Clearance of intracellular aggregation-prone protein fragments

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Chaperones affect intermolecular interactions between aggregated polyQ peptides

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Various neurodegenerative disorders such as Huntington’s disease are caused by the expansion of the polyglutamine (polyQ) tract within the disease-related protein. Protein fragments containing the polyQ tract are thought to initiate aggregation. Other proteins, including proteasomal components and chaperones, are sequestered in these aggregates. To examine whether these sequestered proteins interact with aggregated polyQ fragments, we performed fluorescence lifetime imaging microscopy studies in living cells using polyQ peptides containing a short tetracysteine motif. This motif can be labeled with various small fluorescent dyes prior to imaging, allowing the visualization of polyQ peptide interactions and kinetics in living cells without interference of large fluorescent tags. Here, we show that polyQ-polyQ interactions only occurred when peptides were irreversibly sequestered in aggregates, whereas diffusing expanded polyQ peptides were mostly monomeric. The proteasomal subunits LMP2 and β7 were both sequestered in the core of aggregates. However, aggregated polyQ peptides showed higher FRET efficiencies with β7 than with LMP2. The chaperones DnaJB6 and DnaJB8 are potent inhibitors of polyQ peptide aggregation. Their presence in the core of polyQ peptide-induced aggregates coincided with strong interactions with aggregated polyQ peptides. The chaperones Hsp70, which is found in a ring around trapped polyQ peptides, and Hsp40, that is hardly associated with these aggregates, were less effective in inhibiting polyQ peptide aggregation. These chaperones also did not interact with aggregated polyQ peptides. Surprisingly, Hsp70 and Hsp40 altered the architecture of aggregated polyQ peptides more strongly than DnaJB6 and DnaJB8, despite their relative lack of inhibition of polyQ peptide aggregation.
Polyglutamine (polyQ) disorders are a family of at least nine dominantly inherited neurodegenerative disorders caused by an expansion of the polyQ tract in otherwise unrelated proteins. When exceeding the threshold of 40 glutamines the polyQ-containing protein becomes pathogenic. The onset of the disease is irreversibly correlated with the length of the polyQ tract. Expansion of the polyQ tract is associated with aggregation, loss of cell function and cell death (Orr and Zoghbi, 2007).

A number of studies in post-mortem brains of patients suffering from various polyQ disorders showed that aggregates are predominantly composed of proteolytic fragments containing the expanded polyQ tract (DiFiglia et al., 1997; Goti et al., 2004; Lunkes et al., 2002). These fragments are generated by cleavage by various proteases, such as caspases, calpains and aspartic endopeptidases (Gafni et al., 2004; Goldberg et al., 1996; Graham et al., 2006; Lunkes et al., 2002; Wellington et al., 1998; Young et al., 2007). Proteolytic fragments are more aggregation-prone and more toxic as compared to their full-length analogues. Therefore, these fragments are thought to initiate aggregation and toxicity (Cooper et al., 1998; Ellerby et al., 1999a; Ellerby et al., 1999b; Haacke et al., 2006; Ikeda et al., 1996; Merry et al., 1998; Wellington et al., 2000; Young et al., 2007).

Both wild-type and expanded polyQ-containing proteins can be degraded by proteasomes (Bailey et al., 2002; Iwata et al., 2009; Jana et al., 2005; Michalik and Van Broeckhoven, 2004; Rousseau et al., 2009). However, proteasomes are unable to cleave within polyQ tracts (Holmberg et al., 2004; Venkatraman et al., 2004). As a consequence, polyQ peptides may be released in the cytoplasm after protein degradation. When mimicking proteasomal polyQ peptide generation by expression of polyQ peptides without tags or starting methionine residue, we observed that only polyQ peptides of disease-related lengths accumulate and induce aggregation and toxicity (Raspe et al., 2009). This suggests that there is a common mechanism underlying these neurodegenerative disorders, where small proteolytic fragments containing the expanded polyQ tract initiate aggregation and toxicity.

PolyQ-induced aggregates are decorated with various proteins, including chaperones, ubiquitin (Ub), proteasomal components and polyQ-containing proteins, either wild-type or polyQ-expanded, such as huntingtin (Htt), ataxin 3 (Atx3) and the transcription factors TBP and CBP (Cummings et al., 1998; Davies et al., 1997; Holmberg et al., 2004; Kazantsev et al., 1999; Kim et al., 2002; Matsumoto et al., 2006; Perez et al., 1998; Raspe et al., 2009). PolyQ-containing protein fragments tagged with GFP, such as Httexon1-GFP and truncated Atx3-GFP are often found in a ring around the aggregate core (Chai et al., 2002; Kim et al., 2002; Raspe et al., 2009). These findings are in agreement with the hypothesis that short fragments of Htt and Atx3 containing the expanded polyQ tract initiate aggregation and than sequester the much larger GFP-tagged Htt and Atx3.

Fusion of fluorescent proteins such as GFP with polyQ peptides may affect the behavior of these peptides, because these tags are relatively large when compared to polyQ peptides. To visualize aggregate formation by polyQ peptides in living cells without the interference of large fluorescent tags, we fused the small tetracysteine (C4)-motif to polyQ peptides. These motifs were labeled shortly before microscopical analysis using small fluorescent dyes. Various microscopical techniques such as fluorescence lifetime imaging microscopy (FLIM) and photobleaching assays were used to measure polyQ peptide interactions and kinetics.

**INTRODUCTION**

Hsp70 and Hsp40 affect intermolecular interactions between aggregated polyQ peptides
Here, we show that expanded polyQ peptides were immobilized upon aggregation and interactions between polyQ peptides occurred only when they were trapped in aggregates. PolyQ peptides were found in the core of aggregates, interacting with the proteasomal subunits LMP2 and β7 and the chaperones DnaJB6 and DnaJB8. Ub, Httexon1-Q103 and Hsp70 were found in a ring around the core of polyQ-peptide induced aggregates, resulting in weaker interactions with polyQ peptides. Finally, DnaJB6 and DnaJB8 interacted stronger with trapped polyQ peptides than Hsp70 and Hsp40, but expression of Hsp70 and Hsp40 had a stronger effect on intermolecular interactions of sequestered polyQ peptides.

