The role of the proteasome in Huntington's disease
Krom, Sabine

Citation for published version (APA):
Krom, S. (2013). The role of the proteasome in Huntington’s disease

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: http://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.
MIMICKING PROTEASOMAL RELEASE OF POLYGLUTAMINE PEPTIDES INITIATES AGGREGATION AND TOXICITY

M. Raspe*
J. Gillis*
H. Krol
S. Schipper-Krom
K. Bosch
H. van Veen
E. A. J. Reits

* These authors contributed equally to this work

Department of Cell Biology and Histology, Academic Medical Center, Meibergdreef 15, 1105 AZ Amsterdam, The Netherlands

ABSTRACT

Several neurodegenerative disorders, including Huntington’s disease, are caused by expansion of the polyglutamine (polyQ) tract over 40 glutamines in the disease-related protein. Fragments of these proteins containing the expanded polyQ tract are thought to initiate aggregation and represent the toxic species. Although it is not clear how these toxic fragments are generated, in vitro data suggest that proteasomes are unable to digest polyQ tracts. To examine whether the resulting polyQ peptides could initiate aggregation in living cells, we mimicked proteasomal release of monomeric polyQ peptides. These peptides lack the commonly used starting methionine residue or any additional tag. Only expanded polyQ peptides appear peptidase-resistant, and their accumulation initiated the aggregation process. As observed in polyQ disorders, these aggregates subsequently sequestered proteasomes, ubiquitin and polyQ proteins, and recruited HSP70. The generated expanded polyQ peptides were toxic to neuronal cells. Our approach mimics proteasomal release of pure polyQ peptides in living cells, and represents a valuable tool to screen for proteins and compounds that affect aggregation and toxicity.

INTRODUCTION

Numerous neurodegenerative diseases are manifested by the accumulation and aggregation of intracellular proteins. These diseases include polyglutamine (polyQ) expansion disorders like Huntington’s disease (HD), Spinal Bulbar Muscular Atrophy (SBMA) and various spinocerebellar ataxia’s (SCAs). PolyQ disorders are dominantly inherited and caused by expansions of CAG repeats. Normally, the disease-related proteins involved contain sequences of 6-40 glutamine repeats, while expansion of these tracts to 40-300 repeats leads to disease. The age of onset of the disorder is inversely correlated with the repeat length of the polyQ tracts (reviewed by [1]).

The presence of proteolytic protein fragments harbouring a polyQ tract in aggregates [2-5] has led to the ‘toxic fragment hypothesis’, which states that proteolytic fragments of polyQ-expanded huntingtin [6], androgen receptor [7] or certain ataxins [8-10] initiate protein aggregation and induce neuronal toxicity. Full length polyQ proteins aggregate, but at a much slower rate than their proteolytic fragments [7]. These fragments can be generated by proteases like caspasases, aspartic endopeptidases, calpains and the proteasome [11-15]. Accumulation of these proteolytic fragments may therefore function as a nucleation centre that sequesters full-length polyQ proteins in time. The proteasome can degrade both wild-type and expanded forms of most polyQ proteins, as was demonstrated in cultured cells and animal models [16, 17]. Surprisingly, polyQ-expanded proteins are not degraded to completion by the proteasome both in vitro and in vivo [18, 19]. Venkatraman and colleagues showed that isolated proteasomes cannot digest polyQ tracts present within a protein, which will result in the release of polyQ peptides. While flanking amino acids may be removed by exo-peptidases, the polyQ tracts themselves will accumulate when not efficiently cleared by downstream peptidases.

RESULTS

PolyQ-expanded peptides accumulate and induce intracellular aggregates

To examine the fate of proteasomal-released polyQ-peptides in living cells, we generated fusion proteins of fluorescently-tagged Ub with polyQ peptides of wild-type and disease-related lengths. Upon expression, the C-terminal polyQ peptide will be efficiently released from GFP-Ub by immediate cleavage via ubiquitin C-terminal hydrolases [20]. As a result, the generated polyQ peptide does not contain a starting methionine residue, which may affect degradation properties due to similarities with the N-terminus of a full-length protein [23]. Also, no tags (such as fluorophores or antibody epitopes) were directly attached to the polyQ peptide. As Ub was fluorescently tagged, the fluorescence intensity reflected the amount of generated polyQ peptides. PolyQ peptides of 16, 65 or 112 glutamine residues were fused to GFP-Ub resulting in GFP-Ub-Q16, GFP-Ub-Q65 and GFP-Ub-Q112, respectively (Fig. 1A).

