Identification of Novel Potentially Toxic Oligomers Formed in Vitro from Mammalian-derived Expanded huntingtin Exon-1 Protein

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Huntington disease is a genetic neurodegenerative disorder that arises from an expanded polyglutamine region in the N terminus of the HD gene product, huntingtin. Protein inclusions comprised of N-terminal fragments of mutant huntingtin are a characteristic feature of disease, though are likely to play a protective role rather than a causative one in neurodegeneration. Soluble oligomeric assemblies of huntingtin formed early in the aggregation process are candidate toxic species in HD. In the present study, we established an in vitro system to generate recombinant huntingtin in mammalian cells. Using both denaturing and native gel analysis, we have identified novel oligomeric forms of mammalian-derived expanded huntingtin exon-1 N-terminal fragment. These species are transient and were not previously detected using bacterially expressed exon-1 protein. Importantly, these species are recognized by 3B5H10, an antibody that recognizes a two-stranded hairpin conformation of expanded polyglutamine believed to be associated with a toxic form of huntingtin. Interestingly, comparable oligomeric species were not observed for expanded huntingtin shortstop, a 117-amino acid fragment of huntingtin shown previously in mammalian cell lines and transgenic mice, and here in primary cortical neurons, to be non-toxic. Further, we demonstrate that expanded huntingtin shortstop has a reduced ability to form amyloid-like fibrils characteristic of the aggregation pathway for toxic expanded polyglutamine proteins. Taken together, these data provide a possible candidate toxic species in HD. In addition, these studies demonstrate the fundamental differences in early aggregation events between mutant huntingtin exon-1 and shortstop proteins that may underlie the differences in toxicity.

Huntington disease (HD)3 is an inherited neurodegenerative disorder caused by an expanded polyglutamine (polyQ) region in the N terminus of the HD gene product, huntingtin (htt), a large protein over 3,000 amino acids in length (1–3). Individuals affected with HD have a polyQ region of 36 or more glutamine residues (4), and clinical studies have shown an inverse correlation between polyQ length and age of disease onset (5, 6). Recent work indicates that Htt may undergo proteolysis, generating several truncation products (7, 8). One of the smallest products is an N-terminal fragment that corresponds to the first exon of the HD gene and is comprised of the first 90 amino acids of Htt (based on a polyQ region of 23 glutamine repeats (3)). Htt exon-1 is of particular interest, as it is believed to be a mediator of toxicity in animal models of HD and in HD patients. HD is characterized by the deposition of large intracellular protein aggregates, or inclusion bodies, comprised of N-terminal fragments of mutant Htt. In HD, there is a strong correlation between polyQ repeat length and the threshold for aggregation and disease (4). While inclusions are a pathological hallmark of disease, inclusion formation does not correlate well with pathogenesis in vivo (9–12). The serendipitous development of an HD transgenic mouse expressing human Htt truncated at amino acid 117 by a stop codon, and referred to as shortstop, provided additional support for these earlier observations. Despite the formation of frequent and widespread inclusions, expanded Htt shortstop transgenic mice displayed no evidence of behavioral abnormalities or neurodegeneration.

This article contains supplemental Figs. S1–S7.

The abbreviations used are: HD, Huntington disease; polyQ, polyglutamine; Htt, huntingtin; Aβ, amyloid β; GST, glutathione S-transferase; NEAA, non-essential amino acids; AEBSF, 4-(2-aminoethyl)benzenesulfonfyl fluoride; TEM, transmission electron microscopy; AFM, atomic force microscopy; Hsp70, heat shock protein 70; AGERA, agarose gel electrophoresis for resolving aggregates.

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observed for other HD transgenic mouse models (13). A more recent study in a cell culture model of Htt aggregation and toxicity confirmed these data (14), and, taken together, strongly suggest that inclusion bodies are not the main toxic species in HD. At present, formation of inclusions is thought to represent a cellular protective response to aggregation (15–17).

While there is some controversy, increasing evidence suggests that soluble oligomeric assemblies of Htt formed early in the aggregation process may be responsible for toxicity. Previous studies in a number of other protein aggregation disorders have involved the characterization and isolation of soluble oligomeric assemblies of the respective disease-related protein (18–20). In particular, those formed by amyloid-β (Aβ) peptide in Alzheimer disease were found to cause neuronal death in cell culture and block hippocampal long-term potentiation (21). Soluble assemblies of Htt have been identified and characterized using bacterially expressed recombinant Htt protein in vitro (22–26), and more recently from mammalian cell lines (25, 26), from brain homogenates of HD mice (26, 27), and from human brain extracts (26). In a recent study, globular oligomers isolated from the brains of R6/2 transgenic mice were found to be identical to those formed by recombinant expanded Htt exon-1 in vitro (27). These data provide evidence for Htt oligomers in vivo and support the idea that soluble oligomeric forms of expanded Htt could play a role in HD pathogenesis.

While expanded Htt exon-1 aggregation has been studied extensively in vitro, previous studies have been carried out with bacterially expressed protein. Aggregation of expanded Htt shortstop has not yet been investigated in vitro. In the present study, we have established an in vitro system to generate recombinant Htt exon-1 and shortstop proteins in mammalian cells. To investigate the biochemistry of early stages of Htt exon-1 and shortstop aggregation in vitro, we have used both SDS-PAGE and Blue native-PAGE, a non-denaturing gel analysis system carried out in the absence of SDS. Using these techniques, we have found differences in SDS-stable and native soluble oligomeric aggregation species detected for toxic expanded Htt exon-1 and non-toxic expanded Htt shortstop. In parallel, we have carried out cell toxicity analysis of Htt exon-1 and shortstop proteins in cultured primary cortical neurons and show that expanded Htt shortstop is not toxic. Taken together, these data confirm that expanded Htt shortstop is not toxic and suggest a possible mechanism via differences in oligomer formation during early aggregation events.

