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THE ROYAL SOCIETY

Aggregation of truncated GST–HD exon 1 fusion proteins containing normal range and expanded glutamine repeats

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We have shown previously by electron microscopy that the purified glutathione S-transferase (GST)–Huntington's disease (HD) exon 1 fusion protein with 51 glutamine residues (GST–HD51) is an oligomer, and that site-specific proteolytic cleavage of this fusion protein results in the formation of insoluble more highly ordered protein aggregates with a fibrillar or ribbon-like morphology (E. Scherzinger *et al.* (1997) *Cell* **90**, 549–558). Here we report that a truncated GST–HD exon 1 fusion protein with 51 glutamine residues, which lacks the proline-rich region C-terminal to the polyglutamine (polyQ) tract (GST–HD51ΔP) self-aggregates into high-molecular-mass protein aggregates without prior proteolytic cleavage. Electron micrographs of these protein aggregates revealed thread-like fibrils with a uniform diameter of *ca.* 25 nm. In contrast, proteolytic cleavage of GST–HD51ΔP resulted in the formation of numerous clusters of high-molecular-mass fibrils with a different, ribbon-like morphology. These structures were reminiscent of prion rods and β-amyloid fibrils in Alzheimer's disease. In agreement with our previous results with full-length GST–HD exon 1, the truncated fusion proteins GST–HD20ΔP and GST–HD30ΔP did not show any tendency to form more highly ordered structures, either with or without protease treatment.

Keywords: Huntington's disease; huntingtin; glutamine repeat; aggregation; amyloid fibrillogenesis

1. INTRODUCTION

Huntington's disease (HD) is a progressive neurodegenerative disorder with autosomal dominant inheritance (Harper 1996). The disorder is characterized by selective neuronal cell death, primarily in the cortex and striatum, leading to psychiatric symptoms, choreatic movement disturbances and cognitive decline (Vonsattel *et al.* 1985). Onset of the disease is generally in midlife but can vary from early childhood until well into old age. The mutation causing HD is an expansion of polymorphic CAG repeats located within exon 1 of the *IT-15* gene (Huntington's Disease Collaborative Research Group 1993). The CAG repeat is translated into a polyglutamine (polyQ) stretch. Thus in HD patients huntingtin proteins with 38–182 glutamine residues are expressed, whereas in healthy individuals huntingtin proteins with 8–41 glutamine residues are synthesized (Rubinsztein *et al.* 1996; Sathasivam *et al.* 1997). Furthermore, there is a strong correlation between, on the one hand, the age of onset and the severity of symptoms and, on the other hand, the length of the polyQ repeat expansion. However, the molecular mechanism by which an elongated polyQ stretch leads to selective neurodegeneration is still unknown. Several lines of mice transgenic for the HD mutation have been generated to study the pathomechanism of HD. Mangiarini *et al.* (1996) showed that transgenic mice expressing exon 1 of the human HD gene carrying CAG repeat expansions of 115–156 units exhibited a progressive neurological phenotype with similarities to HD. Electron microscopy revealed that the formation of characteristic neuronal nuclear inclusions (NIIs) containing aggregated huntingtin protein underlies the neurological dysfunction in these mice (Davies *et al.* 1997). In addition, we have shown that a recombinant glutathione S-transferase (GST)–HD exon 1 fusion protein with a polyQ expansion in the pathological range (51 glutamine residues) forms high-molecular-mass protein aggregates *in vitro* after site-specific proteolysis of the fusion protein. These aggregates, which were not detected after proteolysis of shorter fusion proteins (20 or 30 glutamine residues), revealed a fibrillar or ribbon-like morphology similar to that of scrapie prion rods and β-amyloid fibrils in Alzheimer's disease (Scherzinger *et al.* 1997). Recently, high-molecular-mass polyQ-containing protein aggregates were also detected in the brains of patients with HD (Becher *et al.* 1997; DiFiglia *et al.* 1997), SCA1 (Matilla *et al.* 1997; Skinner *et al.* 1997), SCA3 (Paulson *et al.* 1997), SCA7 (Holmberg *et al.* 1998) and dentatorubral–pallidoluysian atrophy (DRPLA) (Becher *et al.* 1997; Igarashi *et al.* 1998), suggesting that all these diseases are the result of a toxic amyloid fibrillogenesis, as has been proposed for Alzheimer's disease and the prion diseases.