**VISUALIZATION OF POLYQ PEPTIDE AGGREGATION**

GFP-Ub-polyQ constructs were used recently to express polyQ peptides in cells. It was shown that polyQ peptides of disease-related lengths are resistant to degradation and induce aggregation and toxicity (Raspe et al., 2009). These polyQ peptides lacked a fluorophore and were visualized with the use of antibodies. Aggregation of polyQ peptides was visualized indirectly by binding of the Q-binding protein 1 (QBP1) (Nagai et al., 2000) or sequestration of GFP-Ub. To enable direct visualization of polyQ peptide aggregation in living cells, we tagged polyQ peptides at their C-terminus with the short tetracysteine C4 motif (FLNCCPGCCMEP; (Martin et al., 2005)), resulting in GFP-Ub-polyQ-C4. When the construct is expressed in cells, polyQ-C4 is released from the GFP-Ub moiety by cleavage by Ub C-terminal hydrolases (Figure 1A) (Johnson et al., 1995). The C4 motif is specifically recognized by membrane-permeable biarsenical dyes, such as FlAsH and ReAsH, which become green or red respectively. These dyes become fluorescent upon binding to the C4 motif (Adams et al., 2002; Griffin et al., 1998). The C4 motif is a small tag of only 1.3 kDa and less likely affects aggregation of polyQ peptides (12.7 kDa for Q99), in contrast with fluorescent protein tags such as GFP (27.1 kDa).

To examine whether the C4 tag affected the behavior of polyQ peptides, we transiently expressed GFP-Ub-polyQ and GFP-Ub-polyQ-C4 peptides and quantified the percentage of cells containing GFP-Ub-positive aggregates (Figure 1B). Expression of either C4-tagged or non-tagged polyQ peptides resulted in a similar percentage of aggregate-containing cells after 72 hours. Therefore, fusion of polyQ peptides to the C4 tag did not affect their aggregation kinetics. Western blot analysis showed that GFP-Ub was efficiently separated from C4-tagged polyQ peptides as GFP-positive bands were present at 36 kDa, representing GFP-Ub, and no uncleaved GFP-Ub-polyQ-C4 was detected (Figure 1C, middle panel). PolyQ-positive bands were only present when peptides of disease-related lengths of more than 40 glutamines were expressed (Figure 1C, upper panel), similar as shown before (Raspe et al., 2009). However, the high molecular weight band that was present when expressing Q104 peptides was absent when C4-tagged Q99 peptides were expressed (Asterisk; Figure 1C, upper panel).

To visualize polyQ peptides directly, HeLa cells expressing GFP-Ub-polyQ-C4 were labeled with ReAsH and either separated on SDS-PAGE or imaged by confocal microscopy. After separation
of ReAsH-labeled polyQ peptides on SDS-PAGE, the ReAsH signal was directly visualized with a Typhoon fluorescent gel imager. Only ReAsH-positive bands were present when polyQ peptides of disease-related lengths were expressed (Figure 1D). These bands ran at similar heights as compared to Western blots stained for 1C2 (Figure 1C).

Expression of non-expanded GFP-Ub-Q17-C4 did not result in ReAsH-positive cells (Figure 1E, upper panel). Because these cells were GFP-positive, indicates that Q17-C4 was efficiently degraded as shown before for non-expanded polyQ peptides (Raspe et al., 2009). Different distribution patterns of GFP-Ub and ReAsH were observed in cells expressing GFP-Ub-Q99-C4, depending on the presence of aggregates. In cells without aggregates, both GFP-Ub and expanded polyQ peptides were present throughout the cytoplasm and nucleus (Figure 1E, lower panel). However, aggregation led to the redistribution of both GFP-Ub and ReAsH-labeled Q99-C4, with ReAsH-labeled Q99-C4 sequestered in the core of aggregates and GFP-Ub in a ring around the core (Figure 1E, lower panel). Together, these data show that only C4-tagged polyQ peptides of disease-related lengths accumulated and initiated aggregation, whereas short C4-tagged polyQ peptides were degraded, similarly to untagged polyQ peptides.

Previously, we have shown that proteasomal components, Ub and different chaperones are recruited at different stages into polyQ peptide-induced aggregates (Gillis et al., manuscript in preparation; Raspe et al., 2009). To investigate whether these components colocalized with aggregated polyQ peptides, we co-expressed these proteins fused to GFP with C4-tagged polyQ peptides and labeled cells with ReAsH. Visualization by confocal microscopy showed that C4-tagged polyQ peptides were present in the core of aggregates and colocalized with the proteasomal subunits LMP2 and β7 and the chaperones DnaJB6 and DnaJB8 (Figure 1F). In contrast, Hsp70 and Ub were present in a ring around the core of aggregates (Figure 1F), similarly as observed when using non-tagged polyQ peptides (Gillis et al., manuscript in preparation; Raspe et al., 2009).

**POLYQ PEPTIDES BECOME IMMOBILIZED AGGREGATES AND STRONGLY INTERACT WITH OTHER SEQUESTERED POLYQ PEPTIDES**

Next, we studied the intracellular dynamics of expanded polyQ peptides in living cells by using the C4 tag to label polyQ peptides prior to imaging. To determine intracellular mobility of polyQ peptides, fluorescence recovery after photobleaching (FRAP) experiments were performed. The rate of recovery after photobleaching of a small area of interest is correlated with the mobility of proteins (Lippincott-Schwartz and Patterson, 2003; Reits and Neefjes, 2001). Q99-C4 peptides that were freely distributed throughout the cytoplasm and nucleus showed a fast recovery after photobleaching, indicating a high mobility of diffuse Q99-C4 peptides (Figure 2A). However, aggregated Q99-C4 peptides hardly showed any recovery (Figure 2A), indicating that polyQ peptides became immobile after aggregation.