**EXPERIMENTAL PROCEDURES**

*Plasmid Construction*—To generate Htt exon-1 44Q for expression as a glutathione S-transferase (GST)-fusion protein in HEK293-FT cells, a cDNA insert was subcloned from pMAL Htt exon-1 44Q bacterial expression construct (22) using BamHI and NotI restriction sites into a modified pEBG-2T vector (generously provided by Dr. Dario Alessi) altered to incorporate these restriction sites. Htt shortstop 44Q was prepared by cloning a synthetic DNA fragment into the modified pEBG-2T vector using BamHI and NotI restriction sites. Htt exon-1 and shortstop constructs with wild-type (9Q) and expanded (82Q) polyQ regions used for cell toxicity studies and confocal microscopy analysis in primary cortical neurons were prepared as previously described (14).

*Antibodies*—Antibodies used for SDS- and Blue native-PAGE include anti-Htt exon-1 (1:2000), MW1 (1:10,000), 3B5H10 (1:2000), anti-Htt amino acid 1–17 (anti-Htt aa 1–17; 1:2500), and anti-Htt amino acid 81–90 (anti-Htt aa 81–90; 1:5000). Anti-Htt exon-1, a goat polyclonal antibody, was generated against an Htt exon-1 internal deletion mutant as previously described (28). MW1, a mouse monoclonal IgG selective for an expanded polyQ domain, was prepared as previously described (29). 3B5H10, a mouse monoclonal IgG selective for expanded polyQ, was described previously (30). Anti-goat, anti-rabbit, and anti-mouse peroxidase-linked secondary antibodies were obtained from GE Healthcare and used at a concentration of 1:5000. For immunocytochemistry studies, a mouse monoclonal IgG generated against Htt amino acids 1–82 (anti-Htt 1–82) was obtained from Millipore and used at a concentration of 1:1500, followed by an anti-mouse-cy3 secondary antibody (1:300).

*Cell Culture and Transfection*—HEK293-FT cells (Invitrogen) were maintained in high glucose Dulbecco’s Modified Eagle Medium (DMEM, Invitrogen), 10% fetal bovine serum, 0.1 mM MEM Non-Essential Amino Acids (NEAA, Invitrogen), 6 mM L-glutamine, 1 mM MEM sodium pyruvate, 1% penicillin-streptomycin, and 500 μg/ml geneticin. Twenty-four hours prior to transfection, cells were seeded to obtain a final confluence of 80–90% in medium lacking geneticin. Transfections were carried out with Lipofectamine 2000 (Invitrogen) using standard manufacturer’s protocols, with the exception that cells were transfected in HEK293-FT maintenance medium lacking geneticin, rather than in Opti-Minimal Essential Medium, and that transfection reagent/DNA mixtures were not removed until the cells were harvested 48 h following transfection.

*Purification of Recombinant Mammalian-derived GST-Htt Exon-1 and Shortstop Fusion Proteins*—HEK293-FT cells transfected with pEBG-2T Htt exon-1 and shortstop plasmids were washed twice with cold PBS and harvested by centrifugation at 1.7 K RPM for 5 min 48 h after transfection. For preparation of GST-Htt fusion proteins under native conditions, cells were resuspended with lysis buffer (1× PBS pH 7.4, 2 mM EDTA, 1% Triton X-100, 1 mM AEBSF, Complete, mini protease inhibitor mixture tablets (1 tablet/17 ml, Roche), and 0.1% β-mercaptoethanol) and clarified by centrifugation at 15K RPM for 15 min at 4 °C. Soluble material was added to a glutathione-Sepharose column (GE Healthcare) equilibrated with lysis buffer. The column was washed once with 1 column volume of wash buffer 1 (1× PBS pH 7.4, 0.05% Tween 20, 0.1% β-mercaptoethanol) supplemented with 5 mM ATP, followed by 2 column volumes of wash buffer 1 without ATP. GST-Htt fusion protein was eluted with 50 mM Tris-HCl, pH 7.4, 20 mM reduced glutathione, 0.05% Tween 20, and 0.1% β-mercaptoethanol. Eluted GST-Htt exon-1 and shortstop fusion proteins were dialyzed against thrombin cleavage buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2.5 mM CaCl₂, 0.1% β-mercaptoethanol) and quantified using SDS-PAGE followed by Coomassie staining using bovine serum albumin (BSA) as a standard. For preparation of GST-fusion proteins under denaturing
conditions, HEK293-FT cell extracts were dialyzed overnight against denaturing equilibration buffer (50 mM sodium phosphate, pH 7.0, 300 mM NaCl, 6 mM guanidine-HCl, pH 7.0). The dialysate was then passed over a talon metal affinity column (Clonetech) and eluted with 45 mM sodium guanidine, pH 7.0, 270 mM NaCl, 5.4 M guanidine-HCl, and 250 mM imidazole. Eluted GST-Htt fusion proteins were then dialyzed in a stepwise manner against thrombin cleavage buffer with decreasing amounts of imidazole (5, 4, 3, 2, and 1 M, 1 h per buffer exchange). A final dialysis step in thrombin cleavage buffer (0 mM imidazole) involved two buffer exchanges lasting 2 h each, followed by a final buffer exchange that continued overnight.

