Mutant Huntingtin Promotes the Fibrillogenesis of Wild-type Huntingtin

A POTENTIAL MECHANISM FOR LOSS OF HUNTINGTIN FUNCTION IN HUNTINGTON’S DISEASE

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Aggregation of huntingtin (htt) in neuronal inclusions is associated with the development of Huntington’s disease (HD). Previously, we have shown that mutant htt fragments with polyglutamine (polyQ) tracts in the pathological range (>37 glutamines) form SDS-resistant aggregates with a fibrillar morphology, whereas wild-type htt fragments with normal polyQ domains do not aggregate. In this study, we have investigated the co-aggregation of mutant and wild-type htt fragments. We found that mutant htt promotes the aggregation of wild-type htt, causing the formation of SDS-resistant co-aggregates with a fibrillar morphology. Conversely, mutant htt does not promote the fibrillogenesis of the polyQ-containing protein NOCT3 or the polyQ-binding protein PQBP1, although these proteins are recruited into inclusions containing mutant htt aggregates in mammalian cells. The formation of mixed htt fibrils is a highly selective process that not only depends on polyQ tract length but also on the surrounding amino acid sequence. Our data suggest that mutant and wild-type htt fragments may also co-aggregate in neurons of HD patients and that a loss of wild-type htt function may contribute to HD pathogenesis.

Huntington’s disease (HD) is an autosomal dominant neurodegenerative disorder characterized by uncontrolled movements, motor impairment, and dementia. The disease is associated with selective neuronal cell death occurring mainly in the cerebral cortex and the striatum (1). The mutation causing HD is an expanded CAG repeat located within exon 1 of the IT-15 gene encoding huntingtin (htt), a 350-kDa protein of unknown function (3). The CAG repeat is translated into a polyglutamine (polyQ) sequence, and the disease appears when the critical length of ~37 glutamines is exceeded (4).

It is generally assumed that the expanded polyQ stretch confers a toxic gain of function to the htt protein. However, the current data do not exclude the possibility that loss of wild-type htt function may also contribute to the disease phenotype in post-mitotic neurons. Htt function is essential for neurogenesis (5) and also important for neuron survival in adult brains (6, 7). Strikingly, deletion of the gene encoding the htt mouse homologue hdh resulted in a progressive neurological phenotype (8). Therefore, it is intriguing to speculate that the toxic gain of function of mutant htt may cause a loss of the essential function of wild-type htt and thereby induce selective neurotoxicity.

However, the molecular basis for such a disease mechanism remains unclear. A characteristic feature of mutant htt proteins with elongated polyQ sequences is that they spontaneously self-assemble into SDS-resistant protein aggregates with a fibrillar morphology in vitro and in vivo (10, 11). This suggests that HD is due to a toxic gain of function that leads to the formation of abnormal protein aggregates in a nucleation-dependent process.

Indeed, the formation of neuronal inclusions (NI) in affected brain regions of patients is a characteristic feature of HD (11, 12), and the formation of NIs and disease progression are linked (13). NIs do not consist solely of htt protein but also of other cellular proteins such as molecular chaperones (14). These observations have led to the hypothesis that cellular toxicity associated with htt aggregation results from sequestration of proteins that are involved in cellular key events. Of particular interest is the finding that NIs also contain other polyQ-containing proteins such as the transcription factors TBP (TATA-binding protein; Ref. 15) and CBP (CREB-binding protein; Ref. 16). This suggests that the polyQ domain in transcription factors mediates protein-protein interactions and associates with the polyQ sequence of htt.

Here, we have studied the co-aggregation of recombinant HD exon 1 proteins with normal and extended polyQ sequences. In addition, we have analyzed the interaction of HD exon 1 proteins with the transcription factors NOCT3 and PQBP1, which are expressed in brain (17, 18). NOCT3 contains a polyQ sequence, whereas PQBP1 does not. However, PQBP1 is known to specifically bind to polyQ-containing proteins (18, 19). We find that mutant HD exon 1 protein rapidly aggregates in vitro and thereby promotes the aggregation of HD exon 1 proteins with polyQ sequences in the normal range. When both wild-type and mutant HD exon 1 proteins are mixed together, SDS-resistant co-aggregates with a fibrillar morphology are formed by a nucleation-dependent process. In contrast, SDS-resistant co-aggregates are not formed between mutant huntingtin and the transcription factors NOCT3 and PQBP1 in vitro, although...
these two proteins do co-localize in inclusion bodies in cell culture models of HD. Together our results suggest that co-aggregation between wild-type and mutant htt fragments may also occur in patient brains and that this may lead to the loss of wild-type huntingtin function in neuronal cells.

