Huntingtin Spheroids and Protofibrils as Precursors in Polyglutamine Fibrillation*

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The pathology of Huntington’s disease is characterized by neuronal degeneration and inclusions containing N-terminal fragments of mutant huntingtin (htt). To study htt aggregation, we examined purified htt fragments in vitro, finding globular and protofibrillar intermediates participating in the genesis of mature fibrils. These intermediates were high in β-structure. Furthermore, Congo Red, a dye that stains amyloid fibrils, prevented the assembly of mutant htt into mature fibrils, but not the formation of protofibrils. Other proteins capable of forming ordered aggregates, such as amyloid β and α-synuclein, form similar intermediates, suggesting that the mechanisms of mutant htt aggregation and possibly htt toxicity may overlap with other neurodegenerative disorders.

Huntington’s disease (HD)1 is an inherited, neurodegenerative disorder resulting from an expanded polyglutamine (poly(Q)) region in the N terminus of huntingtin (htt) (1–4). Individuals affected with HD have a poly(Q) stretch of 36 or more glutamines and an age of disease onset inversely correlated with the length of the expanded poly(Q) region (5). HD post-mortem brain examination reveals intranuclear and cytoplasmic neuronal inclusions and other deposits, consisting of fibrillar huntingtin aggregates (6–9). However, the presence of these large inclusions does not correlate well with neuronal death (10–14), suggesting that they may not be the toxic species. In vitro, expanded htt forms fibrillar aggregates with morphological and biophysical properties similar to those formed by amyloid β (Aβ) peptide (15). The aggregation threshold in vitro mimics the glutamine length threshold for the disease phenotype (16), suggesting that an abnormal conformation of expanded htt plays a role in disease pathogenesis. Recently, Thompson and colleagues (17) have designed polypeptides that bind mutant htt, suppress aggregation in vivo, and reduce pathology in both cell and animal models of poly(Q) disease. It is possible that the pathway from soluble htt to fibrillar aggregate is a multistep process, with the toxic species formed before the mature fiber. To better understand this pathway, we have characterized the morphological and structural features of poly(Q)-mediated htt fibrilization using biochemical and biophysical techniques, including SDS-PAGE, transmission electron microscopy (TEM), Fourier transform infrared (FTIR) spectroscopy, and atomic force microscopy (AFM).

EXPERIMENTAL PROCEDURES

Plasmid Construction—Plasmids encoding huntingtin exon 1 (htt exon1) proteins with either 16 or 44 consecutive glutamine residues were prepared using a multistep PCR-based and synthetic DNA approach (18). The 5′ and 3′ regions of htt exon1 DNA were individually cloned into the Bluescript vector (Stratagene; 5′/H9252 and 3′/H9252) peptide (15). The aggregation threshold for htt exon1 cDNA with a 42-base pair linker between the 5′ and 3′ regions. To generate the 16Q htt exon1 construct, an oligonucleotide comprised of a mixed CAG/CAA sequence and encoding for 14 glutamine residues was inserted into the modified htt exon1 cDNA using the BseRI and BglI restriction sites flanking the synthetic linker. To prepare the 44Q htt exon1 construct, two additional rounds of PCR were carried out to add a total of 30 CAG/CAA codons to the repeat region. The CAG/CAA oligonucleotide was then inserted into this modified vector to yield the final 44Q htt exon1 construct. Finally, both 16Q and 44Q inserts were subcloned into a modified pMAL vector (New England Biolabs) using SfiI and NotI restriction sites to allow for expression as MBP fusion proteins.