Here we show that a truncated GST–HD exon 1 fusion protein with 51 glutamine residues (GST–HD51ΔP), which lacks the proline-rich region located immediately downstream of the glutamine repeat, self-assembles into more highly ordered structures with a fibrillar morphology. Under the same conditions fusion proteins containing only 20 or 30 glutamine residues do not self-aggregate.

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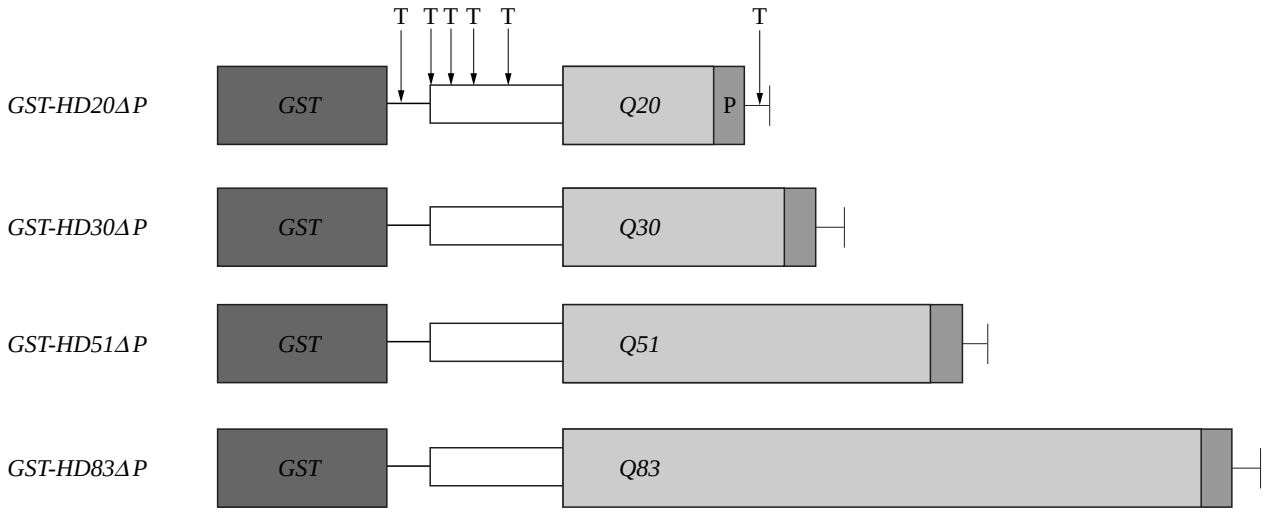

Figure 1. Schematic representation of primary structure of GST–HD fusion proteins. The amino acids corresponding to the N-terminal portion of huntingtin are boxed. Q and P stand for polyglutamine and polyproline extensions, respectively. Arrows labelled T indicate cleavage sites for trypsin.

2. MATERIALS AND METHODS

(a) *Strains and plasmids*

Escherichia coli DH10B (BRL) was used for plasmid construction; *E. coli* SCS1 (Stratagene) was used for the expression of GST–HD fusion proteins. Standard protocols for DNA manipulations were followed. The construction of the plasmids pCAG20ΔP and pCAG51ΔP encoding the fusion proteins GST–HD20ΔP and GST–HD51ΔP, respectively, has been described elsewhere (Wanker *et al*. 1998). *IT-15* cDNA sequences encoding the N-terminal portion of huntingtin, including the CAG repeats, were amplified by PCR with the oligonucleotides ES25 (5′-TGGGATCCGCATGGCGACCCTGGAAAAGCTGATGAAGG-3′) and ES27 (3′-CTCCTCGAGCGGCGGTGGCGGCTGTTGCTGCTGCTGCTG-5′) as primers and the plasmids pCAG30 and pCAG83 as templates (Scherzinger *et al*. 1997). Conditions for PCR were as described (Mangiarini *et al*. 1996). The resulting cDNA fragments were gel-purified, digested with *Bam*HI and *Xho*I and then inserted into the *Bam*HI–*Xho*I site of the expression vector pGEX-5X-1 (Pharmacia), yielding pCAG30ΔP and pCAG83ΔP, respectively. The plasmids pCAG30ΔP and pCAG83ΔP were used for the expression of the truncated HD exon 1 fusion proteins containing 30 and 83 glutamine residues, respectively.