Expression of the different GFP-Ub-polyQ proteins and subsequent release of polyQ peptides were analyzed 48 hours after transfection. Western blot analysis demonstrated the presence of GFP-Ub (36kDa) separated from all polyQ proteins (Fig. 1B, left panel). In addition, a large Ub conjugate was present, as shown before for GFP-Ub [24]. No additional bands were detected that could represent uncleaved GFP-Ub-polyQ proteins. Efficient cleavage was also observed when the Western blot was analysed for Ub (Fig. 1B, right panel). These results indicate that all polyQ peptides were efficiently cleaved from the GFP-Ub protein. Subsequent immunoblotting against polyQ using the antibody 1C2 [25] showed that polyQ peptides were present in the GFP-Ub-Q65 and GFP-Ub-Q112 lanes (Fig. 1C). The mobility on SDS-PAGE of expanded polyQ peptides was different from their
Mimicking proteasomal release of polyglutamine peptides initiates aggregation and toxicity

THREE

Mimicking proteasomal release of polyglutamine peptides initiates aggregation and toxicity

calculated molecular weights, as has been observed before for polyQ-containing proteins [18, 26]. Some additional high molecular weight bands were present, which may represent oligomeric polyQ structures as these bands are GFP and Ub negative (Fig. 1B). The absence of Q16 peptides in cells expressing GFP-Ub-Q16 indicates that small Q peptides are efficiently cleared from the cytoplasm. It is unlikely that small Q peptides are not recognized by the 1C2 antibody, since a Q16-GFP fusion protein was recognized by 1C2 with almost equal efficiency as expanded GFP-polyQ fusions (supplementary Fig. 1A). Accumulation of Q65 and Q112 peptides, but not of Q16 peptides, suggests that expanded polyQ peptides were not efficiently degraded in living cells. To our knowledge, these peptides represent the first group of peptides that are resistant to degradation.

Fig. 1. PolyQ-expanded peptides induce intracellular aggregates.

(A) Schematic representation of GFP-Ub-polyQ (Q16, Q65 and Q112) fusion proteins and the generation of polyQ peptides upon synthesis and cleavage by Ub C-terminal hydrolases. (B) Cytosolic cell lysates of HEK293T expressing the different GFP-Ub-polyQ fusions were immuno-blotted against GFP (left) or Ub (right) 48 hours after transfection. GFP-Ub migrated at the same height for all three fusion proteins, indicating efficient cleavage of polyQ from GFP-Ub. Transfection efficiencies were lower for expanded polyQ peptide constructs. (C) Subsequent staining with an antibody against polyQ (1C2) revealed only the presence of polyQ peptides in cells expressing GFP-Ub-Q65 and GFP-Ub-Q112, and not of GFP-Ub-Q16. The asterix indicates potential oligomeric structures. (D) Confocal images of GFP-Ub and the various GFP-Ub-polyQ distribution in Mel JuSo cells. GFP-Ub-Q16 showed a Ub distribution similar to free GFP-Ub, whereas a high percentage of cells expressing GFP-Ub-Q65 and GFP-Ub-Q112 showed Ub redistribution into aggregates. Scale bar ~5 μm. (E) Percentage of transfected HEK293T cells exhibiting fluorescent aggregate at 48 and 72 h after transfection of cells (data are mean ± SEM of 3 different experiments). The amount of aggregates in cells expressing expanded polyQ peptides increased both in time and with polyQ length. (F) GFP-Ub was present in a ring around the aggregate induced by GFP-Ub-Q112 (left panel) that had a fibrillar structure at the ultrastructural level (middle panel), similar to structures induced by non-cleavable GFP-Q65 (right panel). Scale bar ~5 μm. (G) Filter retardation assay showed entrapment of aggregates in HEK293T cells expressing GFP-Ub-Q65, GFP-Ub-Q112 and Htt exon1-Q203-GFP, after immunostaining using the 1C2 antibody. In contrast, GFP is only present when the non-cleavable fusion protein Htt exon1-GFP is used. (H) Confocal images of cells expressing GFP-Ub or the various GFP-Ub-polyQ constructs after immunostaining using antibodies against polyQ (1C2). Mel JuSo cells expressing GFP-Ub or GFP-Ub-Q16 showed no polyQ staining. Cells expressing GFP-Ub-Q65 and GFP-Ub-Q112 showed cytoplasmic polyQ staining when no aggregates were present. The presence of aggregates depleted the cells of free polyQ peptides, preventing polyQ staining. The arrows indicate an aggregate. Scale bar ~5 μm. (I) Protease K treatment dissolved the protein shells around the polyQ aggregate, resulting in labeling the outside of the aggregation core with the anti-polyQ antibody 1C2. Scale bar ~5 μm. (J) The Q-binding peptide QBP1-CFP was redistributed into aggregates induced by RFP-Ub-Q112. The arrow indicates the presence of a visible aggregate by phase contrast. Scale bar ~5 μm.
Since proteolytic protein fragments containing polyQ tracts are more aggregation prone than the full-length protein, we examined whether the accumulation of Q65 and Q112 peptides initiated aggregate formation. We observed a similar intracellular distribution of GFP-Ub in cells transfected with either GFP-Ub or GFP-Ub-Q16. GFP-Ub was enriched in the nucleus but was also present in the cytoplasm and on vesicles (Fig. 1D), similar to the distribution of endogenous Ub [24, 27]. In contrast, expression of GFP-Ub-Q65 and GFP-Ub-Q112 resulted in the appearance of a distinct intracellular structure decorated with fluorescent Ub in a high percentage of the transfected cells, present in either the nucleus or cytoplasm. The number of cells containing these structures increased both in time and with polyQ length (Fig. 1E). To see whether the length-dependency of aggregate formation would also hold true for polyQ lengths near the threshold, we expressed GFP-Ub fUSED to polyQ peptides of 33 or 48 glutamine residues. Whereas GFP-Ub-Q33 showed no aggregates, cells expressing GFP-Ub-Q48 showed aggregates, although in a much lower percentage of cells than those expressing Q65 or Q112 peptides (supplementary Fig. 1B, data not shown). GFP-Ub fluorescence was usually present in a ring around a dark core indicating that Ub was recruited (Fig. 1F). At the ultrastructural level, this structure showed a radiating dense core similar to aggregates formed by non-cleavable GFP-polyQ fusion proteins (Fig. 1F) and expanded huntingtin [28]. In cells expressing Q65 and Q112 peptides, these dense structures were resistant to SDS and selectively trapped in a filter-retardation assay [29]. Immunostaining using 1C2 showed that the trapped structures contained polyQ peptides (Fig. 1G), similar to huntingtin exon-1 Q103 (httx1-Q103-GFP) [29]. This suggests that expanded polyQ peptides induce intracellular SDS-resistant aggregates. Although httx1-Q103-GFP is also positive for GFP, no GFP is present on filter trap with the GFP-Ub-polyQ constructs, indicating efficient cleavage of the GFP-Ub-polyQ fusion proteins (Fig. 1G). Also, analysis of the soluble and insoluble fraction of cell lysates showed no uncleaved GFP-Ub-Q112 fusion proteins in either fraction (supplementary Fig. 1C).