To visualize aggregation of polyQ peptides in time, HeLa cells expressing Q99-C4 were labeled with FlAsH after 24 hours, which labeled all polyQ peptides generated at that timepoint. After a subsequent 24 hours the pool of newly-synthesized Q99-C4 peptides were labeled with ReAsH. Confocal images of these cells showed the expansion of aggregates in time, with FlAsH-labeled polyQ peptides present in the core, surrounded by sequestered, newly-synthesized...
Figure 1. Tetracystéine (C4)-tagged polyQ peptides behave similarly as non-labeled polyQ peptides. (A) Schematic representation of cleavage of GFP-Ub-polyQ-C4 constructs by Ub C-terminal hydrolases directly after Ub, thereby separating GFP-Ub and polyQ-C4. (B) Percentage of HEK cells that contained fluorescent aggregates at 72 hours after transfection with GFP-Ub-Q16/Q54/Q104 or C4-tagged GFP-Ub-Q17/Q56/Q99 as determined by sequestration of GFP-Ub in polyQ peptide-induced aggregates (data are mean ± SEM of three independent experiments). Similar amounts of aggregates were detected with either non-tagged or C4-tagged polyQ peptides. (C) Western blot analysis of HEK cells expressing GFP-Ub-Q104 or ReAsH-labeled, C4-tagged GFP-Ub-Q17/Q56/Q99 at 72 hours after transfection. Blots were stained for polyQ (upper panel), GFP (middle panel) or actin (lower panel). Asterisk indicates oligomeric structures. Only polyQ-positive bands were detected when expressing polyQ-expanded peptides. (D) SDS-PAGE of ReAsH-labeled HeLa cells expressing C4-tagged GFP-Ub-Q17/Q56/Q99 at 48 hours after transfection. ReAsH signal was imaged by a Typhoon gel imager. Arrows indicate ReAsH-labeled polyQ peptides. (E) Confocal images of ReAsH-labeled HeLa cells expressing C4-tagged GFP-Ub-Q17 or GFP-Ub-Q99 at 48 hours after transfection. ReAsH-positive cells were detected only when disease-related C4-tagged Q99 peptides were expressed (middle panel). The zoom-in shows a GFP-Ub-decorated aggregate (left panel). (F) Confocal images of ReAsH-labeled HeLa cells expressing Ub-Q99-C4 together with indicated GFP-tagged constructs at 48 hours after transfection. The insets in the lower right corners represent a sequential zoom-in of polyQ peptide-induced aggregates. Scalebar: 20 μm
Figure 2. Dynamics of C4-tagged polyQ peptides. (A) FRAP analysis of ReAsH-labeled Q99-C4 peptides. Cells were imaged before photobleaching (pre-bleach) of the indicated area (white circle) and at indicated timepoints after photobleaching. The graph represents mean ± SEM of three different cells. Non-aggregated Q99-C4 peptides showed a rapid recovery after photobleaching, whereas aggregated Q99-C4 peptides became immobilized. Scalebar: 20 µm. (B) Confocal images of Ub-Q99-C4-expressing cells were labeled with FlAsH at 24 hours after transfection. After an additional 24 hours, the same cells were labeled with ReAsH. Arrows indicate aggregates visible by phase-contrast. Scalebar: 5 µm. (C) EM images of ReAsH-labeled Ub-Q99-C4-expressing cells without photoconversion (left panel) or after photoconversion of DAB by ReAsH (right panel). Scalebar: 2 µm. (D) FLIM analysis of FlAsH in HeLa cells expressing Ub-Q99-C4 labeled with either FlAsH alone or both with FlAsH and ReAsH, showing in each column, from left to right, representative graphs of fluorescence intensity, false-color map of the fluorescence lifetime calculated from the phase shift (τφ) and histogram of lifetime distribution with the same false-color scale as the lifetime map. Scalebar: 20 µm. (E) Table summarizes the average phase (τφ ± s.d.), modulation lifetimes (τM ± s.d.) and FRET efficiencies (EFRET) based on phase lifetimes. The number of cells measured is indicated by n. Histogram represents average phase modulation lifetimes (data are mean ± SEM; two-tailed unpaired t-test; *** p<0.001; ** p<0.01; * p<0.05). Only aggregated polyQ peptides showed high FRET efficiencies.
ReAsH-labeled peptides (Figure 2B). The reduced amount of ReAsH-positive peptides in the core of aggregates is in agreement with the FRAP data (Figure 2A), because the FlAsH-labeled polyQ peptides present in the core of aggregates were immobile.

The fluorophore ReAsH can also be used for photoconversion of diaminobenzidine (DAB). Only at sites where ReAsH is present, a local brownish electron-dense precipitate appears that can be detected by electron microscopy (EM). As a result, ReAsH-labeled proteins can be directly visualized by EM without immunogold-labeling (Gaietta et al., 2002). EM analysis showed fibril-rich aggregates in cells that contained ReAsH-labeled polyQ peptide aggregates, particularly after photoconversion of DAB (Figure 2C), similarly as shown before for Q112 peptides and polyQ-expanded Htt (Qin et al., 2004; Raspe et al., 2009).