Htt Exon-1 and Shortstop Aggregation Studies—To remove the GST tag and initiate aggregation of expanded GST-Htt fusion proteins, 0.1 μg/μl of purified fusion protein was incubated at room temperature with thrombin (10 units/50 μg fusion protein). To terminate the cleavage reaction, aliquots were removed over a 10-day period and either flash-frozen in liquid N2 (for transmission electron microscopy and atomic force microscopy), treated with SDS sample buffer (SDS-PAGE), or treated with Blue native sample buffer (Blue native-PAGE).

SDS- and Blue Native-PAGE/Western Blotting—For SDS-PAGE/Western blotting analysis, samples prepared in SDS sample buffer were resolved on a 4–15% Tris-HCl gel and transferred to nitrocellulose using the Bio-Rad Mini Protean system. For Blue native-PAGE/Western blotting analysis, samples prepared in Blue native sample buffer were resolved on a 3–12% (Htt exon-1 44Q) or 4–16% (Htt shortstop 44Q) Bis-Tris gel and transferred to PVDF (Immobilon P, Millipore) using the Invitrogen system.

Transmission Electron Microscopy Analysis—Htt exon-1 and shortstop aggregation reactions were carried out as described above. For each time point analyzed, a 5-μl aliquot was removed, flash frozen in liquid N2, and stored at −80°C. Prior to imaging, freshly thawed aliquots were diluted to a concentration of 0.01 μg/μl, applied to a 400 mesh carbon and parlodion-coated copper grid (Polysciences), and negatively stained with 1% uranyl acetate-0.04% tylose. Grids were viewed on a Hitachi H-7600 transmission electron microscope and digitally captured using an Advanced Microscopy Techniques CCD at 1k × 1k resolution.

Deposition of Protein Samples for Ex Situ AFM Imaging and AFM Imaging Conditions—Samples were deposited on freshly cleaved mica (Ted Pella Inc, Redding, CA). Aliquots of 2.5 μl of Htt exon-1 and shortstop 44Q aggregation time course samples (0 h, 0.5 h, 2 h, 4 h, 6 h, 1 d, 2 d, 3 d, 5 d, 7 d, and 10 d) were deposited onto the mica surface and left on the substrate for ~1 min. The substrate was then washed with 200 μl of ultrapure water. To prevent damage to any fragile aggregates, the substrate was tilted ~45°, and the ultrapure water was applied above the sample, providing a gentle flow over the deposited sample. The sample was dried with a gentle stream of canned compressed gas. Deposited samples were then imaged ex situ with a Nanoscope V MultiMode scanning probe microscope (Bruker, Santa Barbara, CA) equipped with a closed-loop vertical engagement J-scanner. Images were taken with a diving board-shaped oxide-sharpened silicon cantilever (μmasch, San Jose, CA) with a nominal spring constant of 40 N/m and resonance frequency of 325 kHz. Scan rates were set at 1–2 Hz with cantilever drive frequencies 10% below resonance. AFM images were analyzed using Matlab equipped with the image processing toolbox (Mathworks, Natick, MA) as described (31). Images were imported into Matlab, and a flattening algorithm was applied to correct for curvature from the imaging process. A binary map of the location of aggregates was created using a height threshold. Discrete aggregates were located by applying pattern recognition algorithms to the binary map and finally physical properties (including height, volume, and average diameter) were measured automatically. Volume measurements were partially corrected for error associated with the finite size of the AFM probe based on geometric models (32).

These corrected volume measurements were used to estimate the molecular weight of observed aggregates based on the average density of proteins (33, 34). The number of molecules per each oligomer was estimated by dividing the observed corrected volume of each individual aggregate by the estimated volume of a single monomer. This calculation assumes perfect packing of individual monomers within the oligomer and that the density of the proteins is the same in aggregated and non-aggregated forms.

Cell Viability Studies in Primary Cortical Neurons—Primary cortical neurons isolated from embryonic day 17 mice as previously described (35) were transfected with wild-type (9Q) and expanded (82Q) Htt exon-1 and Htt shortstop constructs at DIV7 using Lipofectamine 2000 according to the manufacturer’s protocol. After 24, 36, and 48 h, cells were fixed for 30 min with 4% paraformaldehyde in PBS. After three washes with PBS, cells were treated with 0.8 μg/ml of bisbenzimide (Hoechst 33342, Sigma) and stained with anti-Htt 1–82 monoclonal antibody (1:1500), followed by Alexa fluor 488 secondary antibody (Invitrogen; 1:2000). Cells were automatically analyzed using an inverted fluorescence microscope (Axiovert 200, Zeiss) and images were digitized from 144 independent fields per well. Transfected cells were visualized by immunostaining with anti-Htt 1–82 antibody and quantified for cell survival using the Volocity software (Perkin Elmer) by automated measurement of the average intensity of Hoechst-stained nuclei of transfected cells. Cells were considered as viable when their intensity was lower than 200% of the control intensity. Data reported are an average (± S.E.) of six independent experiments. For confocal microscopy analysis, primary neurons were transfected with Htt exon-1 and shortstop constructs at DIV6, fixed with 4% paraformaldehyde in PBS, and stained with anti-Htt 1–82, Alexa fluor 488, and bisbenzimide as described above. Cells were imaged using a Zeiss LSM510 laser scanning confocal microscope.