To confirm the presence of polyQ peptides in intracellular aggregates, we immunostained cells expressing Q16, Q65 or Q112 peptides with 1C2. As expected, no polyQ peptides were detected in cells expressing GFP-Ub or GFP-Ub-Q16 (Fig. 1H). However, cells transfected with GFP-Ub-Q65 or GFP-Ub-Q112 showed two patterns of polyQ staining, dependent on the presence of aggregates. When aggregates were not present, polyQ staining was mainly cytoplasmic, whereas GFP-Ub localization was predominantly nuclear. By contrast, cells containing polyQ peptide aggregates were not recognized by 1C2 (Fig. 1H, arrows indicate an aggregate). A similar difference in immunostaining was obtained using the anti-polyQ antibody MW1 [30] (supplementary Fig. 1D). The absence of polyQ staining in cells containing aggregates is likely due to the dense aggregate structure and its surrounding protein layers that may shield the polyQ core. Indeed, pretreatment with proteasome inhibitor degrades shielding proteins and resulted in positive immunostaining of polyQ peptide aggregates (Fig. 1I), as has been observed previously for huntingtin aggregates [28]. To further confirm that the aggregates contain polyQ peptides, we used a cyan fluorescent protein (CFP) tagged Q-binding peptide (QBP-3) which selectively binds to polyQ aggregates [31]. QBP-1 showed a cytoplasmic distribution pattern when expressed alone or together with RFP-Ub or RFP-Ub-Q16 (data not shown). However, cells harboring aggregates initiated by Q112 peptides showed binding of QBP-1 to aggregates (Fig. 1J). Taken together, these results indicate that expanded polyQ peptides are not efficiently degraded and subsequently initiate formation of aggregates that display all characteristics of disease-related polyQ aggregates.

### PolyQ peptide aggregates recruit proteasomes, ubiquitin and chaperones

Aggregates formed by expanded polyQ proteins often sequester proteins involved in the ubiquitin proteasome system (UPS) but also chaperones [18, 21]. We examined whether aggregates induced by expanded polyQ peptides showed a similar sequestration of UPS components. GFP-Ub was present in a ring around the aggregates (Fig. 1F). Absence of Ub in the aggregate core can be explained by the lack of lysine residues in polyQ peptides, thereby excluding ubiquitination of the polyQ peptides. The presence of GFP-Ub around the core was not due to inefficient cleavage of GFP-Ub-polyQ, since no uncleaved GFP-Ub-polyQ fusions could be detected by SDS-PAGE (Fig. 1B and supplementary Fig. 1C) and filtertrap (Fig. 1G). In addition, co-expression of GFP-Ub with RFP-Ub-Q112 showed a similar sequestration of both fluorescently-tagged Ub proteins into aggregates (Fig. 2A), indicating efficient cleavage. This suggests that the presence of GFP-Ub is due to ubiquitination of sequestered proteins.

We examined whether proteasomes co-localized with polyQ aggregates in our model, by co-expressing the different RFP-Ub-polyQ constructs with GFP-tagged immuno-proteasomal subunit LMP2. LMP2 is efficiently incorporated into active proteasomes [32]. Notably, LMP2-GFP was present in the core of polyQ aggregates, suggesting that proteasomes were recruited to aggregates even before Ub sequestration (Fig. 2B). A similar recruitment was observed when using the constitutive proteasome subunit β7 (Fig. 2D). This finding most likely reflects a proteasomal attempt to degrade accumulating polyQ peptides. The sequestered proteasomes and Ub seemed irreversibly trapped, which was revealed when Fluorescence Recovery After Photobleaching (FRAP) [33] was applied to determine on/off rates of the sequestered molecules. Upon photobleaching of one half of an aggregate, no exchange between the sequestered proteasomes or Ub and the surroundings was observed (supplementary Fig. 2A). This indicates that the proteasome becomes immobilized, as has been previously observed [18].