(b) *Purification and proteolytic cleavage of GST–HD fusion proteins*

The procedure for the purification of GST–HD fusion proteins has been described elsewhere (Wanker *et al*. 1998). The purified proteins were stored at −70 °C at a concentration of 1 mg ml^{-1}. The GST–HD fusion proteins (2 µg) were digested with modified trypsin (Boehringer Mannheim, sequencing grade) at an enzyme:substrate ratio of 1:20 (w/w). The reaction was performed in 20 µl of 20 mM Tris–HCl (pH 8.0), 150 mM NaCl and 2 mM $CaCl_2$ for 3–16 h at 37 °C.

(c) *Microscopic analysis of GST–HD fusion proteins*

For electron microscopic observation, the native or protease-digested GST–HD fusion proteins were adjusted to a final concentration of 50 µg ml^{-1} in 40 mM Tris–HCl (pH 8.0), 150 mM NaCl, 0.1 mM EDTA and 5% (v/v) glycerol. Samples were negatively stained with 1% (w/v) uranyl acetate and viewed using a Philips CM100 electron microscope.

3. RESULTS

(a) *Purification of GST–HD fusion proteins lacking the proline-rich region*

Previously we have produced GST–HD exon 1 fusion proteins containing polyQ tracts of various lengths and the proline-rich region located C-terminal to the polyQ tract in HD exon 1 (Scherzinger *et al*. 1997). In this study, truncated GST–HD exon 1 fusion proteins with 20 (GST–HD20ΔP), 30 (GST–HD30ΔP), 51 (GST–HD51ΔP) and 83 (GST–HD83ΔP) glutamine residues, lacking most of the proline-rich region, were used. These proteins were purified by affinity chromatography on glutathione–agarose (Smith & Johnson 1988). The structures of the various GST–HD fusion proteins are shown in figure 1. SDS–PAGE analysis of the purified proteins GST–HD20ΔP, GST–HD30ΔP, GST–HD51ΔP and GST–HD83ΔP revealed major bands migrating at about 30, 33, 39 and 45 kDa, respectively (figure 2). These bands were also detected by immunoblot analysis with the anti-huntingtin antibody HD1 (results not shown). All recombinant proteins migrated at a size corresponding nearly to that predicted from their amino-acid sequence. Interestingly, in the protein preparations of GST–HD51ΔP and GST–HD83ΔP an additional band was detected on top of the gel, indicating the presence of insoluble high-molecular-mass protein aggregates in these preparations (Scherzinger *et al*. 1997). Such high-molecular-mass protein bands were never detected in the protein preparations of GST–HD20ΔP or GST–HD30ΔP.

(b) *Self-assembly of GST–HD51ΔP into more highly ordered structures*

To examine the morphology of the aggregated GST–HD51ΔP fusion protein, the high-molecular-mass aggregates were separated from the soluble protein by centrifugation and then analysed by electron microscopy. Ordered protein aggregates with a strikingly uniform