Expression and Purification of Recombinant Proteins—MBP-htt exon1 fusion proteins were expressed in Escherichia coli, grown to an OD600 of 0.6–0.8, induced with 0.3 mM (final concentration) isopropyl-β-D-thiogalactopyranoside (Calbiochem) for 2 h at 28 °C, and harvested by centrifugation for 15 min at 5,000 rpm. Cell pellets were resuspended in lysis buffer (phosphate-buffered saline (PBS), pH 7.4, supplemented with 10 mM methionine, 2 mM EDTA, 5 mM dithiothreitol, 1 mM 4-2-aminoethylbenzenesulfonyl fluoride (ICN Biochemicals), and Complete protease inhibitor mixture (Roche Molecular Biochemicals)), lysed by French pressure, and clarified by centrifugation at 13,000 rpm for 15 min. MBP fusion proteins were purified by incubation of the lysate supernatant with amylose resin (New England Biolabs) in PBS, pH 7.4, for 30 min at 4 °C. Following several washes, MBP fusion proteins were eluted with PBS supplemented with 10 mM maltose and further purified by nickel chelate chromatography. Purified fusion proteins were analyzed by SDS-PAGE and visualized by Coomassie staining.

Antibodies—A goat polyclonal antibody was prepared against the N-terminal htt exon1 fragment as described previously (19). Anti-MBP was purchased from New England Biolabs and expanded polyglutamine

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FIG. 1. A conformational change in htt exon1–44Q following removal of an N-terminal MBP affinity tag. A, schematic of MBP-htt exon1 recombinant proteins. A factor Xa cleavage site is located between the MBP and htt exon1 domains. MBP is not drawn to scale. B, SDS-PAGE analysis of MBP-htt exon1 following bacterial expression and purification. Left panel, purified fusion protein following amylose (1) and nickel (2) affinity chromatography. Middle and right panels, Western blots using anti-htt exon1 or 1C2 antibody. While both normal and expanded htt exon1 fusion proteins are recognized by a htt-specific polyclonal antibody, only the expanded fusion protein is immunoreactive with monoclonal 1C2 antibody. C, Western blot analysis of MBP-htt cleavage reaction (see “Experimental Procedures”). Digested proteins were resolved by SDS-PAGE and visualized by Western blotting using the indicated antibodies and a chemiluminescence detection system (Renaissance enhanced luminol reagent). The antibody to MBP demonstrates that proteolytic cleavage is complete. MBP-cleaved htt exon1–44Q is recognized by a htt antibody. However, 1C2 immunoreactivity is lost following cleavage of the fusion protein. htt aggregates at the top of the gel (arrow) are also recognized by anti-htt but not by 1C2.

FIG. 2. Evidence for a morphological and structural intermediate in poly(Q)-mediated htt fibrilization. A–H, time course of factor Xa cleavage reaction as monitored by TEM. MBP-htt exon1 44Q prior to addition of enzyme (control) is shown in A. Distinct globular features likely to represent htt oligomers are visible after a 30-min incubation with enzyme (not shown) and become abundant at 2 h (B) and 3 h (C) post-MBP cleavage. The smallest particles (arrowheads) are 4–5 nm in diameter. Larger particles are also observed and may represent aggregates of small particles. At 4 h, fibers (arrows) begin to form. Oligomers and fibers co-exist at 4, 5 (E), and 6 (F) h. By 7 and 8 h (G, H) only fibers are present. The fibers appear to take on a more uniform morphology and can be seen bundled together. Bars, 200 nm. I and J, second derivative FTIR spectra of factor X-treated MBP-htt exon1–44Q fusion protein. Fiber formation at 7 h (I) is accompanied by a large increase in β-sheet structure (1,620-cm⁻¹ band, likely resulting from intermolecular β-sheet (23)). A change in poly(Q) structure is also observed at 2 and 3.5 h (J), with the appearance of two distinct IR bands in the 1,620–1640-cm⁻¹ region (indicated by large arrow and resulting from β-sheet and/or β-turn) (23). This change coincides with the appearance of htt oligomers, as shown in C.
selective monoclonal antibody 1C2 was obtained from Chemicon International.

**Preparation of htt Exon1 for Fibrillation Studies**—Purified fusion proteins were dialyzed against 50 mM Tris-Cl, pH 8.0, 100 mM NaCl, 5 mM CaCl₂, and 10% glycerol. Final protein concentrations were estimated using Bio-Rad protein assay with bovine serum albumin as a standard. To remove the MBP affinity tag and induce aggregation of htt exon1–44Q, dialyzed fusion protein (0.25 mg/ml) was digested with factor Xa protease (Novagen; 1 μg of enzyme:25 μg of protein) at room temperature. At various time points, aliquots were removed and mixed with SDS sample buffer to terminate the cleavage reaction. Digest reactions were analyzed by SDS-PAGE (4–15%) and Western blotting using anti-htt exon1, anti-MBP, or 1C2 antibodies.