To examine whether diffusing and aggregated polyQ peptides showed intermolecular interactions, we performed fluorescence resonance energy transfer (FRET) experiments. FRET enables sensitive evaluation of protein-protein interactions in living cells. Only when proteins are in close proximity (<10 nm) FRET can occur and be measured, whereas proteins separated over 10 nm show no FRET (Miyawaki, 2003). FLIM was used to measure FRET, which is detected as a decrease in donor fluorescence lifetime (van Munster and Gadella, 2005). HeLa cells expressing Ub-Q99-C4 were simultaneously labeled with equal amounts of FlAsH and ReAsH at 48 hours after transfection, leading to individual labeling of polyQ peptides with either FlAsH or ReAsH. When polyQ peptides were not clustered in aggregates, no reduction in lifetime of FlAsH was measured (Figure 2D-E), suggesting that polyQ-polyQ peptide interactions hardly occurred when not sequestered in aggregates. Importantly, it indicated that non-aggregated polyQ peptides were primarily monomeric. In contrast, aggregated polyQ peptides showed a remarkable decrease in both phase and modulation lifetimes of FlAsH (Figure 2D-E), which is indicative of FRET. Based on the phase lifetimes, a FRET efficiency of 39.6% was calculated (Figure 2E). Together, these data show that polyQ peptides are mainly monomeric when not present in aggregates, whereas aggregated polyQ peptides are immobile and only then strong polyQ-polyQ peptide interactions are observed.

**POLYQ PEPTIDES INTERACT DIFFERENTLY WITH SEQUESTERED PROTEINS**

The recruitment of proteasomes into the core of polyQ peptide-initiated aggregates suggests that proteasomes recognize polyQ peptides. To measure interactions between sequestered proteasomes and aggregated polyQ peptides, cells were co-transfected with Ub-Q99-C4 and the GFP-tagged proteasomal components β7 or LMP2. Prior to FLIM analysis, polyQ peptides were labeled with ReAsH. Co-expression of Q99-C4 with the proteasomal component β7 caused a decreased lifetime of GFP, resulting in a FRET efficiency of 14.5% (Figure 3A-C). The proteasomal subunit LMP2 showed only a small decrease in lifetime resulting in a FRET efficiency of 5.4% (Figure 3A-C). Although the orientation of GFP in relation to the fused protein may differ between β7 and LMP2, the different subunits seem to interact differently with aggregated polyQ peptides.

The presence of GFP-Ub in aggregates most likely represents sequestered proteins that become ubiquitinated in time. PolyQ peptides are not likely ubiquitinated as no lysine residues are present. We tested whether Ub, present in a ring around aggregated polyQ peptides,
interacts with aggregated polyQ peptides. Because expression of GFP-Ub-Q99-C4 results in an efficient separation of GFP-Ub and Q99-C4 (Figure 1), cells expressing GFP-Ub together with Ub-Q99-C4 or GFP-Ub-Q99-C4 should show similar interactions between polyQ peptides and GFP-Ub. Indeed, FRET efficiencies of approximately 5% were measured in both cases (Figure 3A-C).

Besides proteasomal subunits and Ub, various chaperones are recruited into polyQ-induced aggregates as well. We and others showed before that the chaperones DnaJB6 and DnaJB8, two members of the Hsp40 family, are potent inhibitors of polyQ-induced aggregation (Gillis et al., manuscript in preparation; Hageman et al., manuscript in preparation). The chaperone Hsp40 also reduces aggregation of polyQ peptides, but less efficient, whereas Hsp70 does not affect aggregation at all (Gillis et al., manuscript in preparation). DnaJB6 and DnaJB8 are recruited in the core of polyQ peptide-induced aggregates (Figure 1F). Therefore, we examined whether these chaperones interacted with aggregated polyQ peptides. Both GFP-tagged DnaJB6 and DnaJB8 interacted strongly with polyQ peptides with FRET efficiencies of approximately 16% (Figure 3D-F). However, the recruitment of Hsp70 into polyQ peptide-induced aggregates did not result in a significant reduced lifetime of GFP-tagged Hsp70 (Figure 3D-F), suggesting that Hsp70 does not interact with polyQ peptides. FRET between Hsp40 and aggregated polyQ peptides was undetectable (data not shown), likely because Hsp40 is only weakly associated with aggregates induced by polyQ peptides (Gillis et al., manuscript in preparation). Thus, most proteins that sequester in the core of aggregates, such as β7, DnaJB6 and DnaJB8, interacted with aggregated polyQ peptides. Ub, present in a ring around sequestered polyQ peptides, interacted less strongly with aggregated polyQ peptides, whereas Hsp70 did not interact with sequestered polyQ peptides at all.

**INTERACTION OF HUNTINGTIN WITH POLYQ PEPTIDES AND OTHER PROTEINS SEQUESTERED IN AGGREGATES**

Httexon1 is a polyQ-containing protein fragment that is sequestered into polyQ peptide-induced aggregates (Raspe et al., 2009). Similarly to Ub, Httexon1-Q103-GFP is present in a ring around the polyQ peptide-containing core of larger aggregates (Figure 4A). Httexon1-Q103-GFP is almost absent in the core, suggesting that polyQ-peptides initiate aggregation and larger fragments, such as Httexon1, are sequestered in time. To examine whether polyQ-expanded Httexon1 interacted with ReAsH-labeled Q99 peptides, FLIM experiments were performed. A FRET efficiency of 8.3% was determined (Figure 3A-C), indicating that aggregated polyQ peptides interacted with Htt. Probably, Httexon1 interacts with polyQ peptides due to its polyQ repeat, as polyQ-polyQ interactions are strong. The observed interaction of polyQ peptides with Ub is probably indirectly due to binding of ubiquitinated proteins, because polyQ peptides are not likely to become ubiquitinated.