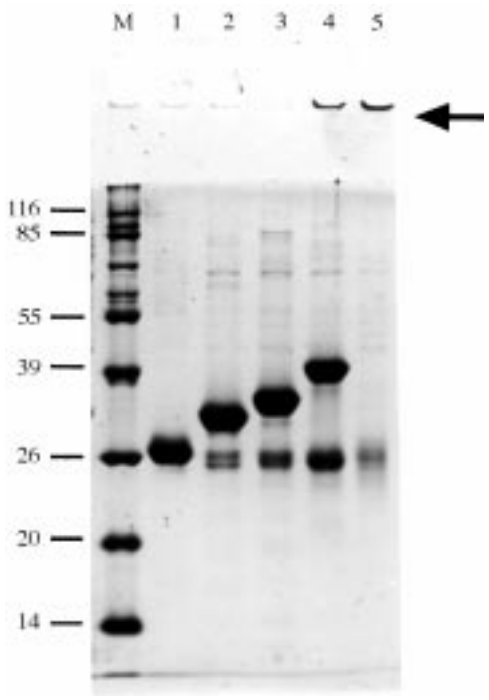

Figure 2. SDS–PAGE analysis of GST–HD fusion proteins. Aliquots (15 µl) of eluates from the glutathione–agarose column were subjected to SDS–PAGE (12.5% (w/v) gel) and analysed by staining with Coomassie blue R. Lanes 1–5 contained GST, GST–HD20ΔP, GST–HD30ΔP, GST–HD51ΔP and GST–HD83ΔP, respectively; lane M contained molecular mass standards (molecular masses are indicated in kDa at the left). The arrow indicates insoluble high-molecular-mass protein aggregates.

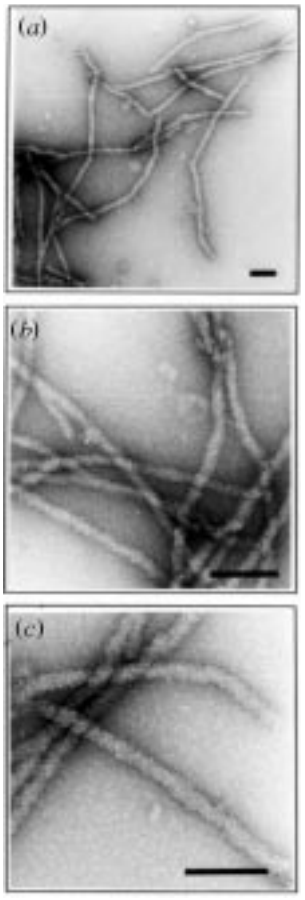

Figure 3. Electron micrographs of aggregated GST–HD51ΔP fusion protein. Aggregated GST–HD51ΔP fusion protein was separated from soluble protein by centrifugation, negatively stained with uranyl acetate, and viewed by electron microscopy. The scale bars in (*a*)–(*c*) are 100 nm.

fibrillar morphology were observed (figure 3). The fibrils had a diameter of 25 nm and a length of several micrometres. At higher magnification, small spherical particles could be detected on the surface of the fibrils, presumably representing the GST tag (Smith & Johnson 1988).

(c) *Proteolytic cleavage of GST–HD30ΔP and GST–HD51ΔP fusion proteins*

In our previous study (Scherzinger *et al*. 1997), we showed that proteolytic digestion of the GST–HD51 fusion protein with trypsin or factor Xa results in the formation of insoluble protein aggregates with a fibrillar or ribbon-like morphology. Such filaments were not produced by proteolysis of the GST–HD20 or GST–HD30 fusion proteins. To examine whether the deletion of the proline-rich region from the C-terminus of the HD exon 1 protein has any effect on protein aggregation and/or the morphology of the resulting fibrils, the purified fusion proteins GST–HD30ΔP and GST–HD51ΔP were digested with trypsin. Electron microscopy of the undigested GST–HD30ΔP and GST–HD51ΔP proteins revealed nearly spherical particles with diameters of 6–7 nm, consistent with an oligomeric form of the fusion proteins (figure 4*a*,*c*). In contrast, the preparations obtained by tryptic digestion of GST–HD51ΔP showed numerous clusters of high-molecular-mass fibrils with a ribbon-like morphology (figure 4*d*). These ribbon-like structures were very similar to the fibrils that have been previously observed after tryptic digestion of full-length GST–HD51 fusion protein (Scherzinger *et al*. 1997). In strong contrast with GST–HD51ΔP, tryptic digestion of GST–HD30ΔP, which contained only 30 glutamine residues, did not result in the formation of any more highly ordered fibrillar structures (figure 4*b*), although clots of various sizes containing small particles were frequently detected in this protein preparation.