We also examined whether chaperones such as HSP70 were interacting with polyQ aggregates, as has been observed in polyQ diseases [21, 34]. Upon co-transfection of the different RFP-Ub-polyQ fusion proteins with GFP-tagged HSP70, we observed an additional ring-like structure of HSP70-GFP around the Ub-positive aggregate (Fig. 2C). This indicates that the proteasome becomes immobilized, as has been previously observed [18].
Mimicking proteasomal release of polyglutamine peptides initiates aggregation and toxicity

THREE

Mimicking proteasomal release of polyglutamine peptides initiates aggregation and toxicity

Mimicking proteasomal release of polyglutamine peptides initiates aggregation and toxicity

Fig. 2. PolyQ peptide aggregates recruit UPS components and chaperones.

Mel Juso cells were transfected with the indicated constructs and imaged 48 hours after transfection. (A) Co-expression of GFP-Ub and RFP-Ub derived from RFP-Ub-Q112 resulted in identical redistribution into aggregates. (B) Proteasomes labeled with LMP2-GFP colocalize with the core of aggregates induced by RFP-Ub-Q112, with RFP-Ub surrounding the core. LMP2-GFP was freely distributed in nucleus and cytoplasm of cells expressing RFP-Ub-Q16. (C) The chaperone HSP70-GFP was redistributed into aggregates induced by RFP-Ub-Q112, and formed an additional ring around the Ub-positive polyQ peptide aggregate. (D) Upon transfection with GFP-Ub-Q112 or httex1-Q103-GFP together with the proteasomal subunit β7-RFP, cells were immunostained for endogenous HSP70. The proteasome was within the aggregate core, surrounded by Ub and an additional ring of chaperones. Scale bar ~5 μm.

Sequestering of glutamine-containing proteins into polyQ peptide aggregates

The presence of httex1-Q103 in ring-like structures around the aggregate and not within the core (Fig. 2D) suggests recruitment of large polyQ fragments into aggregates in a later stage. To examine this hypothesis, we co-expressed RFP-Ub-Q112 and httex1-Q103-GFP. Indeed, we found that httex1-Q103-GFP was sequestered into aggregates induced by polyQ peptides (Fig. 3A). In addition, the aggregation rate of httex1-Q103-GFP was also dramatically increased when Q112 peptides were present (supplementary Fig. 2B), which suggests that polyQ peptides initiate aggregates that accelerate huntingtin aggregation. Similar results were obtained with truncated polyQ-expanded ataxin-3 (atx3-Q85-GFP) and the SBMA-related truncated androgen receptor with a Q84 repeat (AR-Q84-GFP) (data not shown).
Aggregates induced by disease-related polyQ proteins also sequester the wild-type protein expressed by the non-expanded allele [8, 35]. We examined whether polyQ peptide aggregates also sequester non-expanded, wild-type polyQ proteins. The non-expanded httex1-Q25-GFP remained freely distributed in cells that co-expressed either RFP-Ub or RFP-Ub-Q16 (supplementary Fig. 2B). In contrast, httex1-Q25-GFP was recruited into polyQ peptide aggregates when co-transfected with RFP-Ub-Q112 (Fig. 3B and 2B). A similar entrapment of wild-type truncated ataxin-3 (atx3-Q28-GFP) (supplementary Fig. 2C) and the truncated androgen receptor (AR-Q19-GFP) was observed (data not shown). This sequestration of wild-type polyQ proteins may therefore lead to loss of function. Sequestration of non-expanded polyQ proteins was not limited to disease-related proteins, as other polyQ proteins were recruited into aggregates initiated by polyQ peptides, including Q16-GFP (Fig. 3C), and also the Q-tract containing transcription factor TBP1 when nuclear aggregates were present (Fig. 3D).

**PolyQ peptides induce aggregates and toxicity in neuronal cells**

To examine whether polyQ peptides also initiate aggregate formation in neuronal cells, we transiently transfected N2A neuroblastoma cells with the various GFP-Ub-polyQ constructs. N2A cells transfected with either GFP-Ub-Q65 or GFP-Ub-Q112 developed aggregates similar to those present in non-neuronal cells (Fig. 4A), whereas GFP-Ub-Q16 expressing cells showed an Ub distribution comparable to GFP-Ub alone. Since HD mostly affects striatal cells, we also used immortalized STHdh+/+ striatal cells [36] which similarly generated intracellular aggregates when transfected with GFP-Ub-Q65 or Q112 (Fig. 4A). Many cells rounded up after expression of expanded polyQ peptides, suggesting toxicity, although this did not correlate with the presence of GFP-Ub positive aggregates. To determine whether the expressed polyQ peptides were toxic, the viability of transfected N2A cells was tested using propidium iodide (PI). Expression of expanded polyQ peptides resulted in increased numbers of PI-positive cells (data not shown). However, hardly any double-positive cells were observed. This is presumably explained by the fact that uptake of PI into polyQ peptide expressing cells was often preceded by loss of GFP fluorescence (Fig. 4B) as observed previously [37]. Because loss of fluorescence seemed to be associated with cell death, we used another approach to quantify polyQ peptide induced toxicity. To determine changes in the number of GFP-Ub positive cells in time, we used FACS analysis and compared cell populations expressing the different GFP-Ub-polyQ proteins at 24 and 48 hours after transfection. There was no difference in GFP-Ub fluorescence between cells expressing either GFP-Ub or GFP-Q16 in time. However, a significant decrease in fluorescence was observed in cells expressing GFP-Ub-Q112 when compared to GFP-Ub or GFP-Ub-Q16 (p<0.05), indicating that expression of Q112 peptides induced cell death (Fig. 4C). GFP-Ub-Q65 had a mild, although not significant, effect on cell death. Taken together, these results showed that expanded polyQ peptides form aggregates and become toxic to neuronal cells.