PolyQ peptides are present in the core of polyQ-induced aggregates and Httexon1-Q103-GFP is found in an additional ring. Therefore, interactions between sequestered proteins and Httexon1-Q103 may be different as compared to their interactions with polyQ peptides. In order to perform FLIM analysis between Htt and sequestered GFP-tagged proteins, C4-tagged Htt was generated. Confocal analysis of cells co-expressing Httexon1-Q103-C4 and Httexon1-Q103-GFP
Figure 3. The chaperones DnaJ6 and DnaJ8 and the proteasomal subunits LMP2 and β7 interact with aggregated polyQ peptides. (A) FLIM analysis of GFP in HeLa cells expressing ReAsH-labeled Ub-Q99-C4 together with indicated GFP-tagged constructs at 48 hours after transfection, first panel is not labeled with ReAsH to determine lifetime GFP. Showing in each column, from left to right, representative graphs of fluorescence intensity, false-color map of the fluorescence lifetime calculated from the phase shift (τφ) and histogram of lifetime distribution with same false-color scale as the lifetime map. Scalebar: 20 µm. (B) Table summarizes the average phase (τφ ± s.d.), modulation lifetimes (τM ± s.d.) and FRET efficiencies (E_FRET) based on phase lifetimes. The number of cells measured is indicated by n. Histogram represents average phase modulation lifetimes (data are mean ± SEM; two-tailed unpaired t-test; *** p<0.001; ** p<0.01; * p<0.05). (C) FRET efficiencies (mean ± SEM) are calculated based on the phase lifetimes. FLIM analysis resulted in high FRET efficiencies between aggregated Q99-C4 peptides and the proteasomal subunit β7. (D) FLIM analysis of GFP in cells expressing ReAsH-labeled Ub-Q99-C4 together with indicated GFP-tagged chaperones at 48 hours after transfection. Scalebar: 20 µm. (E) Table summarizes the average phase (τφ ± s.d.), modulation lifetimes (τM ± s.d.) and FRET efficiencies (E_FRET). The number of cells measured is indicated by n. Histogram represents average phase modulation lifetimes (data are mean ± SEM; two-tailed unpaired t-test; *** p<0.001; ** p<0.01; * p<0.05). (F) FRET efficiencies (mean ± SEM) are calculated based on the phase lifetimes. FLIM analysis resulted in high FRET efficiencies between aggregated polyQ peptides and DnaJ6 and DnaJ8. No FRET was detected between aggregated polyQ peptides and Hsp70.
Figure 4. Aggregated Htt exon1 strongly interacts with DnaJB6, whereas only moderate interactions were observed between aggregated Htt and DnaJB8 or Hsp70. (A) Confocal images of HeLa cells co-expressing ReAsH-labeled Ub-Q99-C4 and Htt exon1-Q103-GFP (Htt-GFP) labeled at 48 hours after transfection, showing sequestration of Htt exon1 around aggregated polyQ peptides. Scalebar: 20 µm. (B) Confocal images of HeLa cells co-expressing Htt exon1-Q103 tagged with either GFP or C4 and labeled with ReAsH at 48 hours after transfection. Whereas ReAsH-labeled Htt exon1 was present in the core of aggregates, GFP-labeled Htt was present either in the core (upper panel) or in a ring around aggregated C4-labeled Htt (lower panel). Scalebar: 20 µm. (C) FLIM analysis of FlAsH (upper two panels) or GFP (lower 5 panels) in HeLa cells expressing Ub-Q99-C4 together with indicated constructs labeled with either FlAsH (upper panel), FlAsH and ReAsH (2nd panel), or ReAsH alone (lower 4 panels) at 48 hours after transfection, showing in each column, from left to right, representative graphs of fluorescence intensity, false-color map of the fluorescence lifetime calculated from the phase shift (τφ) and histogram of lifetime distribution with same false-color scale as the lifetime map. Scalebar: 20 µm. (D) Table summarizes the average phase (τφ ± s.d.), modulation lifetimes (τM ± s.d.) and FRET efficiencies (EFRET) based on phase lifetimes. The number of cells measured is indicated by n. Histogram represents average phase modulation lifetimes (data are mean ± SEM; two-tailed unpaired t-test; *** p<0.001; ** p<0.01; * p<0.05). (E) FRET efficiencies (mean ± SEM) are calculated based on the phase lifetimes. Similar FRET efficiencies were measured between aggregated FlAsH- and ReAsH-labeled Htt and between aggregated GFP- and ReAsH-labeled Htt. High FRET efficiencies were measured between aggregated Htt and DnaJB6, whereas only modest FRET efficiencies were measured between aggregated Htt and DnaJB8 or Hsp70.
showed that C4-tagged Htt exon1-Q103 labeled with ReAsH was present in the core of aggregates, whereas GFP-tagged Htt was found either in the core of smaller aggregates or in an additional ring at a later stage (Figure 4B). This interesting result may be explained by the different sizes of C4-tagged and GFP-tagged Htt, ~22.5 kDa and ~48.1 kDa, respectively. The smaller C4-tagged Htt may represent a fragment that is more aggregation-prone, resulting in its presence in the core of aggregates, whereas the larger Htt exon1-Q103-GFP is sequestered later.

Interactions between ReAsH-labeled Htt exon1-Q103 and GFP-tagged chaperones were measured. Interactions between DnaJB6 and Htt seemed somewhat stronger ($E_{FRET} = 19.3\%$; Figure 4C-E) when compared to the interactions between DnaJB6 and aggregated polyQ peptides. In contrast, interactions between DnaJB8 and Htt were weaker ($E_{FRET} = 8.8\%$; Figure 4C-E) when compared to interactions between DnaJB8 and aggregated polyQ peptides. Although Hsp70 does not interact with polyQ peptides, a FRET efficiency of 3.6% was measured between Hsp70-GFP and Htt exon1-Q103-C4 (Figure 4C-E). This indicates that Htt interacted weakly with Hsp70. Together, these data suggest that the interactions between aggregated Htt exon1 and DnaJB6 and DnaJB8 are different when compared to the interactions of aggregated polyQ peptides with these chaperones.

