E. coli* with either an *HA*-tagged Htt exon 1 with 20Q or a *myc*-tagged point mutant of CRABP, consisting each of the domains of the chimera (Figure 1B). The expression of CRABP Htt53 chimera was induced by adding IPTG for various periods and was terminated by depletion of the IPTG-inducer by changing to fresh medium. The *T7* promoter is almost completely repressed once IPTG is depleted (Supporting Information Figure 1A,B). Subsequent to turning-off the expression of the full-length chimera, the probe fragments were pulse-induced with arabinose. and their incorporation in the intact CRABP Htt53 aggregates was detected using the immunoreactivity of the corresponding tag. The expression strength of the P_{BAD} promoter ensures a relatively high synthesis pulse and allows a rapid increase of the concentration of either of the probe fragments within the test time of 30 min (Supporting Information Figure 1C). From the earliest time points tested, CRABP Htt53 aggregates efficiently recruited the CRABP species (anti-*myc* reacting), with the highest activity at the earliest time-point (60 min) (Figure 2). In contrast, the early aggregates showed almost no ability to stimulate polyQ-mediated elongation as evidenced by the lack of recruitment of the Htt exon 1 with 20Q (anti-*HA* positive species). The later time point CRABP Htt53 aggregates (240 min), for which we have detected a fibrillar phenotype (*34*), showed the opposite trend. They significantly enhanced the deposition of the polyQ-containing fragments (*HA*-positive) and were not able to recruit the *myc*-tagged CRABP mutant (Figure 2). Unlike the pathological variant with 53Q, the Htt exon 1 with 20Q is not aggregation-prone and remains

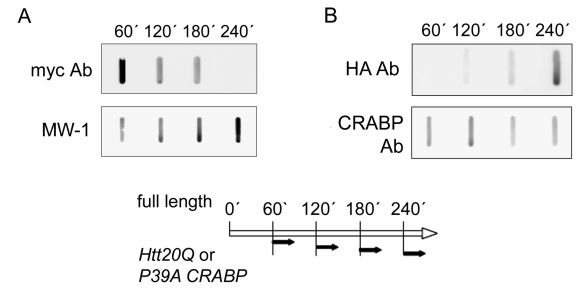

FIGURE 2: In the cellular environment, the seeding ability of aggregates formed by the CRABP Htt53 chimera evolves over time. *E. coli* BL21(DE3) cells were cotransformed with CRABP Htt53 and either P39A CRABP (A) or Htt exon 1 with 20Q. (B) At different times after induction (as indicated in the expression schemes), the expression of the CRABP Htt53 chimera was turned off, and probe fragments were pulse-expressed for 30 min. The recruitment of either P39A CRABP or Htt exon 1 with 20Q domain into the CRABP Htt53 aggregates was detected by immunoreactivity using *myc* or *HA*-antibodies, respectively. The charged membrane retained both soluble (oligomeric) species and insoluble aggregates. The same blots were also stripped and reprobed with CRABP antibody or the polyQ specific antibody MW-1 to validate that the aggregates arose from the CRABP Htt53 chimera. Although equal amounts of the aggregates CRABP Htt53 (3 μM) isolated at different times were loaded onto the membrane, their immunoreactivity towards the CRABP and MW-1 antibodies varies. We consistently observed lower MW-1 reactivity of the early species and lower anti-CRABP reactivity for aggregates isolated at later time, probably because of variations in the exposure of both epitopes ((*34*) and unpublished results). (Note that MW-1 binds preferably to elongated polyQ stretches and does not recognize short polyQ segments.)

soluble during the entire cycle of expression (data not shown). Therefore, its detection in the aggregates reflects their intrinsic ability to recruit polyQ species. Similarly, while the aggregation-prone mutant of CRABP partitions between the soluble and insoluble fraction at later time points of expression, in the first 60 min after induction, it is only present in the soluble fraction (*33*). [N.B. Soluble *HA*-tagged CRABP lacking the point mutation can be recruited into the aggregates with a CRABP core as well. However, the intensity of the resulting signal is lower (data not shown). The point mutation of CRABP used is P39A, which has been previously shown to cause an intermediate with delayed β-barrel closure to be populated and is as a result aggregation-prone (*36*). The mutant protein is, therefore, more efficiently recruited into the aggregates raising stronger readout signal.]