**DISCUSSION**

Proteolytic fragments containing expanded polyQ tracts are more aggregation-prone than original full-length proteins, as has been shown for huntingtin [6], androgen receptor [7], ataxin-3 [8] and ataxin-7 [9]. Recently, it was also postulated that an expanded polyQ fragment was expressed in SCAB due to anti-sense transcription resulting in polyQ inclusions [38]. These data suggest that polyQ fragments may be fundamental in initiating aggregation. It has however been shown that expanded polyQ proteins are efficiently targeted to the proteasome [18], which can degrade entire proteins with the exception of polyQ tracts [19]. Degradation by the proteasome may also release polyQ peptides, whose flanking amino acids may be removed by exo-peptidases. It is unknown whether the resulting pure polyQ peptides are rapidly degraded by peptidases. If resistant, their subsequent accumulation may initiate aggregation and toxicity as observed in polyQ disorders. In order to examine this toxic fragment hypothesis, we mimicked intracellular proteasomal polyQ peptide generation as closely as possible by fusing pure polyQ peptides to GFP-tagged Ub. While Ub-polyQ fusions have been used before, these polyQ fragments also included either GFP tags.
Mimicking proteasomal release of polyglutamine peptides initiates aggregation and toxicity

[39, 40] or additional amino acids including a starting methionine residue [41]. Expression of our constructs resulted in the efficient release of “naked” polyQ peptides due to immediate cleavage by Ub C-terminal hydrolases. This was shown both by SDS-PAGE that showed a band at equal height for GFP-Ub irrespective of the original construct (Fig. 1B), analysis of the filtertrap assay (Fig. 1G) and the insoluble fraction (supplementary Fig. 1C) and different intracellular locations of GFP-Ub and polyQ peptides (Fig. 1H). Since the released polyQ peptides do not contain a starting methionine or additional tags, they closely resemble peptide generation by the proteasome. All previous studies have relied on expression of polyQ fusions that did include such features, which can significantly alter the in vivo behavior of polyQ fragments. Starting methionines will lend the peptides resemblance to the N-terminus of proteins, possibly affecting the rate of degradation [23]. Fluorescent tags contain lysine residues, which can serve as targets for ubiquitination and subsequent degradation by the proteasome. The intracellular release of monomeric polyQ peptides is also closer to the in vivo situation than the addition of synthesized polyQ peptide aggregates to cells [42].

We showed that only polyQ peptides with Q repeat lengths similar to disease-related peptides accumulated in the cell and initiated aggregation. The characteristics of aggregates induced by expanded polyQ peptides were similar to aggregates initiated by expression of expanded polyQ-containing proteins [18, 21, 22, 28]. These characteristics include sequestration of proteasomes, ubiquitin and other polyQ containing proteins such as TBP, and the presence of HSP70. While previous studies only speculated on the effect of proteasomal release of polyQ peptides in living cells, we show here that ‘proteasomal-derived’ expanded polyQ peptides by themselves are sufficient to accumulate and initiate aggregation. Accumulation of expanded polyQ peptides is toxic to neuronal cells, but it remains to be established which particular step in aggregate formation is toxic. The toxicity seems to be induced by necrosis instead of apoptosis, as no apoptotic markers such as annexin-5 or activated caspases were detected (data not shown). The toxic species may be either small polyQ peptide oligomers or large polyQ aggregates. Further studies are required to determine whether the proteasome can indeed generate similar polyQ peptides from different polyQ proteins. If so, these released polyQ peptides may be the common feature of the different polyQ disorders.

Based on our findings, we propose a model in which expanded polyQ peptides are degradation-resistant, and their accumulation leads to intracellular polyQ aggregates (Fig. 5). Proteasomes are rapidly recruited into the polyQ core, possibly in a final attempt to degrade the expanded polyQ peptides. Subsequently, other proteins are sequestered and ubiquitinated, perhaps due to (partial) unfolding. These events also lead to the binding of chaperones like HSP70 that may recognize denatured proteins. All these events result in concentric ring-like structures formed around the aggregate (Fig. 5). Essential proteins are depleted from the cell, contributing to cellular dysfunction. We conclude that polyQ peptides may be fundamental in initiating aggregation and sequestration of different types of proteins including polyQ proteins. Whereas FRAP experiments indicated that UPS components can still be solubilized.