**HSP40 AND HSP70 REDUCE INTERMOLECULAR INTERACTIONS OF TRAPPED POLYQ PEPTIDES**

Both DnaJB6 and DnaJB8 strongly reduce aggregation of polyQ peptides (Gillis et al., manuscript in preparation) and interact with polyQ peptides in the core of aggregates. These interactions may affect aggregation of polyQ peptides directly. To examine whether chaperones were able to reduce the strong interactions observed between polyQ peptides, we co-expressed Q99-C4 peptides in combination with pIRES DsRed, containing DnaJB6, DnaJB8, Hsp40 or Hsp70. Double-labeling of C4-tagged Q99 peptides with FlAsH and ReAsH resulted in a FRET efficiency of 53.9% (Figure 5A-C). This FRET efficiency was higher than measured earlier (Figure 2E), which may be due to the lower phase lifetime of FlAsH in the presence of DsRed. Expression of DnaJB6 or DnaJB8 reduced interactions between aggregated polyQ peptides, as FRET efficiencies of 24.6 and 27.7%, respectively, were measured (Figure 5A-C). Surprisingly, Hsp40 and Hsp70 were more effective in reducing the interactions between aggregated polyQ peptides, as FRET efficiencies of 13.5% and 8.3%, respectively, were measured (Figure 5A-C). Together, these data suggest that these chaperones reduce intermolecular interactions of aggregated polyQ peptides, where Hsp40 and Hsp70 were more effective than DnaJB6 or DnaJB8 in disrupting polyQ-polyQ interactions.
In the present study, the small C4 tag was used to visualize dynamics and interactions of polyQ peptides in living cells. Earlier studies fused fluorescent proteins such as GFP to the relative small polyQ peptides to study their aggregation and dynamics in living cells (Brignull et al., 2006; Desai et al., 2006; Holmberg et al., 2004; Kim et al., 2002). However, the size of GFP compared to the polyQ peptides is relatively large, which may affect aggregation kinetics and behavior of polyQ peptides. This effect was also suggested by the comparison of the behavior of GFP-tagged Httexon1 and C4-tagged Httexon1. The smaller C4-tagged Httexon1 was detected only in the core of aggregates, whereas the larger GFP-tagged Httexon1 was sequestered around the core. We assume that fragments rather than proteins containing the expanded polyQ tract initiate aggregation. GFP-tagged peptides may behave as a polyQ-containing protein, being sequestered into aggregates instead of initiating aggregation.

Another advantage of C4-tagged polyQ peptides is the possibility to perform double labeling of peptides with different biarsenical dyes, either simultaneously for FLIM analysis or sequential for pulse-chase experiments. Double labeling of polyQ peptides with FlAsH and ReAsH was used to study interactions between polyQ peptides and the effects of chaperones on these interactions. Sequential labeling of polyQ peptides revealed an increase in size of aggregates in time due to sequestration of newly-synthesized polyQ peptides. Sequestered polyQ peptides became immobilized.

Interactions between polyQ peptides were only detected when these peptides were present in aggregates. This data is in agreement with studies that reported interactions between aggregated fluorescently-tagged polyQ peptides by measuring FRET between donor and acceptor (Brignull et al., 2006; Desai et al., 2006; Holmberg et al., 2004; Kim et al., 2002). PolyQ peptides are primarily monomeric instead of small oligomers when freely diffusing within cells. We previously showed that polyQ peptides can easily diffuse between cytoplasm and nucleus (Gillis et al., manuscript in preparation). This supports our finding that non-sequestered polyQ peptides are monomeric, as only proteins up to 60 kDa can diffuse through nuclear pores (Gorlich, 1998). Together, these data indicate that dimerization and subsequent oligomerization and aggregation of polyQ peptides occurs rapidly. We cannot exclude that a small proportion of diffusing polyQ peptides are present in an oligomeric form, as it may be beyond detection levels of FLIM or Western blotting.

It should be noted that high molecular weight bands were not present on Western blots when expressing C4-tagged polyQ peptides, irrespective of labeling cells with ReAsH (data not shown). This indicates that even the small C4 motif influences oligomerization of polyQ peptides, although similar percentages of aggregate-containing cells were present at 72 hours after transfection.

Recruitment of proteasomal components into polyQ-induced aggregates (Chai et al., 1999; Cummings et al., 1998; Holmberg et al., 2004; Raspe et al., 2009; Stenoien et al., 1999; Verhoeof et al., 2002), has led to the hypothesis that sequestered proteasomes may become clogged in the attempt to degrade polyQ-containing proteins (Goellner and Rechsteiner, 2003; Holmberg et al., 2004; Venkatraman et al., 2004). Depletion of functional proteasomes may subsequently contribute to cytotoxicity. Morimoto and colleagues (Holmberg et al., 2004) reported
interactions between the proteasomal subunit LMP2-YFP and CFP-tagged polyQ peptides by FRET analysis. Whereas we observed a low FRET efficiency between LMP2 and aggregated polyQ peptides, a stronger FRET efficiency was found between aggregated polyQ peptides and the proteasomal subunit ß7. Although we cannot exclude that that different FRET efficiencies were measured due to different orientations of fluorophores, it may mean that different proteasomal subunits have different affinities for polyQ peptides. Changing the subunit composition of proteasomes (Kloetzel, 2001) may be an approach to increase degradation of aggregation-prone polyQ tracts. Whether a higher affinity of the subunits for polyQ peptides is also correlated with the ability of these subunits to degrade polyQ peptides has to be examined.

DnaJB6 and DnaJB8 are potent inhibitors of polyQ peptide aggregation (Gillis et al., manuscript in preparation). These chaperones colocalized with polyQ peptides in the core of aggregates and seem to have stronger interactions with aggregated polyQ peptides as compared...
to other sequestered proteins. No interaction between the more classical chaperones Hsp70 or Hsp40 and aggregated polyQ peptides was observed. Despite the stronger interactions of DnaJB6 and DnaJB8 with aggregated polyQ peptides, DnaJB6 and DnaJB8 were less effective in reducing the intermolecular interactions of aggregated polyQ peptides than Hsp70 and Hsp40. A reason could be that DnaJB6 and DnaJB8 only solubilize monomeric polyQ peptides, thereby keeping these peptides in a degradation-competent state. Once an aggregate has been formed, DnaJB6 and DnaJB8 are sequestered in the core, but are then unable to alter the composition of polyQ peptides within aggregates. In contrast, Hsp70 did not affect aggregation of expanded polyQ peptides (Gillis et al., manuscript in preparation) and no interactions between Hsp70 and aggregated polyQ peptides were observed, which is in agreement with other studies that also did not detect interactions between Hsp70 and fluorescently-tagged, aggregated polyQ peptides or polyQ-containing proteins (Kim et al., 2002; Matsumoto et al., 2006). Because Hsp70 has a high on/off rate when associated with polyQ-induced aggregates (Kim et al., 2002; Raspe et al., 2009), they may be only temporarily recruited in aggregates but thereby still able to modify polyQ peptide binding. This may be similar for Hsp40, which is not sequestered into polyQ peptide-induced aggregates. In conclusion, it can be stated that Hsp70 and Hsp40 are less efficient in reducing aggregation of polyQ peptides compared to DnaJB6 and DnaJB8, but they do affect the composition of sequestered polyQ peptides. Whether it alters their cytotoxicity remains to be investigated.