**TEM**—MBP-htt digest reactions were carried out as described above. For each time point analyzed, a 5-μl aliquot was removed and flash frozen in liquid N₂. Prior to TEM analysis, freshly thawed samples were applied to a carbon-coated copper grid and negatively stained with 2% uranyl acetate. TEM imaging was carried out using a Zeiss-EM10 equipped with a Gantan CCD camera.

**FTIR Spectroscopy**—A Nicolet model 8210 FTIR spectrometer equipped with a zinc selenide attenuated total reflectance accessory and deuterated triglycerine sulfate (DTGS) detector was used to detect spectral recordings at room temperature. The spectrometer was purged with liquid nitrogen for 4 h before recording spectra. For each spectrum, a 256-scan interferogram was collected at a resolution of 4 cm⁻¹. For each reading, the single beam spectrum of the buffer and protein solutions were divided by the background single beam spectrum, before conversion to absorbance spectra. The final protein spectra were obtained by subtraction of the buffer spectra and smoothed using a 7-point Savitsky-Golay algorithm. For all protein spectra, the area of amide I region (1,800–1,700 cm⁻¹) was normalized to one. To compare secondary structural changes in different samples, a second derivative calculation was performed using Grams/386 software (Galactic Industries Corp.) as described previously (20).

**AFM**—Prior to analysis, a 5-μl aliquot of digested 44Q protein was deposited onto freshly cleaved ruby mica (Mica, New York) and allowed to adsorb for 30 s. The sample was washed with double distilled water, dried with nitrogen gas, and imaged in air with a Nanoscope III microscope (Digital Instruments, Inc., Santa Barbara, CA) operating in tapping mode as described previously (21). Images were flattened with a first-order or second-order fit using Digital Instruments software.

**RESULTS**

Recombinant htt Expression and Purification—For these studies, we prepared MBP-tagged htt exon1 fusion proteins with a normal length (16Q) and a pathological length (44Q) poly(Q) region (Fig. 1A). A His₉ tag was engineered at the C terminus of the fragment to allow isolation of full-length exon 1 protein. SDS-PAGE followed by Coomassie staining confirmed that the recombinant htt exon1 fusion proteins were >99% pure following the second chromatography step (Fig. 1B, left panel, lane 2). Western blot analysis demonstrated that both normal length and pathological length htt exon1 proteins were recognized by an anti-huntingtin antibody (Fig. 1B, middle panel). However, only the htt exon1–44Q protein was immunoreactive with 1C2 (Fig. 1B, right panel), an antibody previously shown to selectively recognize expanded poly(Q) tracts.