4. DISCUSSION

In this study we analysed by SDS–PAGE and electron microscopy the aggregation of truncated GST–HD exon 1 fusion proteins, which lack the proline-rich region at the C-terminus of HD exon 1. We found that the truncated fusion protein GST–HD51ΔP readily self-assembled into fibrillar protein aggregates without prior proteolytic cleavage, whereas the fusion protein GST–HD51, containing the proline-rich region, showed little or no tendency to self-aggregate (Scherzinger *et al*. 1997). This indicates that the presence of the proline-rich region located C-terminal to the polyQ tract increases the solubility of the fusion protein.

Electron micrographs of the GST–HD51ΔP protein aggregates revealed a fibrillar morphology that was different from that obtained after proteolytic cleavage of GST–HD51ΔP (figure 4*d*). All GST–HD51ΔP fibrils seemed to have a uniform structure and a diameter of 25 nm. No cross-links between single fibrils were detected. In contrast, the protein fractions obtained by proteolytic

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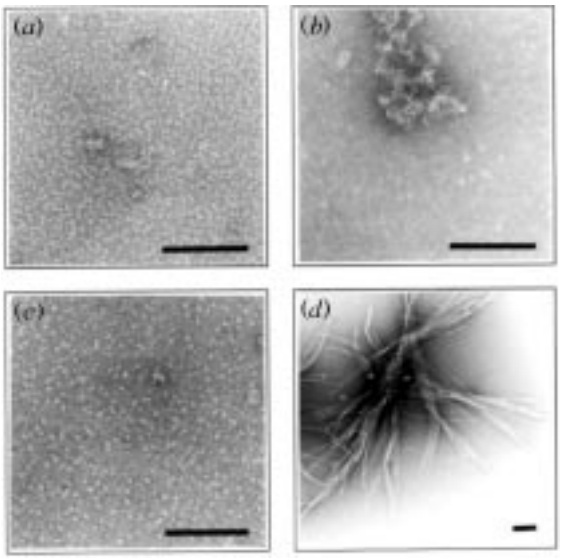

Figure 4. Electron micrographs of native GST–HD fusion proteins and their trypsin cleavage products. Purified GST fusion proteins were treated with protease, negatively stained with uranyl acetate and viewed by electron microscopy. The undigested GST–HD30ΔP (*a*) and GST–HD51ΔP (*c*) molecules appear as a homogeneous population of small, round particles. Digestion of GST–HD30ΔP with trypsin shows no evidence for the formation of ordered structures (*b*). In contrast, proteolytic cleavage of GST–HD51ΔP results in the formation of more highly ordered amyloid-like fibrils (*d*). The scale bars in (*a*)–(*d*) are 100 nm.

digestion of GST–HD51ΔP showed large clusters of high-molecular-mass fibrils with a typical ribbon-like morphology similar to that previously observed for trypsin-digested GST–HD51 (Scherzinger *et al*. 1997). This indicates that the GST tag present in the GST–HD51ΔP protein aggregates has a strong influence on the aggregation behaviour of the HD exon 1 protein. At higher magnification, we detected tightly packed spherical particles with a diameter of *ca*. 6–7 nm on the surface of the GST–HD51ΔP fibrils. This suggests that the soluble oligomeric GST–HD51ΔP particles (figure 4*c*), are assembled into ordered fibrillar structures owing to the formation of hydrogen-bonded hairpins between the polyQ chains within HD exon 1 (Perutz 1996). In good agreement with our previous results, the undigested or trypsin-digested GST–HD20ΔP and GST–HD30ΔP fusion proteins did not form any ordered high-molecular-mass protein aggregates. These results substantiate our previous findings that only polyQ tracts beyond a critical length might be capable of forming polar zippers (Perutz 1996), resulting in the formation of amyloid-like fibrils. Experiments to determine the critical length of the glutamine repeat necessary for the self-assembly of HD exon 1 proteins into more highly ordered structures are in progress.

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