MATERIALS AND METHODS

Antibodies—Commercially available antibodies were mouse monoclonal HA antibody (Berkley Antibody, Richmond, CA), mouse monoclonal FLAG antibody (Roche Applied Science), polyclonal rabbit c-Myc antibody (Santa Cruz Biotechnology, Santa Cruz, CA), polyclonal rabbit FLAG antibody (Sigma), secondary donkey anti-mouse and anti-rabbit antibodies (Roche Applied Science), and immunogold-labeled (5–10 nm) secondary anti-mouse and anti-rabbit antibodies (British BioCell, Cardiff, UK). The polyclonal huntingtin-specific antibody CAG53b has been described (11).

Plasmid Construction—The pGEX-6P-FLAG-1 plasmid used for subcloning of HD exon 1 fragments was generated from the pGEX-6P-1 vector (Amersham Biosciences) as follows. Downstream of the sequence coding for the PreScission protease cleavage site an adapter carrying an NsiI restriction site and the FLAG tag (F, peptide sequence DYD- DDDK) was inserted into the multicloning site. DNA fragments coding for HD exon 1 proteins with 20, 32, 37, 39, 45, or 53 CAG repeats were excised by restriction endonucleases EcoRI and NcoI from pTL-HD exon 1 plasmids (10) and subcloned yielding pGEX-6P-F-HDQ20, -Q32, -Q37, -Q39, -Q45, and -Q53. The construction of plasmids coding for Myc- and HA-tagged HD exon 1 HD exon 1 fragment (Fig. 1A) was reported previously (22, 23). The plasmids pEGFP-HDQ20 and pEGFP-HDQ17 were described under the names pEGFP-CAG27 and pEGFP-CAG17, respectively (23). The coding sequences for NOCT3 and PQBP1 were amplified by PCR from the full-length cDNA plasmids pBS-NOCT3 and pBS-PQBP1 (kind gifts from A. Fontana and N. Bonini) and subcloned into pGEX-6P-1-FLAG. Subsequently, NOCT3 and PQBP1 were subcloned as BamHI-XhoI and EcoRI-NcoI fragments, respectively, into pTL1-HA2 (23). All of the constructs were confirmed by DNA sequencing.

Protein Expression and Purification—Expression of GST fusion proteins in Escherichia coli DH10B was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (Sigma) at 37 °C for 3.5 h. The proteins were affinity purified on glutathione-agarose (Sigma) as described (10) and thiogalactopyranoside (Sigma) at 37 °C for 3.5 h. The proteins were incubated at concentrations of 50, 100, 200, and 400 μg/ml with PreScission protease for 3 h at 8 °C to remove the GST tag, which itself hinders aggregate formation. The unique PreScission protease site in the fusion proteins is located between the GST and the HD exon 1 fragment (Fig. 1A). After cleavage, the samples were incubated for 16 h at 37 °C to permit accumulation of SDS-resistant polyQ-containing protein aggregates. Formation of insoluble aggregates was quantified using a filter retardation assay (10). We found that both F-HDQ39 and F-HDQ53 formed SDS-insoluble high molecular weight aggregates in vitro, whereas F-HDQ20, F-HDQ32, and F-HDQ37 did not (Fig. 2A). Similar results were obtained when the aggregation of the protease-treated Myc-tagged fusion proteins with 20, 32, 37, 39, 45, and 52 glutamines was analyzed (data not shown). SDS-resistant aggregates could not be detected for any of the fusion proteins prior to incubation at 37 °C.