RESULTS

Mammalian-derived Recombinant Htt Fusion Proteins—For these studies, we prepared GST-tagged Htt fusion protein constructs encoding exon-1 (amino acids 1–90) and shortstop (amino acids 1–117) with a pathological length (44Q) polyQ region for expression in HEK293FT cells (Fig. 1A). These constructs were designed with an N-terminal GST tag, which allows for expression and native purification of soluble...
**Novel Mammalian-derived Expanded Htt Exon-1 Oligomers**

Expanded Htt protein, and a C-terminal 6-residue histidine tag, which was used for purification of the fusion proteins under denaturing conditions. GST-Htt fusion proteins were purified under native conditions using glutathione-Sepharose chromatography (as described in “Experimental Procedures”) and analyzed by SDS-PAGE followed by Coomassie staining (Fig. 1B). Both expanded GST-Htt exon-1 and shortstop fusion proteins were co-purified with a second protein that migrated around 70 kDa. Mass spectrometry analysis confirmed that this 70-kDa protein was Hsp70 (data not shown). Densitometry analysis indicated that purified GST-Htt exon-1 contained ~5–10% Hsp70, while GST-Htt shortstop contained ~40% Hsp70.

Because stringent ATP washes did not alter the levels of co-purified Hsp70, a parallel protocol was developed to purify GST-Htt shortstop under denaturing conditions. This purification was carried out in 6 M guanidine hydrochloride using metal chelate chromatography and the C-terminal histidine tag (Fig. 1A) and effectively removed >99% of co-purified Hsp70 (Fig. 1B). A minor contaminant migrating at ~100 kDa in this preparation (Fig. 1B) lacked GST reactivity (supplemental Fig. S1) and may represent a non-related mammalian protein with a high affinity to the metal affinity resin. Western blotting confirmed that GST-Htt exon-1 (Fig. 1C) and GST-Htt shortstop purified under both native (Fig. 3A) and denaturing conditions (Fig. 1C) were recognized by anti-Htt exon-1 antibody (28).

**Expanded Htt Exon-1 Protein Generated in Mammalian Cells Forms SDS-stable and Native Oligomeric Complexes**

A number of previous and more recent studies using bacterially-expressed recombinant Htt exon-1 MBP- or GST-tagged fusion proteins with an expanded polyQ region have demonstrated the formation of oligomeric aggregation species upon removal of the N-terminal affinity tag (22, 24, 26). To investigate the behavior of mammalian-derived expanded GST-Htt exon-1, fusion protein was treated with thrombin to induce cleavage of the N-terminal GST tag. The cleavage reaction was monitored over a 10-day period by SDS-PAGE and Blue native-PAGE, followed by Western blotting. SDS-PAGE/Western blot analysis using anti-Htt exon-1 antibody demonstrated the presence of an SDS-stable species migrating at ~75 kDa that first appeared 1 day following thrombin-induced GST cleavage (Fig. 2A). This species was also recognized by expanded polyQ selective antibodies 3B5H10 and MW1, but not by an antibody directed against the first 17 amino acids of Htt exon-1 (Fig. 2A), suggesting that this region was either masked or in an altered conformation. An additional possibility is that the N-terminal 17 amino acid region has undergone nonspecific proteolytic cleavage and is not present. This 75-kDa species was Htt-derived, as it was not recognized by antibodies to GST or Hsp70 (supplemental Fig. S2), and was no longer detectable after 5 days. Further analysis of the cleavage reaction showed that SDS-insoluble material reactive with anti-Htt exon-1 antibody was detectable beginning at 5 days and persisted throughout the 10-day time course (supplemental Fig. S3). These data demonstrate that mammalian-derived expanded Htt exon-1 forms high molecular weight SDS-PAGE aggregates that are comparable to those formed by the bacterial version of the protein.

**Using the Blue native-PAGE technique, an 1100-kDa Htt-derived oligomeric species was detected with anti-Htt exon-1, expanded polyQ selective 3B5H10 and MW1, and anti-Htt aa 81–90 antibodies (Fig. 2B). This species was initially observed at 1 d post thrombin cleavage, and was no longer detected after the 3 day time point. This higher molecular weight species was not recognized by anti-Htt aa 1–17 (Fig. 2B), suggesting that this domain was masked or in an altered conformation (or possibly not present). This Htt oligomeric species was also not recognized by anti-GST or anti-Hsp70 antibodies (supplemental Fig. S2), demonstrating that it was Htt-derived. In contrast, a smaller native Htt oligomer with an apparent molecular mass between 242 and 480 kDa was recognized by all anti-Htt and anti-GST antibodies (Fig. 2B).**