We were able to detect expanded polyQ peptides containing Q65 or Q112 on Western blot and by immunostaining in fixed cells, but we were unable to detect any Q16 peptides. These short polyQ peptides are most likely rapidly degraded by downstream peptidases like PSA [43] that can digest short polyQ peptides and perhaps also extended peptides with less efficiency. Alternatively, a technical explanation for this result might be poor staining by IC2. It has been suggested that anti-polyQ antibodies do not detect the polyQ peptide itself, but interact with the secondary structure created by the expanded polyQ peptide [44]. Nonetheless, we showed that the IC2 antibody was able to recognize a Q16 peptide fused to GFP with almost equal efficiency as GFP-Q65 and GFP-Q112 proteins. Similarly, the polyQ-antibody MW1 was able to detect a Q16-GFP fusion protein but no Q16 peptides derived from GFP-Ub-Q16 (data not shown). This shows our inability to detect Q16 peptides is not likely to be caused by the intrinsic inability of IC2 to recognize this peptide species. Thus, the inability to detect any Q16 peptides in cells expressing GFP-Ub-Q16 is most likely due to rapid and efficient degradation of non-expanded polyQ peptides. During the preparation of this article, it has been suggested that isolated proteasomes are able to cleave multiple times within a short polyQ-containing peptide [45]. They argued that Venkatraman and colleagues [19] underestimated the amount of cleaved polyQ-fragments as a consequence of their mass-spectrometry methods. However, their conclusion was also based on other experiments such as Western blot analysis of polyQ protein products generated by proteasomes, and are in line with the conclusions by Holmberg and colleagues [18]. The observation by Pratt and Rechsteiner [45] was done in the presence of a mutated PA28γ subunit, which alters proteasomal access and

Fig. 5. Model of polyQ peptide aggregate formation and sequestering of UPS components.

Upon proteasomal degradation of polyQ proteins, pure polyQ peptides are released into the cytoplasm, where peptidases should recycle them into amino acids. Expanded polyQ peptides show resistance to degradation, leading to accumulation and initiation of aggregate formation. Proteasomes are rapidly recruited in an attempt to degrade the fragments. In time, other proteins including various polyQ.
Mimicking proteasomal release of polyglutamine peptides initiates aggregation and toxicity

specificity to peptides. In addition, although isolated proteasomes may be to cleave short polyQ peptides, our observation that Q65 and Q112 peptides readily aggregate suggests that the proteasome cannot efficiently degrade expanded polyQ peptides and thus cannot prevent their accumulation.

PolyQ aggregation is commonly visualized using full-length or truncated polyQ-proteins that are GFP-tagged, which therefore represent proteins that require degradation by the proteasome. When such GFP-tagged proteins are degraded by the proteasome, this results in the release of non-fluorescent polyQ peptides that will initiate aggregation and subsequently sequester GFP-tagged fragments or full-length polyQ proteins in time. Visualization of aggregation using GFP-tagged polyQ proteins thus represents a later stage in aggregate formation and does not reveal much about the initiation of aggregation. Long- or short-lived polyQ proteins have been used to link degradation to aggregation kinetics, where long-lived GFP-polyQ [40, 46] and GFP-tagged polyQ-expanded htt fragment [39] fusion proteins were compared to short lived variants. In these studies proteasomal degradation of short-lived expanded polyQ proteins resulted in reduced formation of GFP positive aggregates compared to their long-lived counterparts. Strikingly, toxicity was higher in cells expressing the short-lived expanded htt fragment when compared to long-lived version [39]. Our model can explain this unexpected finding: short-lived polyQ proteins are more rapidly degraded than long-lived proteins, resulting in aggregation-prone and toxic polyQ peptides. However, such aggregates remain invisible as GFP fluorescence of the short-lived peptides is lost due to rapid breakdown, preventing its incorporation in the aggregates. Consequently, these results have likely led to an underestimation of the real number of aggregates formed by short-lived proteins in these studies. The increased toxicity was in fact presumably caused by higher levels of generated polyQ peptides. The reduced toxicity in GFP positive cells by Verhoef and colleagues [40] may similarly be explained by preferential loss of fluorescence by toxic fragments, since only toxicity of GFP-positive cells was analyzed.

Our method mimicking proteasomal release of polyQ peptides is also a valuable tool to investigate a number of important questions concerning the role of polyQ peptides in HD and related neurodegenerative disorders. It enables us to identify proteases or peptidases that can target intracellular polyQ peptides in vivo, providing a strategy to prevent accumulation of toxic polyQ peptides. Similarly, the role of alternative degradation pathways, such as autophagy, in clearance of polyQ aggregates can be investigated. Our approach may also be useful to screen for compounds that affect aggregation and decrease toxicity. We expect that the outcome of such studies using this tool that express polyQ peptides in living cells holds true for all expanded polyQ disorders.