Next we explored the morphology of the aggregates formed in vitro from FLAG or Myc-tagged fusion proteins. GST-F-HDQ20 and GST-F-HDQ53 were used for aggregation assays, and aliquots thereof were subjected to electron microscopy. As expected, the F-HDQ53 aggregates obtained after GST-F-HDQ53 cleavage showed a distinct fibrillar morphology, whereas no such fibrils were observed after cleavage of GST-F-HDQ20 or for the uncleaved GST fusion proteins (Fig. 2B). The same results were obtained with the corresponding Myc-tagged fusion proteins. Thus, the presence of both the FLAG and Myc epitope (7 and 10 amino acid residues, respectively) does not seemingly hinder the process of aggregation and allows the formation of fibrils similar to those observed for the non-tagged HD exon 1 proteins (10).

We found that self-assembly of polyQ-containing htt fragments strongly depends on protein concentration and occurs only when a critical threshold of polyQ-length is exceeded (Fig. 2A). Compared with previous in vitro models of htt aggregation
using recombinant proteins (10, 15, 20), our assay bears two major advantages. First, because of the introduction of a unique PreScission protease site between the GST tag and the epitope-tagged htt fragment, it is possible to separate the processes of proteolytic removal of the GST tag and subsequent aggregation of the htt portion of the fusion protein. Thus, aggregation can be followed independently from the proteolytic cleavage step. Second, differential labeling of htt fragments with the FLAG or Myc epitope allows to follow the fate of different moieties of purified HD exon 1 proteins in the same aggregation reaction. This renders our assay an excellent tool to study co-aggregation of htt molecules with normal and extended polyQ stretches.

Kinetic Studies of HD Exon 1 Protein Aggregation in Vitro—A time course of F-HDQ53, F-HDQ20, and F-HDQ32 aggregation is shown in Fig. 2C. Following removal of the GST tag from the fusion proteins, samples were incubated at 37 °C to permit aggregate formation. Aliquots were taken at the indicated times, and SDS-resistant aggregates were detected by the filter trap assay. Formation of SDS-resistant F-HDQ53 aggregates started after a lag phase of 2–3 h and reached near saturation after 8–11 h, whereas as expected no SDS-resistant aggregates were observed with the F-HDQ20 and F-HDQ32 proteins (Fig. 2C).

In the experiment of Fig. 2D, we examined the effect of seeds on the kinetics of F-HDQ53 aggregation. Aggregates produced from GST-M-HDQ52 were sonicated to disrupt them into small pieces that were visible by electron microscopy but were too short to be detected by the filter trap assay (data not shown). These fragments then were used to seed the aggregation of F-HDQ53. The reactions were set up with GST-F-HDQ53 in the presence of various amounts of seeds (1–5% of input fusion protein). At various times aliquots were removed and analyzed by the filter retardation assay using the anti-FLAG antibody. The results shown in Fig. 2D demonstrate that the addition of increasing amounts of seeds to the aggregation reaction of F-HDQ53 leads to the elimination of the lag phase at a seed concentration corresponding to about 2.5% of the total amount of input protein. Thus, aggregation of FLAG-tagged htt protein requires the formation of nuclei and starts when their concentration exceeds a critical value.

Co-aggregation of FLAG- and Myc-tagged HD Exon 1 Proteins in Vitro—Increasing evidence suggests that besides aggregation of mutant htt, loss of normal htt function also contributes to the phenotype of HD (9). Previous in vitro studies and post-mortem examination of brains from HD patients and transgenic mice have led to the speculation that aggregates of mutant htt may sequester wild-type protein and interfere with its normal function (15, 21). To investigate this hypothesis in more detail, we performed co-aggregation studies using GST-HD fusion proteins with normal or elongated polyQ sequences.

FLAG-tagged fusion proteins with 20, 32, or 37 glutamines were mixed with equal amounts of the Myc-tagged protein GST-M-HDQ52. Samples were treated with PreScission protease, followed by incubation at 37 °C to permit aggregation. In parallel, co-aggregation assays were carried out in which GST-M-HDQ52 was replaced by GST-M-HDQ20 that harbors a normal polyQ tract. Aggregates were captured by filtration and detected with the anti-FLAG (Fig. 3A) and anti-Myc antibodies (Fig. 3B). We found that FLAG- and Myc-tagged proteins form mixed SDS-insoluble high molecular weight aggregates in vitro provided that one of the proteins contains an elongated polyQ tract (>37 glutamines). The extent of aggregate formation is strongly influenced by attributes of the co-aggregating polyQ fragments that cannot trigger aggregation on their own. The highest amount of co-aggregates was formed when M-HDQ52 was combined with F-HDQ37. Significantly fewer co-aggregates were observed after combination of M-HDQ52 with F-HDQ32 or F-HDQ20 (Fig. 3, A and B). Thus, the total amount