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**FIGURE 1. Mammalian-derived expanded Htt GST-fusion proteins for in vitro aggregation studies.** A, schematic representation of expanded Htt exon-1 and shortstop fragments, indicating polyQ region (red), Pro-repeat regions (yellow), and P/Q-rich region (purple). GST-tag (orange; not drawn to scale) is located at the N terminus and is followed by a thrombin cleavage site. A His<sub>6</sub> tag (blue) was engineered at the C terminus. B, SDS-PAGE/Coomassie analysis of expanded GST-Htt exon-1 and shortstop following expression in and purification from HEK293-FT cells. Left panel, Htt exon-1. Middle panel, Htt shortstop purified under native conditions. Right panel, Htt shortstop purified under denaturing conditions. Red arrow denotes co-purified Hsp70 present in native preparations of Htt exon-1 and Htt shortstop. A minor contaminant migrating at ~100 kDa in the Htt shortstop sample purified under denaturing conditions was not reactive with an antibody to GST (supplemental Fig. S1) and likely represents a non-related mammalian-protein that binds non-specifically to the metal affinity column. C, Western blots of purified GST-fusion proteins using anti-Htt exon-1 antibody.
anti-polyQ antibodies (Fig. 2B), as well as by anti-GST and anti-Hsp70 antibodies (supplemental Fig. S2), and likely represents a complex formed between Hsp70 and an uncleaved GST-Htt exon-1 fusion protein with an exposed Htt N-terminal 17-amino acid domain. While an Htt exon-1 protein with a non-pathological polyQ length of 16Q formed a similar higher molecular weight native Htt complex, this species persisted throughout the 10-day time course (supplemental Fig. S3). B, native Htt-derived 1100-kDa species (arrow) initially detected at 1 day following thrombin cleavage is recognized by anti-Htt and anti-polyQ antibodies, but not by Htt aa 1–17. This species is transient, and no longer observed after 3 days. A smaller ~400 kDa species (arrowhead) is comprised of Htt, GST (supplemental Fig. S2), and Hsp70 (supplemental Fig. S2) and likely represents GST-Htt exon-1 fusion protein in a complex with Hsp70.

Expanded Htt Shortstop Oligomeric Aggregation Complexes Are Not Detected by SDS-PAGE or by Blue Native PAGE—Previous studies have shown that an N-terminal fragment of expanded polyQ Htt comprised of the first 117 amino acids and referred to as Htt shortstop was non-toxic in a transgenic model of HD, despite its ability to form visible aggregates in situ (13). To investigate the aggregation pathway of expanded Htt shortstop in vitro, GST–Htt shortstop 44Q purified under native conditions was treated with thrombin and monitored over a 10-day period by SDS-PAGE and Blue native-PAGE, followed by Western blotting. SDS-PAGE/ Western blotting analysis failed to detect an SDS-stable complex for Htt shortstop 44Q using anti-Htt or anti-polyQ antibodies (Fig. 3A), despite efficient

**FIGURE 2.** Expanded Htt exon-1 protein generated in mammalian cells forms SDS-stable and native oligomeric complexes. Time course of GST-Htt exon-1 44Q thrombin cleavage reaction resolved by SDS-PAGE, A, or Blue native-PAGE, B, followed by Western blotting. A, an SDS-stable 75-kDa species is detected by anti-Htt exon-1 antibody and by polyQ-selective antibodies 3B5H10 and MW1 (arrow). This species is not detected by anti-Htt aa 1–17, suggesting that this region is masked, in an altered conformation, or not present. High molecular weight aggregates are present beginning at 5 days and persist throughout the reaction time course (supplemental Fig. S3). B, native Htt-derived 1100-kDa species (arrow) initially detected at 1 day following thrombin cleavage is recognized by anti-Htt and anti-polyQ antibodies, but not by Htt aa 1–17. This species is transient, and no longer observed after 3 days. A smaller ~400 kDa species (arrowhead) is comprised of Htt, GST (supplemental Fig. S2), and Hsp70 (supplemental Fig. S2) and likely represents GST-Htt exon-1 fusion protein in a complex with Hsp70.
cleavage of the GST affinity tag (supplemental Fig. S6). Similarly, the 1100-kDa Blue native-PAGE oligomeric species observed for Htt exon-1 44Q (Fig. 2B) was not seen with Htt shortstop 44Q using anti-Htt (Fig. 3B) or anti-polyQ (data not shown) antibodies. This difference in SDS- and Blue native-PAGE results between expanded Htt exon-1 and shortstop cannot be due to the higher levels of Hsp70 in the GST-shortstop 44Q preparation, as Htt shortstop 44Q purified under denaturing conditions (and lacking appreciable amounts of Hsp70) behaved in a similar manner (data not shown). A smaller native Htt oligomer with an apparent molecular mass between 242 and 480 kDa was observed for natively-purified Htt shortstop 44Q up to 6 h following addition of thrombin (Fig. 3B), and was reactive with both anti-GST and anti-Hsp70 antibodies (supplemental Fig. S6). This species was also observed for Htt exon-1 (Fig. 2B), though appeared to be less prevalent in the Htt shortstop sample. Taken together, these data suggest that while expanded Htt exon-1 and shortstop proteins displayed differences in observable SDS-stable and higher molecular weight Htt-derived native complexes, both proteins have detectable levels of a GST-Htt/Hsp70 complex at early time points in the aggregation time course before thrombin-induced cleavage of GST is complete.