**MATERIALS AND METHODS**

**Plasmid Constructs.** Ub was generated by PCR from GFP-Ub [24] with forward primer 5’-CCGAGCC-TCAGATGCAATCTTCGAGAAG-3’ and reverse primer 5’-CTCGGGGCTCTCACCACCTCTGAGAAGGG-3’ and ligated into EGFP-C1 (Clonetech). The resulting construct GFP-Ub was again generated by PCR with forward primer 5’-CGGGATCTGAGGACGAGACGGCAG-3’ and a reverse primer 5’-CCGGGATCTGAGGACGAGACGGCAGGAGG-3’ and ligated into Ub-x-GFP-Q65/Q112 [40] where the Ub-x-GFP insert was replaced for GFP-Ub, resulting in GFP-Ub-Q65/Q112. This procedure was required to remove the restriction site PstI present between GFP and Ub, since PstI was also required for Ub-polyQ ligation. The usage of restriction sites required the presence of some flanking amino-acids, resulting in an N-terminal residue and a Glu-Thr-Ser-Pro-Arg sequence at the C-terminus. GFP was exchanged for mRFP to generate the different RFP-Ub-polyQ fusions. The alternative polyQ peptide lengths of Q33 and Q48 were generated by re-formation of GFP-Ub-Q65, leading to altered polyQ lengths. Q16-GFP was generated by inserting a Q16 repeat (derived from Ub-M-GFP-Q16) in front of GFP. Htt exon-1 was kindly provided by Ron Kopito (Stanford University, USA), atx3 by Henry Paulson (University of Iowa, USA), AR by Paul Taylor (St. Jude Children's Research Hospital, Memphis, USA), GFP-Ub, RFP-Ub, Ub-M-GFP-polyQ (used to express GFP-polyQ) and β7-RFP by Nico Dantuma (Karolinska Institute, Sweden), HSF70-GFP by Harm Kampinga (UMC Groningen, The Netherlands), TBP1 by Rick Morimoto (Northwestern University, USA) and QBP1-CFP by Yoshitaka Nagai (Osaka University Graduate School of Medicine, Japan).

**Transfections, cell-culture and toxicity assay.** Human embryonic kidney cells (HEK293T) and Mel JuSo fibroblast cells were cultured in Iscove’s Modified Dulbecco’s Medium (IMDM; Gibco) supplemented with 10 % FCS and penicillin/streptomycin/L-glutamine. The cells were transiently transfected with Fugene6 (Roche) and analyzed at indicated time-points after transfection. Mouse ST8hnA(Q7) cells (kindly provided by Marcy MacDonald) [36] and N2A neuroblastoma cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10 % FCS and penicillin/streptomycin/L-glutamine. Neuronal cells were transiently transfected with Lipofectamine 2000 (Invitrogen). Mouse ST8hnA(Q7) cells were incubated at 32°C. For toxicity measurements, N2A cells were analyzed by FACS LSRII for GFP fluorescence 24 or 48 hours after transfection, and the percentage of GFP-positive cells was quantified.

**Western blot.** Cytosolic extracts were generated by lysing cells with 0.1 % Triton X-100 for 30 minutes one ice, and the supernatant was used after spinning down the lysate. 20 µg of cytosolic protein lysates were separated by 18 % SDS-PAGE and transferred to Protran nitrocellulose membranes. Membranes were blocked in 5 % dry milk in TBS containing 0.3 % tween and probed with 1:1000 anti-GFP (Molecular Probes), 1:100 anti-Ub (SIGMA) or the anti-Polyglutamine 1C2 (MAB1574, Millipore). Polyclonal Horseradish Peroxidase (HRP) conjugated secondary antibodies, anti-rabbit (Sigma) or anti-mouse (Dako) were used 1:10000 to detect the primary antibodies via LuminolPlus westernblotting substrate (Roche). Preparation of SDS-soluble and SDS-insoluble protein fractions was described before [47]. Briefly, cells were trypsinized, homogenized, and heated for 10 min at 99°C in sample buffer (70 mM Tris pH 6.8, 1.5 % SDS, 20 % glycerol) supplemented with 50 mM DTT 72 hours after transfection. Cell lysates were centrifuged for at least 30 minutes at 20,800 g at room temperature. Supernatants were used as SDS-soluble fraction to which 0.05 % bromophenol blue was added. Pellets represented SDS-insoluble fractions and were dissolved in 100 % formic acid, incubated 30 minutes at 37°C, lyophilized overnight in a speed vac (Eppendorf),
Mimicking proteasomal release of polyglutamine peptides initiates aggregation and toxicity