Fig. 1. Structure and purification of HD exon 1 proteins. A, structure of FLAG- and Myc-tagged GST-HD exon 1 fusion proteins. The arrows labeled PP indicate the PreScission protease recognition sites. B, SDS-PAGE and Western blot analysis of FLAG-tagged HD exon 1 fusion proteins with 20–53 glutamine residues. C, SDS-PAGE and Western blot analysis of Myc-tagged HD exon 1 fusion proteins with 20–52 glutamine residues. D, Western blot of Myc- and FLAG-tagged HD exon 1 fusion proteins. The anti-Myc and anti-FLAG antibodies recognize specifically the Myc and FLAG epitope, respectively. For SDS-PAGE analysis 15–30 µg of protein sample were separated on a 12% gel and stained with Coomassie Blue R-250.
of aggregates formed correlates with the polyQ tract length even when the number of glutamines in the co-aggregating partner does not exceed the critical threshold.

We then addressed the question of whether HD fusion proteins with normal polyQ sequences are able to aggregate in the presence of preformed fibrils. GST-F-HD fusion protein with 20, 32, or 44 glutamines was mixed with seeds (2%) derived from M-HDQ52, treated with PreScission protease, and then incubated at 37 °C to allow aggregation. F-HDQ44, F-HDQ32, and F-HDQ20 do not form SDS-insoluble aggregates at the concentration used in the absence of seeds. At various times aliquots were taken and subjected to the filter trap assay. We found that in the presence of seeds F-HDQ44 with a polyQ sequence in the pathological range and also F-HDQ32 with a polyQ sequence in the normal range forms SDS-insoluble aggregates within 22 h, whereas no aggregates were observed with the F-HDQ20 protein (Fig. 3C). Interestingly, F-HDQ44 aggregates appeared almost immediately, whereas F-HDQ32 aggregates were detected after a lag phase of 2–3 h, indicating that the rate of co-aggregation in vitro is dependent on the length of the polyQ tract in the FLAG-tagged proteins.

**Structural Analysis of Co-aggregation Products—** We wondered whether the stability of co-aggregates formed by different HD exon 1 proteins is based on a specific structural organization. To address this question we sought to identify the different protein moieties in mixed aggregates by immunoelectron microscopy. Equal amounts of GST-M-HD-Q52 and GST-F-HD-Q20 were mixed and incubated at 37 °C after treatment with PreScission protease. A second sample containing GST-M-HDQ52 alone was processed the same way. Aggregates were incubated with both anti-FLAG (from rabbit) and anti-Myc antibody, stained, and subjected to electron microscopy.

Representative electron micrographs are shown in Fig. 3D. Both co-aggregates and aggregates obtained from M-HDQ52 alone formed characteristic fibrils. Fibrils obtained from pure M-HDQ52 protein were labeled exclusively by small gold particles (Fig. 3D, left panel), indicative of the Myc epitope. The bigger particles (black arrows), specifying the FLAG tag, lay randomly distributed in the background (Fig. 3D, right panel). In contrast, even single fibers of the co-aggregates were marked by gold particles of both sizes (Fig. 3D, right panel). Hence, HD exon 1 fragments with expanded and normal polyQ stretches are able to co-aggregate in vitro, forming fibrils in which both the shorter and the longer htt fragments are integral components.