This approach is amenable to simultaneous pulse-induction of both probe fragments, provided that they bear different selection markers (as schematically represented in Figure 1A). Simultaneous induction of both probe fragments by adding arabinose (*HA*-tagged Htt exon 1 with 20Q bearing a kanamycin resistance and *myc*-tagged P39A CRABP with a chloramphenicol resistance) and subsequent parallel probing of the aggregates with *myc* or *HA*-antibodies showed the same trend observed for a single-expressed probe fragment with the CRABP Htt53 chimera (data not shown). In this case, finding only one of the two probe fragments in the CRABP Htt53 aggregates clearly reflects the intrinsic ability of the aggregate to recruit soluble species with related sequence.

This new approach to *in vivo* analysis of aggregate properties is based on pulse-expression of fragments com-

prising parts of the full-length aggregating protein, here Htt exon 1 with 20Q and a point mutant of CRABP. In order for this method to be valid, there must be sufficient expression of the probe fragments. The expression levels of both fragments and the full-length CRABP Htt53 chimera were assessed by SDS−PAGE (Supporting Information Figure 2). The use of strong promoters yielded substantial expression in a short time. Longer induction times are not recommended for the probe fragments, both because the morphology of the aggregate species could change within the time span of the expression and because longer expression may lead to aggregation of the probes themselves. The fusion with polyQ in the nonpathological range (CRABP Htt20) does not aggregate in the *E. coli* expression system (*32*), and its coexpression under conditions identical to those for the CRABP Htt53 with either of the fragments did not yield any detectable aggregates (data not shown).

The orthogonal cross-seeding experiments developed here strongly suggest that different regions of the full-length CRABP Htt53 chimera are transiently forming the aggregate core at different stages in the aggregation process. Thus, the primary player in the aggregate core evolves from CRABP in the in the early stages into polyQ in the late-forming fibrillar aggregates. Thus, the results presented here are fully consistent with our previous *ex vivo* limited trypsinolysis analysis, which we used to map the flexibility and accessibility of various regions of the CRABP Htt53 aggregates (*34*). The CRABP domain was more protected from proteolysis in the early, detergent-labile species, whereas the polyQ segment was less labile in the aggregates isolated at the later time point (*34*). Interestingly, these results are also entirely consistent with observations from two other laboratories on ataxin-3 aggregation (*15, 37*), establishing that polypeptide segments involved in the core aggregate structure can change during the time course of aggregation.

Specificity of the Cross-Seeding Approach. We next explored the promiscuity of the orthogonal cross-seeding approach and asked whether aggregates of a sequence-unrelated amyloidogenic protein, β_2-microglobulin, which is amyloidogenic and forms fibrils in patients suffering from hemodialysis-related amyloidosis (*38*), could also recruit the probe fragments. At different time points of expression of the aggregating β_2m protein under the control of the *T7* promoter (*4*), we pulse-expressed the probe fragments (Htt exon 1 with 20Q or P39A CRABP) for 30 min. Neither fragment was recruited into the β_2m aggregates, arguing that sequence similarity is a prerequisite for recruitment into aggregates (Figure 3).