Mammalian-derived Htt Exon-1 44Q and Shortstop 44Q Form Fibrillar Structures as Detected by Transmission Electron Microscopy—Previous studies have shown that expanded Htt exon-1 fragments generated by peptide synthesis (36, 37) or in bacterial expression systems (22, 24, 38–40) form fibrillar aggregates in vitro. In addition, while expanded Htt shortstop was shown to form large aggregates in transgenic mouse models of HD (13, 41), the ability of this protein to form amyloid-like fibrils has not been determined. To qualitatively assess morphological features of mammalian-derived expanded Htt exon-1 and shortstop aggregates, thrombin-treated GST fusion proteins were analyzed by transmission electron microscopy (or TEM) (Fig. 4). Prior to GST-Htt exon-1 and shortstop 44Q cleavage, small amorphous structures were visible (Fig. 4, 0 h). At 4 h following addition of thrombin (Fig. 4), amorphous aggregates of both expanded Htt exon-1 and shortstop were observed. At later time points, for both Htt exon-1 and shortstop 44Q proteins, fibrillar structures were observed and co-existed with the amorphous aggregate species. For both proteins, fibril structure became more complex over time, with a significant amount of bundling and branching observed (Fig. 4, 5, 7, and 10 d). These structures were often associated with amorphous aggregate species (Fig. 4A, 10 d; Fig. 4B, 5, 7, and 10 d). As expected, no evidence of fibrillar structure was observed for non-expanded Htt exon-1 16Q (data not shown), suggesting that these structures represent multimeric forms of the fusion protein driven by self-association of GST, a known dimer in solution. At 4 h following addition of thrombin (Fig. 4), amorphous aggregates of both expanded Htt exon-1 and shortstop were observed. At later time points, for both Htt exon-1 and shortstop 44Q proteins, fibrillar structures were observed and co-existed with the amorphous aggregate species. For both proteins, fibril structure became more complex over time, with a significant amount of bundling and branching observed (Fig. 4, 5, 7, and 10 d). These structures were often associated with amorphous aggregate species (Fig. 4A, 10 d; Fig. 4B, 5, 7, and 10 d). As expected, no evidence of fibrillar structure was observed for non-expanded Htt exon-1 16Q (data not shown). These data demonstrate that recombinant expanded Htt exon-1 protein generated in a mammalian system forms fibrillar structures comparable to those formed by bacterial and synthetic forms of the protein. The data also show that expanded Htt shortstop is capable of
forming fibrils, much like those formed by expanded Htt exon-1.

Mammalian-derived Expanded Htt Exon-1 and Htt Shortstop Form a Variety of Aggregates Based on Atomic Force Microscopy—To carry out a more quantitative analysis of Htt exon-1 and Htt shortstop aggregation, we examined aggregation samples using ex situ atomic force microscopy (AFM) over the 10-day reaction time course (supplemental Fig. S7). To quantify the relationship between oligomer and fibril formation, the number of each respective aggregate type per \( \mu \text{m}^2 \) was determined (Fig. 5A). For this analysis, aggregate types were defined strictly by morphological features. Oligomers were defined as aggregates that were at least 1 nm in height and had an aspect ratio (longest distance across to shortest distance across) of less than 2.5, which indicated a predominantly round, globular structure. Fibrils were defined as aggregates with heights larger than 1 nm and an aspect ratio greater than 2.5. Oligomeric aggregates were initially observed for both Htt exon-1 44Q and Htt shortstop 44Q proteins and continually increased in
number during the 10-day period. However, the population of oligomers for Htt exon-1 44Q eventually became much larger in comparison to Htt shortstop after 5 days. Short, putative fibril aggregates formed after 1 day of incubation for both proteins. While the number of fibrils gradually increased for Htt exon-1 44Q through 10 days, the population of fibrils abruptly increased on the third day of incubation for Htt shortstop 44Q and remained relatively stable on subsequent days. This abrupt increase in the number of fibrils for Htt shortstop 44Q may have contributed to the smaller population of oligomers observed after 5 days in comparison with Htt exon-1 44Q, as the formation of fibrils would reduce the available protein for oligomer formation. Despite the difference in apparent rate of formation of fibrils, typical fibril morphology was not drastically different between the two proteins with typical height of fibrils being 6–10 nm along the contour (Fig. 5B). Fibrils observed for both proteins also became longer and began to intertwine into bundles with longer incubation times.

As the Blue native-PAGE analysis indicated that Htt exon-1 44Q formed a unique oligomeric species that was not observed for Htt shortstop 44Q, we performed further size analysis on the oligomer subset of aggregates observed by AFM. The average oligomer diameter appeared to be highly correlated with height during the initial stages of aggregation (Fig. 5C, exemplified in the 6 h time point), and these correlations were indistinguishable for both Htt exon-1 44Q and shortstop proteins. From 1 to 3 days, a second population of oligomers with a different correlation between diameter and height was observed for Htt exon-1 44Q, but not for shortstop (Fig. 5C). The diameters of this second population of Htt exon-1 44Q oligomers were larger in comparison to the more commonly observed population of oligomers. This second population was also transient, as it was no longer apparent in correlation plots after 5 days. This larger, transient population of oligomers only observed for Htt exon-1 44Q was consistent with data obtained from Blue native-PAGE analysis (Fig. 2), and may represent the larger 1100-kDa Blue native-PAGE oligomers. Further, volume measurements of oligomers were partially corrected for the contribution due to the finite size of the probe (32). Assuming that the oligomer density is similar to measured protein densities (33, 34), the partially corrected volume measurements were used to estimate the molecular weight of the observed oligomers. The mode of the oligomer molecular weight at all time points for both Htt exon-1 44Q and shortstop was 420–560 kDa. This molecular weight corresponds well to the 242–480 kDa oligomeric species observed for both proteins by Blue native-PAGE analysis. Molecular weight calculations could not be done on the larger Htt exon-1-specific AFM oligomers, as the population of these species was small compared with the 420–560 kDa AFM oligomers observed for both exon-1 and shortstop.