DNA constructs. Generation of GFP-Ub-polyQ constructs was described before (Gillis et al., manuscript in preparation). To add a C4-motif to GFP-Ub-PolyQ constructs a BamHI site was introduced at the C-terminus of polyQ peptides using Quikchange II Site directed Mutagenesis (Stratagene) with forward primer 5’-AGCAGCAGCAGCAGCAGGATCTAGCCCAAGGTAAGC-3’ and reverse primer 5’-GCTTACCTGGGCTAGGATCTCTGTGCTGCTGCTGCTGCT-3’. The C4 tag containing the following sequence FLNCCPGCCMEP (Martin et al., 2005) was obtained by annealing of forward oligo 5’-GATCTGTTTCTTAATTGTTGTCCTGGTTGTTGTATGGAACCTTAAT-3’ and reverse oligo 5’-CTAGATTAAGGTTCCATACAACACAGGACAACATTAGAGAAA-3’. In this way, overhangs were generated compatible with BamHI at the N-terminus and XbaI at the C-terminus. C4-tag was inserted into GFP-Ub-polyQ, thereby generating GFP-Ub-Q17-C4, GFP-Ub-Q56-C4, and GFP-Ub-Q99-C4, respectively. Ub-polyQ-C4 constructs were generated by replacing M-GFP-Q65 for polyQ-C4 in Ub-M-GFP-Q65 (Kindly provided by Nico Dantuma; Karolinski Institutet, Stockholm, Sweden) (Verhoef et al., 2002), resulting in Ub-Q17-C4, Ub-Q56-C4, and Ub-Q99-C4, respectively.

Httexon1-Q103-C4 was obtained by replacing GFP in Httexon1-Q103-GFP (kindly provided by Ron Kopito; Stanford University, Stanford, USA) by a similar C4 tag as described above. The annealed C4 motif was inserted into Httexon1-Q103-GFP generating Httexon1-Q103-C4. β7-GFP was obtained by PCR from β7-mRFP (kindly provided by Nico Dantuma) (Verhoef et al.) using forward primer 5’-GCGGAATTCCACATGGAAGCGTTTTTGGG-3’ and reverse primer 5’-GGGCCCTTCAAAGCCACTGATCATG-3’. PCR product was subsequently cloned in EGFP-N2 (Invitrogen) using EcoRI and Apal, thereby generating β7-GFP. DnaJB6-GFP, DnaJB8-GFP and Hsp40-GFP were generated by cleavage of pRES-DsRed2-DnaJB6, pRES-DsRed2-DnaJB8 and pRES-DsRed2-Hsp40 (Gillis et al., manuscript in preparation) with BamHI and EcoRI. DnaJB6, DnaJB8 and Hsp40 were subsequently cloned into EGFP-C3 (Invitrogen). Constructs were obtained as described before: LMP2-GFP (Reits et al., 1997), GFP-Ub (Raspe et al., 2009). GFP-Hsp70 was kindly provided by Harm.
Kampinga (University Medical Center Groningen, Groningen, the Netherlands).

**Cell culture and transfection.** Human HeLa cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM; Gibco) supplemented with 10% fetal calf serum (FCS), penicillin-streptomycin-glutamine (Gibco) and maintained at 37°C in an atmosphere of 10% CO₂. Human Embryonic Kidney (HEK293T) cells were cultured in Iscove’s Modified Eagle Medium (IMDM; Gibco) supplemented with 10% FCS, 25 mM Heps and penicillin-streptomycin-glutamine and maintained at 37°C in 5% CO₂. For microscopol analysis, HeLa cells (0.25x10⁶) were plated onto glass coverslips (24 mm; Fisher Scientific) in a 6-well and were transiently transfected after 24 hours using Lipofectamine 2000 (Invitrogen). HEK293T cells (0.1x10⁶) were seeded in a 6-well plate and transiently transfected with FuGene6 (Roche) at 24 hours after plating.

**Biarsenical labeling.** HeLa cells were transfected with various GFP-Ub-polyQ-C4 constructs and biarsenical labeling was performed at 24 or 48 hours after transfection, as described before (Martin et al., 2005). Briefly, 1 mM FlAsH or ReAsH was pre-incubated in 10 mM 1,2-ethanedithiol (EDT; Sigma) in DMSO for 10 minutes. Cells were labeled with 1 μM FlAsH or ReAsH in 10 μM EDT in OptiMEM for 45 minutes at 37°C, 10% CO₂ in the dark and subsequently washed several times with 1 mM EDT in OptiMEM containing 10% FCS during 30 minutes at room temperature to remove unbound dyes. For labeling simultaneously with FlAsH and ReAsH, cells were labeled with a mixture of 0.5 μM FlAsH and 0.5 μM ReAsH. For pulse-chase experiments, cells were labeled with FlAsH at 24 hours and with ReAsH at 48 hours after transfection.