**Co-aggregation Studies with Transcription Factors NOCT3 and PQBP1 in Vitro—** Having studied co-aggregation of normal and mutant HD proteins, we sought to find out whether other proteins can also be recruited by mutant htt. For analysis we chose the transcription factors PQBP1 (polyQ-binding protein 1) and NOCT3, both of which are expressed in brain (17, 18). NOCT3 harbors a polyQ tract of 21 glutamines that potentially could mediate co-aggregation with mutant htt. For PQBP1,
We generated plasmids for expression of the transcription factors as GST fusion proteins that also contain the FLAG epitope. The structures of the resulting proteins, GST-F-NOCT3 and GST-F-PBP1, are shown in Fig. 4A. Both fusion proteins contain a unique PreScission site, which allows removal of the GST moiety by incubation with protease. Proteins were isolated by affinity chromatography and analyzed by SDS-PAGE and Western blotting (Fig. 4B). Prominent bands migrating at 80 and 50 kDa were detected with the anti-FLAG antibody, corresponding to full-length GST-F-NOCT3 and GST-F-PBQ1, respectively.

Using the purified proteins, we performed co-aggregation studies analogous to those carried out for the various GST-HD exon 1 fusion proteins. Preliminary experiments indicated that neither F-NOCT3 nor F-PBP1 form SDS-stable aggregates by themselves (data not shown). Co-aggregation assays were set up by mixing GST-F-NOCT3, GST-F-PBQ1, or GST-F-HDQ20 with equal amounts of GST-M-HDQ20 and GST-M-HDQ52, respectively. After removal of the GST tag and subsequent incubation at 37 °C, aggregates were captured by filtration and visualized by immunodetection with anti-FLAG antibody. Co-aggregates were formed by M-HDQ52 and F-HDQ20 (positive control), whereas no such aggregates could be detected for samples in which the Myc-tagged HD proteins had been incubated together with F-NOCT3 or F-PBP1 (Fig. 4C), indicating that these proteins do not co-aggregate with htt. It should be noted, however, that our filtration assay detects only aggregates that are resistant to boiling in SDS.

Co-localization of HA- and GFP-tagged Fusion Proteins in Mammalian COS1 Cells—To verify the above in vitro findings for living cells, we switched to a cell culture model of HD. To monitor intracellular protein–protein interactions, we fused HD exon 1 proteins with polyQ tracts of 20 and 32 glutamines as well as the transcription factors NOCT3 and PBP1 with a HA tag, generating the fusion proteins HA-HDQ20, HA-HDQ32, HA-NOCT3, and HA-PBP1. Plasmids that express HD exon 1 protein with 17 or 72 glutamines fused to GFP or its modified version EGFP have been described previously (22, 23) and are henceforth referred to as GFP-HDQ17 and GFP-HDQ72. All of the constructs are schematically depicted in Fig. 5A. COS1 cells were transfected and grown for 40–44 h. Following cell lysis the whole protein extracts were used for SDS-PAGE and Western blot analysis (Fig. 5B). All of the constructs were translated into fusion proteins of the expected size and could be detected with specific antibodies (anti-HA or anti-CAG53b).

The subcellular distribution of various fusion proteins in COS1 cells was analyzed by fluorescence microscopy. Representative micrographs are shown in Fig. 6. htt fusion proteins with normal polyQ lengths generally showed a diffuse cytoplasmatic distribution (Fig. 6, A, C, and F). In contrast, GFP-HDQ72 with an extended polyQ tract formed distinct nuclear and cytoplasmatic inclusions with an average diameter of 1–2 μm (Fig. 6B). In agreement with previous observations (24, 25), we attributed the formation of GFP-HDQ72 inclusions in COS1 cells to the expanded polyQ tract in the htt protein. The HA-tagged transcription factors were found distributed mainly in the nucleus, although HA-PBP1 was also localized in small cytoplasmatic and nuclear aggregates (Fig. 6L), which fits data published previously (26).

We then explored the interplay of various fusion proteins in COS1 cells. Co-expression of HA-HDQ20 or HA-HDQ32 with GFP-HDQ17 did not affect their distribution pattern (Fig. 6, D