A Conformational Change in 44Q Monomer following Removal of MBP Affinity Tag—Previous studies have shown that following removal of the N-terminal glutathione S-transferase tag, a htt exon1 fragment with an expanded poly(Q) region aggregates in a time- and poly(Q) length-dependent manner (16). MBP-htt exon1 fusion proteins were treated with factor Xa to remove the affinity tag and induce expanded htt aggregation. The cleavage reaction was monitored by Western blotting as shown in Fig. 1C. Analysis of the digests with an anti-htt exon1 antibody showed that the htt 44Q protein formed high molecular weight SDS-insoluble aggregates at later time points in the cleavage reaction while the 16Q protein did not. These observations are consistent with previous studies and confirm that expanded but not normal length poly(Q), in the context of htt exon1, aggregates following removal of an affinity tag. Interestingly, reactivity to 1C2 was eliminated after MBP cleavage, even though the cleaved htt protein is still recognized by an anti-htt antibody. This change in immunoreactivity is unaffected by boiling in SDS and suggests that a
Coincident Increase in β-Structure—To assess morphological and structural changes in the expanded poly(Q) region of htt exon1, we monitored the MBP cleavage reaction of 44Q by TEM and FTIR spectroscopy (Fig. 2). As early as 30 min following addition of protease, small globular assemblies with a diameter of 4–5 nm were visible by TEM (not shown). These globular features increase in number at longer incubation times of 2 and 3 h (Fig. 2B, and C, respectively). At later time points between 4 and 6 h (Fig. 2, D–F), amyloid-like fibers were visible and co-exist with the spherical assemblies. Interestingly, the number of globular features decreased with a coincident increase in fibers. By 7 h post-MBP cleavage (Fig. 2G), only fibers were visible. Neither globular assemblies nor fibers were observed in a control sample not treated with factor Xa (Fig. 2A). FTIR analysis of the cleavage reaction products revealed no evidence of the β-structure within 44Q in the 1,620–1,640-cm⁻¹ region (22, 23) at time 0 (Fig. 2I). However, a large increase in the 1,620-cm⁻¹ band, likely representing intermolecular β-structure (23), was observed 7 h following addition of enzyme. This β-structure coincides with the assembly of htt fibers. At earlier time points when there were exclusively or predominantly oligomers (2 and 3.5 h, respectively; Fig. 2J), two distinct IR bands were observed in the β-region (1,620–1,640 cm⁻¹). These peaks may arise from an increase in β-sheet and or β-turn structure (23), suggesting that at a time coinciding with the appearance of globular oligomers, 44Q adopts a β-structure, which may differ from that of the fibers.

Congo Red Prevents the Formation of htt Fibers, Leading to Accumulation of Protodisribil—Previous work has demonstrated that Congo Red (CR), a dye used to assay for amyloid fibers, is also an inhibitor of poly(Q)-mediated htt fibrillization (24). To determine the effect of CR on the fibrillization of htt exon1-44Q, we treated the purified protein with increasing concentrations of CR 30 min after addition of factor Xa protease. Aliquots were removed at 4, 8, and 24 h, subjected to SDS-PAGE, and visualized by Coomassie staining (Fig. 3A). Monomeric htt exon1 44Q was visible in all samples 4 and 8 h after the addition of enzyme. After overnight incubation with 2.5 and 10 μM CR, monomeric 44Q protein was visible even after an overnight incubation with factor Xa, demonstrating that SDS-insoluble htt fibers were not formed under these conditions. These observations support previous data that CR blocks poly(Q)-mediated fibrilization (24). In contrast, no monomeric htt was detected in the control or 0.25 μM CR conditions. By this time, all monomeric 44Q protein had presumably been incorporated into SDS-insoluble fibers and remained at the origin of electrophoresis (data not shown).

To visualize these effects at a morphological level, we imaged CR-treated 44Q samples by TEM (Fig. 3B and C). Large fiber bundles were visible in the control sample (Fig. 3B). In contrast, numerous small individual htt fibrils were visible in the CR-treated sample (Fig. 3C). Based on SDS-PAGE analysis, these fibrils were not resistant to SDS denaturation. Moreover, the individual fibrils were thinner than those making up the fiber bundle, with a diameter of 4–5 nm, compared with 10–11 nm for the control. Upon closer inspection of the control sample, we observed that at the ends of some fiber bundles were small fibrils 4–5 nm in diameter. These 4–5-nm fibrils may represent incomplete growth at the end of the fiber bundle. A possible explanation for these observations is that fiber assembly is preceded by the formation of smaller fibrils or SDS-sensitive protodisribil.