Expanded Htt Shortstop Is Not Toxic When Expressed in Cultured Primary Cortical Neurons—The original expanded Htt shortstop mouse showed no evidence of neurodegeneration or a behavioral phenotype, despite the presence of a long polyQ region comprised of 128 glutamines (13). To directly compare the toxicity of expanded Htt exon-1 and shortstop, we have developed a nuclear condensation assay in embryonic primary cortical neurons. Primary cultures grown for 7–10 days were transfected with either Htt exon-1 or Htt shortstop constructs encoding for expanded (82Q) and normal (9Q) length polyQ regions. The expanded polyQ region in these proteins was longer than in the GST-fusion protein constructs to allow for sufficient toxicity within the time frame of the experiment. Primary cells were fixed 24, 36, or 48 h following transfection, stained with both Hoechst and a monoclonal antibody to amino acid residues 1–82 of Htt, and imaged by immunofluorescence microscopy. Our results indicate that the toxicity of expanded Htt shortstop at the longest time point of 48 h was ~2-fold less than that for expanded Htt exon-1, and comparable to that observed for both non-expanded versions of Htt exon-1 and Htt shortstop (Fig. 6A, 48 h), despite similar levels of protein expression (Fig. 6B). Analysis at earlier time points indicated an absence of Htt exon-1 82Q toxicity (Fig. 6A, 24 h) or a smaller degree of toxicity (Fig. 6A, 36 h) compared with the 48 h time point. Confocal analysis of Htt exon-1 and shortstop-transfected primary neurons demonstrated the presence of cytoplasmic and nuclear aggregates for both expanded proteins at 48 h (Fig. 6C). While the majority of neurons containing aggregates of Htt exon-1 82Q had condensed nuclei characteristic of a dead or dying cell, most Htt shortstop 82Q-expressing cells with visible aggregates at this time point contained normal, healthy nuclei (Fig. 6C). In summary, these data are consistent with the absence of a toxic phenotype observed for the original mutant Htt shortstop mouse (13), as well as with previous toxicity data on expanded Htt shortstop carried out in a mammalian cell line (14), and suggest that Htt shortstop with an expanded polyQ is not toxic, despite its ability to form aggregates.

DISCUSSION

Using an in vitro system to express recombinant GST-Htt fusion protein in mammalian cells, we have generated two expanded polyQ N-terminal fragments of Htt. The 90-residue exon-1 fragment corresponds to a toxic fragment both in vitro and in vivo (14, 35, 42–44) while the 117-residue “shortstop” fragment was initially identified in a transgenic HD mouse by its ability to aggregate, yet lack a toxic phenotype (13). In the present studies, we have used SDS-PAGE and Blue native-PAGE, a type of non-denaturing gel analysis, to monitor aggregation of Htt. We identified novel oligomeric aggregation species formed by expanded Htt exon-1, but not detected for expanded Htt shortstop, that may correspond to a toxic form of Htt. These potentially toxic oligomers were also detected for expanded Htt exon-1, but not for expanded shortstop, by AFM analysis. Using AFM, we show that morphological differences exist in expanded Htt exon-1 and shortstop aggregation pathways. Finally, we have developed a nuclear condensation assay to compare toxicity of expanded Htt exon-1 and shortstop proteins in cultured primary cortical neurons. We show that whereas expanded Htt exon-1 is toxic relative to non-expanded exon-1, expanded shortstop is significantly less toxic than expanded exon-1, and no different than non-expanded exon-1 or non-expanded shortstop. Confocal analysis further demonstrates that while expanded Htt shortstop forms intracellular aggregates, much like expanded Htt exon-1, primary neurons...
containing these aggregates are visibly healthy. Taken together, these data confirm previous results demonstrating that expanded Htt shortstop is not toxic (13, 14), and suggest a possible mechanism via differences in early stages of Htt aggregation, and in particular, the presence of potentially toxic oligomeric aggregation species detected for expanded Htt exon-1 but not expanded shortstop protein.

The Blue native-PAGE system is an attractive alternative to other methods used to study Htt aggregation species. One such method is the dot blot, or filter trap assay, which involves adsorption of oligomeric species onto a membrane, followed by antibody staining (45). This assay is fast, but does not allow one to resolve individual oligomeric species. The Agarose Gel Electrophoresis for Resolving Aggregates (AGERA) technique has been used successfully to analyze complex samples prepared from transgenic mouse brain extracts (26, 46). These complexes must be SDS-stable and large, as the AGERA technique is carried out with small amounts of SDS and using agarose gels.
which are comprised of large pores designed for resolving larger species. In contrast, Blue native-PAGE is run in the absence of SDS, so is truly a “native” technique, and is performed with polyacrylamide gels, allowing for higher resolution of oligomeric species.