Fluorescence, confocal and electron microscopy. HEK293T cells were transfected with the indicated constructs and the percentages of aggregates were scored using an inverted fluorescence microscope (Leica DMR). For imaging, Mel Juso cells were transiently transfected with the indicated constructs and images were obtained using a confocal microscope (Leica SP2) using a 63x objective. Note that some pictures show ‘over-exposed’ fluorescent aggregates in order to visualize non-sequestered, cytoplasmic staining. For immunostaining, Mel Juso cells were fixed with 4 % paraformaldehyde and permeabilized using 0.1 % triton in PBS containing 1 % FCS and stained with the primary antibodies 1C2 or MW1 [30] (1:1000), followed by goat anti-mouse Cy3 labeling (Jackson ImmunoResearch Laboratories). The MW1 antibody developed by Ko, J., Ou, S., and Patterson, P.H. (University of Iowa, USA) was obtained from the Developmental Studies Hybridoma Bank under the auspices of the NICHD and maintained by the University of Iowa. For endogenous HSP70 labeling, Mel Juso cells were stained against HSP70/Hsc70 (Calbiochem, 1:200) followed by anti-mouse AlexaFluor 633 (Invitrogen). For electron microscopy, Mel Juso cells were embedded in situ. Preceding fixation, cells were washed briefly in 20 mM PBS (pH 7.4). Fixation was done in a mixture of 4 % paraformaldehyde, 1 % glutaraldehyde in 0.1 M Phosphate Buffer (pH 7.4) for 60 minutes. After fixation cells were washed in distilled water, osmicated for 60 minutes in 1 % OsO4 in water, washed again in distilled water, dehydrated through a series of ethanol baths and embedded in LX-112. After polymerization the plastic was removed and small parts of the epon block containing the cells were prepared for ultra-thin sectioning. Ultra-thin sections were cut, collected on formvar coated grids and stained with uranyl acetate and lead citrate. Sections were examined with a Philips EM-420 electron microscope.

Filter retardation assay. Filter retardation assay was performed as described before [29]. Briefly, 72 hours after transfection, HEK293T cells were lysed for 30 minutes on ice in Nondinet P-40 (NP-40) buffer (100 mM TrisHCl, pH 7.5, 300 mM NaCl, 2 % NP-40, 10 mM EDTA, pH 8.0, supplemented with complete mini protease inhibitor cocktail (Roche) and phosphatase inhibitor cocktail (Sigma). After centrifugation 15 minutes at 20,800 g at 4°C, cell pellets were resuspended in benzonase buffer (1 mM MgCl2, 50 mM Tris-HCl, pH 8.0) and incubated for 1 hour at 37°C with 250U benzonase (Merck). Reactions were stopped by adding 2x termination buffer (40 mM EDTA, 4 % SDS, 100 mM DTT). Aliquots of 30 μg protein extract were diluted into 2 % SDS buffer (2 % SDS, 150 mM NaCl, 10 mM Tris pH 8.0) and filtered through a 0.2 μm cellulose acetate membrane (Schleicher and Schuell) pre-equilibrated in 2 % SDS buffer. Filters were washed twice with 0.1 % SDS buffer (0.1 % SDS, 150 mM NaCl, 10 mM Tris pH 8.0) and subsequently blocked in 5 % nonfat milk (Protifar Plus, Nutricia) in TBS. Captured aggregates were detected by incubation with 1C2 antibody and further treated like western blots. Alternatively, GFP fluorescence of trapped aggregates was analysed by LAS3000.

Acknowledgements

We would like to thank Silvia Coolen and Suzanne van der Horst for assisting experiments, and Derk Amsen, Jacob Aten, Ron van Noorden, Sean Diehl and Dineke Verbeek for carefully reading the manuscript. This study was funded by a grant from the Hereditary Disease Foundation, a VENI grant from NWO-ZonMW, a grant from the Hersenstichting and the Dutch Cancer Foundation KWF.

Supplementary Fig. 1. (A) The anti-polyQ antibody LC2 recognizes both short (Q16-GFP and GFP-Q16) and expanded (GFP-Q65 and GFP-Q112) polyQ-tracks fused to GFP with almost equal efficiencies. (B) Whereas the expression of GFP-Ub-Q33 did not induce aggregates, the expression of GFP-Ub-Q48 led to aggregate formation in a low percentage of cells after 72 hours of transfection. (C) Whereas polyQ peptides derived from GFP-Ub-Q112 are present in both the soluble and insoluble fraction of transfected cells, these peptides are not positive for GFP, indicating efficient cleavage of GFP-Ub-Q112. (D) Cells transfected with GFP-Ub-Q112 showed only immunostaining with the anti-polyQ antibody MW1 when no aggregates were present, similar as observed with LC2 (Fig. 1E). Scale bar ~5 μm.
Mimicking proteasomal release of polyglutamine peptides initiates aggregation and toxicity


Supplementary Fig. 2.

(A) FRAP analysis of polyQ-induced aggregates show recovery of fluorescence upon photo-bleaching of HSP70, indicating a high on/off rate, whereas Ub and proteasomes are irreversibly sequestered. (B) The rate of aggregation of httex1-Q103-GFP was enhanced when co-expressed with RFP-Ub-Q112 (left panel). While httex1-Q25-GFP is freely distributed in cells co-expressing RFP-Ub or RFP-Ub-Q16, httex1-Q25-GFP was redistributed to aggregates initiated by RFP-Ub-Q112 (right panel). A representative graph is showing the percentage of cells containing huntingtin-positive aggregates at three time points after transfection. (C) Ataxin-3-Q28-GFP distribution was affected by the presence of polyQ peptide aggregates, leading to sequestration into aggregates induced by RFP-Ub-Q112. Scale bar ~5 μm.