**AFM Analysis of htt Fibrilization**—To further investigate the poly(Q)-mediated htt fiber assembly pathway, we carried out AFM analysis of MBP-htt exon1–44Q before and after removal of the affinity tag. Before MBP cleavage, the fusion protein

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**Fig. 4.** AFM analysis of htt fibers provides further evidence for an oligomer–protodisribil–fiber pathway of assembly. A and B, prior to addition of factor Xa, the fusion protein appears as a small, globular feature (MBP; height = 1.2 nm) with an extended tail (arrows), likely to represent htt exon1–44Q (400 nm scan size) (A). Pictured in B is MBP-htt exon1–44Q following an overnight incubation with enzyme. Two large fiber bundles are shown (2 μm scan size). **Inset:** AFM surface plot depicting end of fiber bundle (200–500 nm scan size). Individual fibers (arrows) have a height of 10–11 nm and display a segmented morphology. At the end of some fibers were thinner fibrils (arrows) with a height of 4–5 nm.
appear by AFM as a globular feature with a height of 1.2 nm, corresponding to the MBP affinity tag, and a "tail" feature likely to represent the htt exon1 fragment (Fig. 4A, arrows). After an overnight incubation with factor Xa, large fiber bundles were visible by AFM (Fig. 4B). These were not observed for the htt exon1–16Q fragment, consistent with previous findings that a poly(Q) tract of 16Q does not form amyloid fibers in the context of the htt exon1 fragment. Individual fibers with a height of 10–11 nm were visible at the ends of the fiber bundle, some of which displayed a segmented "beads on a string" morphology (Fig. 4B, inset, arrowheads). Upon careful inspection of the close-up images, we observed thinner fibrils at some of the bundle ends with a height of 4–5 nm (Fig. 4B, inset, arrows). These height data are consistent with the data observed for the close-up images, we observed thinner fibrils at some of the bundle ends with a height of 4–5 nm (Fig. 4B, inset, arrows).

**DISCUSSION**

A possible model, based on the current data, is shown in Fig. 5. Before removal of the affinity tag, htt exon1 is soluble with an unstructured poly(Q) region. These observations are consistent with previous structural studies suggesting that poly(Q) is unstructured in solution (25–27). After removal of the affinity tag, the expanded htt construct begins to adopt β-structure via conformational changes in the expanded poly(Q) and forms globular assemblies likely to be intermediates in the fibrilization pathway. Over time, the globular oligomers can associate linearly to form single protofibrils. These protofibrils are not visible as isolated entities, unless Congo Red is present to prevent their association. Importantly, the protofibrils are SDS-soluble, unlike mature htt fibers. Mature fiber formation may result from the association or concurrent growth of two protofibrils or by addition of oligomers to a single protofibril; our data cannot distinguish between these possibilities.

In the present studies, we show by electron microscopy and AFM that expanded poly(Q) in the context of htt forms globular and protofibrillar species. Such observations are reminiscent of previous studies on other amyloidogenic proteins such as Aβ, α-synuclein, and yeast (prion) Sup35 NM peptide (28–30). Morphological characteristics of these species are remarkably similar despite the variation in amino acid composition and molecular size among the different proteins. Recently, Wetzel and colleagues (31) have generated two monoclonal antibodies that recognize fibrillar Aβ as well as poly(Q) and other amyloid-like fibers. Taken together, these data suggest that similar structures are formed upon conversion from the normal to the mutant state and are likely to form by similar mechanisms. Globular oligomers may represent early prefibrillar intermediates of fibrilization. By FTIR spectroscopy, we detected the appearance of β-structure that coincides with the appearance of globular oligomers, demonstrating that htt adopts secondary structure early in the fibrilization pathway. Congo Red, a dye commonly used to assay for amyloid fiber formation, prevented the assembly of mature htt fibers, possibly by binding to the β-structure of the protofibril.

Studies of both Aβ and α-synuclein toxicity have suggested that globules and/or protofibrils may be more toxic than mature fibers (32) and that stabilization of protofibrillar intermediates may promote disease pathogenesis (33). Recent experiments show that oligomers or other prefibrillar aggregation intermediates are toxic in several models (34, 35). Regardless of the toxic species, there is evidence that structural changes take place before the appearance of mature fibers and that a strong correlation exists between these alterations and neurotoxicity. Based on the similarities between htt fibrillation and that of other amyloid-forming proteins, we propose that HD toxicity is also governed by such structural alterations. Characterization of these changes may allow for the rational design of therapeutics at multiple steps in htt fibrillation and, by analogy, for other neurodegenerative diseases.

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