Control experiments indicated that there were no nonspecific cross-seeding activities of both fragments (i.e., P39A CRABP monomers recruited into aggregates with polyQ-dominated aggregate cores, or vice versa). We prepared seeds from P39A CRABP or Htt exon 1 with 53Q *in vitro* and used them in cross-seeding reactions *in vitro*. P39A CRABP aggregates failed to shorten the lag time of aggregating Htt exon 1 with 53Q, and preformed aggregates of Htt exon 1 with 53Q did not seed P39A CRABP aggregation (Supporting Information Figure 3). In addition, the coexpression of both P39A CRABP and Htt exon 1 with 20Q led to no cross-reaction between the two species or formation of aggregates containing both proteins (data not shown). This strongly argues that sequence equivalence to the aggregate core is required for the probe fragment to elongate any of the aggregate species. Promiscuous cross-seeding would suggest substantial heterogeneity of the aggregate core within a single fibril. More broadly, such a result could account for how one amyloidogenic disease might influence or even promote another. The protein deposits, however, are typically dominated by a single primary amyloidogenic protein, which is consistent with the idea that the forces driving amyloid formation are highly discriminating.

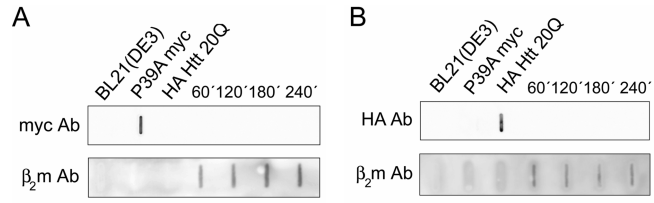

FIGURE 3: Aggregates of β_2m protein cannot recruit soluble P39A CRABP or Htt exon 1 with 20Q. *E. coli* BL21(DE3) cells were cotransformed with β_2m and either P39A CRABP (A) or Htt exon 1 with 20Q (B), and the same expression pattern of the constructs as in Figure 2 was used. The slot blots with the intact aggregates were probed with *myc* and *HA*-antibodies and reprobed after stripping with β_2m antibody. Cells expressing only P39A CRABP (depicted as P39A myc) or Htt exon 1 with 20Q (HA Htt20Q) were induced for 30 min by the addition of 0.2% arabinose, and total lysates were loaded as positive controls. Lysates of nontransformed *E. coli* BL21(DE3) cells (marked BL21(DE3)) served as a negative control.

CONCLUSIONS

Here, we describe an approach for the determination of the active components of the aggregate core of an amyloidogenic protein directly in cells. Our approach offers several advantages over the analysis of aggregates *ex vivo* (after isolation) or of *in vitro* grown aggregates: (1) the direct mapping of the time evolution of the nature of the aggregate core in the natural environment increases the likelihood of detecting metastable species whose transient nature hinders their detection *ex vivo*; (2) the crowded cellular environment may influence the dynamics of the physiologically relevant species, and it is difficult to reconstitute these effects in test tube experiments; (3) the cellular environment is dynamic and actively responds to environmental changes which could introduce new aggregate species or change the aggregation pathway. Finally, this cross-seeding method can be applied to study the active components of aggregates forms of large or unstable proteins whose purification is very inefficient.

We have optimized the orthogonal cross-seeding approach for the *E. coli* system where readily controlled, rapid, and high expression of the target proteins is straightforward. Nonetheless, this approach is not limited to the bacterial system. It can be transferred to mammalian cells so long as tightly controlled promoters are used, a sufficient amount of the proteins to be tested can be expressed in a short-time, and expression can be turned on and off effectively. In addition to the use of whole domains, we envision that shorter peptides can be also coexpressed to precisely map the residues comprising the aggregate core. The resulting insight into the active regions of an aggregate and the mechanism of recruitment into aggregates are crucial to identifying toxic species and pathological mechanisms. Elucidation of these active species and their mode of action opens new doors for developing therapeutic approaches to ameliorate misfolding and aggregation disorders. Short peptides

have been shown to be an attractive therapeutic strategy for the treatment in misfolding diseases (*39*). The method we have developed has the potential to be used to screen for compounds that modulate the aggregate core and redirect the aggregation process to the formation of alternative, nonamyloidogenic species.

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SUPPORTING INFORMATION AVAILABLE

Experimental methods and results about the expression level of the constructs, tightness of the regulation of the *T7* and P_{BAD} promoters, and nonspecific cross-seeding abilities of both fragments. This material is available free of charge via the Internet at http://pubs.acs.org.

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