**Photoconversion of ReAsH-labeled cells.** HeLa cells expressing various C4-tagged proteins were cultured on 35 mm glassbottom dished with grid (Mattek) and labeled with ReAsH before photoconversion of diaminobenzidine (DAB), which was performed as described before (Gaietta et al., 2002). Briefly, cells were fixed with 2% glutaraldehyde in cacodylate buffer (0.1 M Na-cocadylate; pH 7.4) for 15 minutes on ice. After washing cells 5 times with cacodylate buffer, cells were briefly rinsed in blocking buffer (50 mM glycine, 5 mM KCN, 0.001% H₂O₂ in 0.1 M Na-cocadylate; pH 7.4) and subsequently incubated for 30 minutes on ice in the same buffer to reduce nonspecific background. Subsequently, cells were imaged using confocal microscopy (Leica TCS SP2) in the presence of DAB solution (6 mM DAB in oxygenated blocking buffer) and cells were locally illuminated at 568 nm until a brownish reaction product became visible. Cells were subsequently prepared for EM analysis. Cells were washed in cacodylate buffer, post-fixed in osmium tetra-oxide (1% osmium tetra-oxide in 0.1 M Na-cacodylate; pH 7.4) for 30 minutes on ice and washed in distilled water. For contrast enhancement in EM, cells were block-stained overnight in 2% uranyl acetate at 4°C and washed again in distilled water. Dehydration was performed through a series of ethanol solutions and cells were embedded in epon LX-112 (Ladd) by putting a capsule filled with epon on top of the cells. After polymerization, the capsule with epon, containing the cells and an imprint of the grid, was removed from the glassbottom dish. Ultrathin sections of 80 nm were cut on a Reichert EM UC6 ultramicrotome with a diamond knife, collected on formvar coated grids and stained with uranyl acetate and lead citrate. Sections were examined with a Fei Technai-12 EM.

**Confocal and FRET microscopy.** HEK293T cells were transfected with GFP-Ub-polyQ-C4 constructs and percentages of transfected cells containing aggregates were determined at 72 hours after transfection using a Leica IRB inverted fluorescence microscope. Confocal images were obtained at 48 hours after transfection using a Leica TCS SP2 confocal system and a 63x objective. Note that some images show ‘over-exposed’ fluorescent aggregates, but this was done to enable visualization of diffuse cytoplasmic and nuclear staining. For FRAP analysis, a part of the cell containing either ReAsH-labeled free polyQ peptides or ReAsH-labeled aggregated polyQ peptides was bleached at full laser power and fluorescence recovery was measured in the bleached area at low laser power.

FLIM was carried out after biarsenical labeling of living cells. To reduce interference of autofluorescence by DMEM, a special medium was added to cells (20 mM Heps; pH 7.4, 137 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl₂, 0.8 mM MgCl₂ and 20 mM glucose). FLIM was performed using the wide-field frequency domain approach on a home-build instrument (Van Munster and Gadella, 2004a) using a RF-modulated image intensifier (Lambert Instruments II18MD) coupled to a CCD camera (Photometrics HQ) as detector. A 40x objective was used for all measurements. The modulation frequency was set to 75.1 MHz. Eighteen phase images with an exposure time of 20-200 ms were acquired in a random recording order to minimize...
artifacts due to photobleaching (van Munster and Gadella, 2004b). Every FLIM measurement was followed by a reference measurement. From the phase sequence an intensity (DC) image and the phase and modulation lifetime image were calculated using Matlab macros. From this data, the lifetime of individual cells was determined using ImageJ (http://rsb.info.nih.gov/ij/). Subsequently, average phase and modulation lifetimes (± s.d.) were calculated. For the presentation of lifetime maps, a 3x3 smooth filter was applied to the lifetime data. The false-color lifetime maps and 1D and 2D histograms were generated by an ImageJ macro.

The FRET efficiency E was calculated according to: \( E = \left(1 - \frac{\tau_{DA}}{\tau_D}\right) \times 100\% \) in which \( \tau_{DA} \) is the fluorescence lifetime of the donor in presence of the acceptor (i.e. samples labeled with both FlAsH or GFP and ReAsH) and \( \tau_D \) is the fluorescence lifetime of the donor (i.e. FlAsH only or GFP only) in absence of the acceptor. Frequency domain FLIM yields a phase lifetime (\( \tau_\pi \)) and a modulation lifetime (\( \tau_M \)). Since \( \tau_\pi \) is more sensitive than \( \tau_M \), the FRET efficiency was calculated on the basis of \( \tau_\pi \).

**Immunoblotting.** Cytoplasmic cell extracts were generated by lysing cells with 0.1% Triton X-100 in PBS for 30 minutes on ice supplemented with mini protease inhibitor cocktail (Roche). Lysates were centrifuged for 15 minutes at 14,000 rpm at 4°C and protein concentrations of supernatants were determined using Bradford protein assay (BioRad). Twenty µg of cytoplasmic protein lysates were boiled in sample buffer (3 mM Tris-HCl pH 6.8, 5% glycerol, 1% SDS, 2.5% 2-mercaptoethanol, 0.05% (w/v) bromophenol blue) for 5 minutes and separated on 12.5% SDS-PAGE. After electrophoresis, proteins were transferred onto a 0.2 µm pore size nitrocellulose membrane filter (Schleicher & Schuell) and blocked in 5% dry milk (Nutricia) in Tris-buffered saline (TBS). Western blots were incubated with primary antibodies against polyQ (1C2; 1:1.000; MAB1574, Millipore), GFP (1:1.000; Invitrogen), or β-actin (1:10,000; Sigma) in TBS containing 0.1% Tween-20 and subsequently with secondary antibodies IRDye 680 or IRDye 800 (1:10,000; LI-COR Biosciences). Signal was detected using the Odyssey imaging system (Westburg). For detection of ReAsH-labeled polyQ peptides, cytoplasmic cell lysates were not boiled before loading on 12.5% SDS-PAGE gels, as this affected the conformation of ReAsH. After running SDS-PAGE gels were scanned using a Typhoon Trio imager (GE Healthcare) at 720 nm.

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