![Fig. 3. Co-aggregation of Myc- or FLAG-tagged GST-HD exon 1 fusion proteins. A and B, fusion proteins GST-M-HDQ20 and GST-M-HDQ52 (final concentration, 50 μg/ml) were mixed with equal amounts of GST-F-HDQ20, GST-F-HDQ32, or GST-F-HDQ37. After removal of the GST tag by treatment with PreScission protease samples were incubated at 37 °C for 16 h. Samples of 100 or 200 ng of protein were subjected to the filter retardation assay and detected with anti-FLAG (A) or anti-Myc antibody (B). C, cross-seeding of HD exon 1 protein aggregation with preformed fibrils derived from M-HDQ52. Proteins GST-F-HDQ20, GST-F-HDQ32, or GST-F-HDQ44 (final concentration, 50 μg/ml) were mixed with 2% ammonium molybdate, samples were visualized by electron microscopy. Small gold particles of 5 nm diameter (white arrows) are indicative of the secondary anti-Myc antibody, and 10 nm gold particles (black arrows) are indicative of the secondary anti-FLAG antibody.](image-url)
and G). Co-expression of HA-NOCT3 or HA-PQBP1 with GFP-HDQ17 led to enhanced localization of the transcription factors in the cytoplasm (Fig. 6, J and M). Strikingly, when co-expressed with GFP-HDQ72, both HA-HDQ20 and HA-HDQ32 as well as the transcription factors HA-NOCT3 and HA-PQBP1 were dislocated and sequestered in perinuclear inclusions, in which GFP- and HA-tagged proteins co-localized (Fig. 6, E, H, K, and N). These data clearly underscore that in intact cells htt fragments with an elongated polyQ stretch are able to recruit wild-type htt fragments and the transcription factors NOCT3 or PQBP1 as well. An association of these two transcription factors with htt in mammalian cells has not yet been described. Conceivably, sequestration of NOCT3 and PQBP1 in polyQ containing aggregates in neurons might interfere with their transcriptional activity, which could be detrimental to the cell.

Co-aggregation of HA- and GFP-tagged Fusion Proteins in COS1 Cells—The nature of the polyQ-mediated interaction between mutant htt and other proteins was further examined. Using a cell culture model of HD Kazantsev et al. (24) showed that inclusions in which htt fusion proteins with 25 and 104 glutamines co-localize are remarkably stable when incubated with SDS/Triton X-100 at 37 °C. This observation prompted us to apply the filter retardation assay to test whether the protein associates formed in COS1 cells are stable even under more stringent conditions.

GFP- and HA-tagged fusion proteins were expressed either alone or in combination in COS1 cells. Transfected cells were grown for 40–44 h and lysed, and whole cell extracts were prepared. 30 μg of each protein extract were separated by SDS-PAGE and blotted onto a nitrocellulose membrane. The proteins were detected with the anti-HA or the anti-CAG53b antibody as indicated.
was replaced by GFP-HDQ72 (Fig. 7). Interestingly, these aggregates could be split into two classes. Aggregates obtained from cells co-expressing GFP-HDQ72 and HA-NOCT3 or HA-PQBP1 were recognized only by the anti-CAG53b antibody (Fig. 7B) and hence consisted solely of GFP-HDQ72. In contrast, co-expression of GFP-HDQ72 and HA-HDQ20 or HA-HDQ32 yielded SDS-stable co-aggregates that were recognized by the anti-CAG53b and the anti-HA antibody as well (Fig. 7A).

Together, these findings demonstrate that the interaction of mutant htt with unrelated proteins differs from that occurring between mutant and wild-type htt. Although NOCT3 and PQBP1 are sequestered into polyQ containing htt inclusions, they are not tightly bound and can be released by treatment with detergent. In contrast, htt fragments of normal polyQ

**Fig. 6. Co-localization of GFP- and HA-tagged fusion proteins.** COS1 cells expressing either one or two of the indicated fusion proteins were cultivated for 40–44 h and then immunolabeled with Cy3-labeled antibodies directed against the HA tag. Hence, red indicates HA-HDQ20 in C–E, HA-HDQ32 in F–H, HA-NOCT3 in I–K, and HA-PQBP1 in L–N. GFP-tagged proteins GFP-HDQ17 (A, D, G, J, and M) and GFP-HDQ72 (B, E, H, K, and N) are visible directly by their intrinsic green fluorescence. Nuclei were stained blue with 4’,6-diamidino-2-phenylindole.
length are stably integrated into GFP-HDQ72 aggregates, indicating that htt sequences other than the polyQs are required for the formation of stable co-aggregates.