Previous studies have used a number of techniques to characterize Htt oligomers in vitro (22, 24–26), in mammalian cell extracts (25, 26), and in transgenic mouse (26, 27) and HD brain extracts (26). In one particular study, the investigators examined aggregation of an Htt exon-1 fragment expressed as a fusion protein with a monomeric cyan fluorescent protein (Cerulean fluorescent protein) in extracts from cultured neuroblastoma cells (25). Sedimentation velocity analysis was used to identify a population of expanded Htt exon-1-Cerulean species roughly equivalent to ~200 particles (25). While it is difficult to determine the exact number of particles present in the Htt exon-1 44Q 1100-kDa Blue native-PAGE oligomers identified in the present study, it is likely that they are significantly smaller than those characterized by sedimentation velocity. This difference may be explained by the differences in protein tags, or by differences between the two techniques. Future studies will include purification of the Htt exon-1 44Q 1100-kDa Blue native-PAGE oligomers to allow for direct analysis of its components as well as direct testing in cell and animal models of HD.

Analysis of a non-expanded polyQ Htt exon-1 fragment (Htt exon-1 16Q) following GST removal demonstrated that this protein formed an Htt-derived oligomeric species detected by Blue native-PAGE (supplemental Fig. S4). Importantly, this species persisted throughout the 10-day time course. By contrast, the expanded Htt exon-1 1100-kDa species was transient, and no longer detectable after 3 d (Fig. 2B). Interestingly, a transient population of oligomers was also detected for expanded Htt exon-1 by correlation analysis of AFM images (Fig. 5C). This difference suggests that soluble oligomers formed by mutant (expanded polyQ) and wild type (non-expanded) Htt exon-1 proteins are not identical. One possible explanation for these results is that the expanded Htt exon-1 44Q 1100-kDa oligomer transitions into fibrils after 3 days, which would not be expected to occur with non-expanded Htt exon-1 oligomeric species. Consistent with this possibility, a previous atomic force microscopy study demonstrated that while both expanded and non-expanded Htt exon-1 formed morphological oligomers, only those oligomers formed by the expanded protein were able to transition into fibrils, suggesting that structural differences existed between both sets of Htt exon-1 oligomeric species (26).

The mammalian-based system used to generate recombinant expanded polyQ Htt-fusion protein for in vitro aggregation assays provides an advantage over bacterial systems or synthetic peptide-based assays, as it allows for potentially relevant post-translational modifications that would not occur in either of these two systems. Such modifications may include phosphorylation of Thr-3, Ser-13, or Ser-16, all shown to occur in vivo and affect pathogenic properties of mutant Htt (47, 48). While mammalian-derived expanded Htt exon-1 forms fibrillar aggregates that are indistinguishable from those formed by the bacterial counterpart, it does so much more slowly (days versus hours; Fig. 4 and Ref. 22). These differences may be explained by post-translational modifications of the mammalian recombinant protein and will be explored further in future studies.

The nuclear condensation assay used in these studies demonstrates that expanded Htt shortstop is not toxic in primary cortical neurons. These data are consistent with the original Htt shortstop transgenic mouse model (13), as well as a more recent study carried out in a mammalian cell line (14). These observations are not consistent with a newly developed Htt shortstop transgenic mouse model designed to co-express eGFP and shown to display severe behavioral and pathological abnormalities (41). The reason for the disparity between the two Htt shortstop mouse models and the lack of consistency of the new mouse model with the cell culture models remains unclear. One possible explanation is the eGFP co-expressed in the newer transgenic mouse may compromise cell viability in this model. Consistent with this possibility, an eGFP-Htt N586 mouse with an expanded polyQ region comprised of 82 glutamine residues was found to display profound ataxia-like abnormalities as well as cerebellar atrophy (49) not observed in a mutant Htt N586 model incorporating the prion promoter (50).

Heat shock proteins have been shown to play a major role in suppression of toxicity in aggregation-based neurodegenerative diseases (51). In particular, loss of Hsp70 was shown to exacerbate the pathogenic phenotype in a transgenic mouse model of HD (52), demonstrating a role for this chaperone in protecting against Htt-mediated toxicity. In the present in vitro studies, while both natively purified Htt exon-1 and shortstop fusion proteins were co-purified with Hsp70 (Fig. 1B), GST-Htt shortstop preparations contained higher levels of Hsp70 protein (40% versus 5–10% for GST-Htt exon-1). Purification of GST-Htt shortstop under denaturing conditions dramatically increased the yield of fusion protein obtained in the preparation, suggesting that Hsp70/GST-Htt shortstop association may prevent binding of the fusion protein to glutathione-Sepharose under native conditions. Taken together, these observations suggest a stronger and more pronounced Hsp70/Htt shortstop association, as compared with Htt exon-1, in our mammalian-based expression system. It is tempting to speculate a role for this strong Hsp70/Htt shortstop association in protecting cells from expanded polyQ toxicity in Htt shortstop cell and animal models of HD.

To better understand the nature of expanded Htt shortstop protection, we used an in vitro approach and mammalian-expressed recombinant fusion proteins to compare early stages of aggregation of expanded polyQ Htt exon-1 and shortstop N-terminal fragments. We identified a novel expanded Htt-derived transient oligomeric species visible by Blue native-PAGE for exon-1 that is not detected for Htt shortstop. Using correlation analysis of AFM images, we also detected a transient population of oligomers for expanded Htt exon-1 but not for expanded Htt shortstop. In the present study, the ability to form transient oligomers, as detected by Blue native-PAGE and AFM correlation analysis seems to correlate with toxicity, suggesting fundamental differences at the structural level between expanded shortstop and exon-1. Future studies will be aimed at investigating these differences and should help elucidate the mechanism of expanded Htt shortstop protection. Taken
together, these studies may provide insight into the nature of the toxic species and could provide a therapeutic target to treat HD.

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