**DISCUSSION**

The formation of neuronal inclusions with aggregated mutant htt is a characteristic feature of HD (11, 12, 14, 27). Currently, the relationship between the formation of neuronal inclusions and cell death is not clear. It has been suggested that both aggregation of mutant htt and pathogenesis may occur independently, and inclusions may be mainly protective (28–30). In contrast, recent reports indicate that aggregates are indeed deleterious for cells (31), and it was shown for mouse and fly models of HD that inhibition of aggregate formation leads to the reversal of the mutant phenotype (32, 33).

However, a toxic gain of function of mutant htt associated with protein aggregation may not be the only cause of the disease. Recent findings indicate that a potential loss of function of wild-type htt possibly contributes to the disease phenotype. Wild-type htt is involved in several essential cellular functions (for review see Ref. 9). It behaves as an anti-apoptotic protein and reduces the toxicity of mutant htt in cell culture models as well as in vivo (6, 34). It was therefore suggested to regard HD as a disease caused by a toxic gain as well as a loss of htt function (7). However, the underlying molecular mechanisms are still unclear.

To clarify these issues, we have compared the interaction of recombinant htt fragments with expanded or normal polyQ tracts to the interaction with other polyQ-containing or polyQ-binding proteins. With our *in vitro* co-aggregation assay we could show that the aggregation process of HD proteins into fibrils is polyQ length-dependent. Moreover, the fibrils obtained *in vitro* are similar to those isolated from HD patients or transgenic mice (10–12). In contrast to other models (10, 15, 20, 35), this assay allows easy discrimination between HD proteins with different polyQ stretches by the specific detection of the small Myc or FLAG epitope tag, respectively, that also had been fused to the HD exon 1 protein sequence. Hence, our model can be used to address the influence of different polyQ domains on the co-aggregation of htt fragments. We found that htt fragments with normal length polyQ tracts (20–37 glutamines) are readily incorporated into SDS-resistant aggregates of mutant htt and contribute to the formation of fibrils. In contrast, neither the polyQ-binding transcription factor PQBP1 nor the polyQ protein NOCT3 formed SDS-stable co-aggregates with mutant HD exon 1 protein. Similarly, TBP with a polyQ stretch of 40 residues is not recruited into SDS-insoluble aggregates *in vitro* (15). These are important results, because they point to qualitative differences in the binding of mutant htt to the wild-type protein as opposed to the interaction of mutant htt with other polyQ-containing proteins. EM analysis revealed that the specific interaction between HD exon 1 proteins with normal and expanded polyQ sequences allows an intimate association of both protein moieties even in single fibrils. In contrast, such mixed fibrils were not observed, when mutant htt was co-aggregated with the polyQ-containing transcription factor NOCT3 or PQBP1 (data not shown).

To confirm our findings in cell-based assays, we next studied co-aggregation of htt exon 1 proteins in a mammalian cell model system of HD. In agreement with previous results, we found that the expansion of the polyQ tract in the HD exon 1
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protein leads to the formation of perinuclear inclusions, which can easily be detected by immunofluorescence microscopy (10, 35). Moreover, co-localization experiments confirmed that mutant htt fragments sequester cytosolic htt fragments with non-expanded polyQ tracts into these inclusions (15, 21, 24, 36). These data support the notion that a polyQ expansion in the pathological range recruits polyQ tracts in the normal range into htt aggregates and that this interaction, most likely facilitated by a polar zipper (37), is necessary and sufficient for the formation of insoluble protein aggregates in vitro and in vivo. Like the nonexpanded HD exon 1 proteins, the polyQ-containing transcription factor NOCT3 and the polyQ-binding protein PQBP1 were also sequestered into perinuclear inclusions by mutant htt, suggesting that wild-type htt, NOCT3, and PQBP1 form similar protein associates with mutant htt in vivo. However, because of the limited resolution of immunofluorescence microscopy, co-localization studies are unsuitable for analyzing the nature of polyQ-mediated protein-protein interactions. To examine in more detail the characteristics of polyQ-containing associates in mammalian cells, we applied the filter retardation assay to extracts of COS1 cells co-expressing the transcription factors PQBP1 or NOCT3 with mutant htt and to extracts containing mutant and wild-type htt proteins with varying polyQ sequence lengths. With this assay only very stable fibrillar htt aggregates can be detected, whereas less stable oligomeric structures and amorphous aggregates, which are not resistant to SDS treatment, cannot be identified. Neither PQBP1 nor NOCT3 formed SDS-stable co-aggregates with mutant HD exon 1 protein, whereas SDS-resistant co-aggregates of mutant and wild-type htt were clearly detectable in cells. Thus, the insoluble aggregates of mutant and wild-type htt are more stable and have different biochemical properties than co-aggregates formed of mutant htt and, e.g., the polyQ-containing transcription factor NOCT3. We suggest that mutant and wild-type htt form ordered fibrillar co-aggregates in vivo, whereas co-aggregates consisting of mutant htt and the transcription factors PQBP1 or NOCT3 are unordered. An analysis of COS1 cells expressing polyQ-containing proteins with varying polyQ lengths by immunoelectron microscopy is in progress.

Whether mixed fibrils containing both mutant and wild-type htt are formed in patient brains or HD transgenic animals is yet unclear. Our in vitro results indicate that heterologous htt fibrils consisting of both mutant and wild-type htt are morphologically indistinguishable from homologous aggregates consisting only of mutant htt. Thus, electron microscopy analysis of fibrillar structures purified from brain extracts of patients or HD transgenic mice may not allow morphological differentiation between mixed and homogenous htt fibrils. We suggest that additional studies using transgenic mice co-expressing epitope-tagged htt fusions with polyQ tracts in the normal and pathological range are required to address the question of co-aggregation in vivo.

In patients the formation of heterologous htt fibrils could be facilitated by proteolysis of both mutant and wild-type htt (36). htt is cleaved by caspase-3 at positions 513 and 530 (38) and by aspartic endopeptidases between amino acids 104–114 (39). Experimental evidence has been presented that N-terminally truncated htt fragments but not the full-length protein form insoluble aggregates and accumulate in neuronal nuclear inclusions (12). Thus, it seems likely that proteolytic cleavage of both wild-type and mutant htt occurs in neurons, and liberation of short N-terminal fragments is critical for the accumulation of mixed protein aggregates in vivo.

Recently, data have been provided that polyQ-containing suppressor peptides selectively bind to htt and are able to interfere with the aggregation process in vitro and in vivo (33). Thus, the binding of polyQ-containing proteins to mutant htt may modulate its aggregation kinetics. In this study a potential inhibitory effect of wild-type htt and NOCT3 on mutant htt aggregation was not examined. We found that both proteins associate with mutant htt, but only wild-type htt is integrated into mixed fibrils. Preliminary studies indicate that wild-type htt stimulates the fibrillogenesis of mutant htt in vitro, whereas NOCT3 and PQBP1 have a reverse effect. Thus, NOCT3 and PQBP1 may trigger the accumulation of amorphous aggregates and thereby behave similarly to heat shock proteins (22) or the polyQ-binding antibody 1C2 (40).

Our findings provide the basis to understand how both toxic gain of mutant htt and loss of neuroprotective wild-type htt function may be connected. Based on our in vitro and in vivo results, we propose the following pathomechanism for HD (Fig. 8): the polyQ expansion in mutant htt favors the formation of fibrillar aggregates when a critical protein concentration is exceeded. Although different polyQ-containing or even polyQ-binding proteins are able to associate with mutant htt, only wild-type htt is incorporated into fibrillar co-aggregates. PolyQ-mediated co-aggregation of mutant and wild-type htt into fibrillar structures may cause a loss of wild-type htt function in vivo, because the protein is removed from its natural environment and trapped in very stable mixed fibrils. It seems plausible to speculate that a progressive depletion of wild-type htt by irreversible sequestration into fibrils may contribute to the disease phenotype. Whether wild-type htt is trapped in fibrils, protofibrils, or even soluble oligomers remains elusive. The formation of toxic oligomeric Aβ intermediates has been described in cell-free and cell-based model systems of Alzheimer’s disease (41–44), suggesting that similar structures may also form in HD. However, whether they actually exist in patient brains and whether such structures contribute to the disease development needs further investigation.

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