The role of the proteasome in Huntington's disease

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DYNAMIC RECRUITMENT OF ACTIVE PROTEASOMES INTO POLYGLUTAMINE INITIATED INCLUSION BODIES

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ABSTRACT

Neurodegenerative disorders such as Huntington’s disease are hallmarked by neuronal intracellular inclusion body formation. A controversial topic in these protein misfolding disorders is the observed irreversible sequestration of proteasomes into inclusion bodies. These proteasomes may become clogged by the aggregated protein fragments, leading to impairment of the ubiquitin-proteasome system. Here, we show by fluorescence pulse-chase experiments in living cells that proteasomes are dynamically and reversibly recruited into inclusion bodies. As these recruited proteasomes remain catalytically active and accessible for substrates, our results challenge the concept of proteasome sequestration and impairment in Huntington’s disease, and support the reported absence of proteasome impairment in mouse models for Huntington’s disease.

INTRODUCTION

Huntington’s disease (HD) is caused by a polyglutamine (polyQ) expansion in the disease-related huntingtin (Htt) protein, and is hallmarked by inclusion body (IB) formation in neurons [1]. N-terminal fragments of the mutant Htt (mHtt) protein containing the expanded polyQ repeat are thought to initiate IBs that subsequently sequester components of the ubiquitin-proteasome system (UPS) [1, 2]. The observed proteasome sequestration into IBs is considered to contribute to HD pathology due to disruption of proper UPS function [3, 4]. Fluorescence recovery after photobleaching (FRAP) experiments suggest an irreversible sequestration of fluorescently-tagged proteasomes into IBs [5]. In addition, in vitro data suggests that proteasomes cannot cleave within polyQ repeats in polyQ-expanded proteins. As a consequence, pure polyQ fragments are released in the cell that may subsequently act as initiators of aggregation [6, 7]. UPS impairment which is observed in both cell cultures and HD patient material may indeed be caused due to sequestration of proteasomes into IBs initiated by expanded polyQ fragments [6, 8, 9]. However, robust proteasome impairment has not been detected in HD mouse models after IB formation [10, 11]. To examine whether proteasomes are indeed irreversibly sequestered into polyQ IBs, we visualized dynamics of proteasomes and polyQ fragment in IBs by fluorescent pulse-chase experiments. In addition, we used proteasome activity based probes to visualize proteasome accessibility and activity in IBs of individual cells and in brains of HD mice.

RESULTS

Proteasomes directly interact with polyQ peptides when recruited into IBs

To examine the distribution patterns of intracellular proteasomes within polyQ IBs, we initiated aggregation using the N-terminal exon1 fragment of polyQ-expanded mHtt (mHtt(Q97)exon1), which represents the N-terminal cleavage product that is found in brain tissue of HD patients [1, 12]. In addition, pure polyQ peptides, representing products downstream of the proteasome, were used as initiators of aggregation, since it is considered that proteasomes are unable to degrade the polyQ tract [6, 7]. The Ub-Q99 constructs release Q99 peptides directly upon synthesis, as ubiquitin is cleaved off by ubiquitin C-terminal hydrolases [13]. The GFP-tagged proteasomal subunit PSMB4 (B7) was used to visualize proteasomes in living cells. This subunit is efficiently incorporated in both 20S and 26S proteasomes, as shown by complex separation on native gel and subsequent analysis of fluorescence (Supplementary Fig. S1). Recruitment of proteasomes into mHtt initiated IBs was observed when the PSMB4-GFP was co-expressed with tetracysteine-tagged mHtt (mHtt(Q97) exon1-C4). mHtt(Q97)exon1-C4 was labeled with the bisaninul fluorophore ReAsH [14] and was found to be present in the core of IBs. However, dependent on IB size, proteasomes were differently distributed (Fig. 1A). Proteasomes colocalized with mHtt(Q77exon1-C4 in smaller IBs (Fig. 1A, upper panel), but were located in the outer layers of the larger IBs, forming a ring-like structure (Fig. 1A, lower panel). A similar redistribution of proteasomes was observed in cells stably expressing α7-GFP, this efficiently incorporated subunit was used as an alternative proteasomal subunit (data not shown). When C4-tagged polyQ peptides were used (Ub-Q99-C4), a similar proteasomal distribution pattern was observed (Fig. 1B). These findings suggest that proteasomes are not irreversibly trapped, but remain at the periphery of expanding IBs.

To determine whether these proteasomes interact directly with polyQ peptides, both in the core and also in the periphery, we used fluorescence lifetime imaging microscopy (FLIM) to study proteasome-polyQ interactions. Previously, we have shown by wide-field FRET-FLIM microscopy that GFP-tagged proteasomes, ubiquitin and the chaperone HSP70 were all recruited into polyQ aggregates but only GFP-tagged proteasomes directly interacted with polyQ fragments. Whereas GFP-ubiquitin and HSP70-GFP only showed moderate reductions in fluorescence lifetime [15]. By using confocal FRET-FLIM, we could distinguish between protein interactions in de core of the IB and at the periphery of IBs. Co-expression of ReAsH-labeled UbQ99-C4 and GFP-tagged PSMB4 showed a significant decrease in GFP donor lifetime compared to the negative control, both when proteasomes were present in the core or in the ring-like structures of IBs (Fig. 1C). This indicates that while chaperone HSP70 does not act on polyQ peptides in IBs, proteasomes continuously interact with polyQ fragments both at early stages of IB formation as well as during later stages.

Dynamic recruitment of active proteasomes into polyglutamine initiated inclusion bodies

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Proteasomes are dynamically recruited into polyQ initiated IBs

To further determine proteasome dynamics in IBs, we performed a fluorescence pulse-chase experiment. Since FRAP studies did not show fluorescence recovery of proteasomes within IBs over a time span of minutes [5], we studied protein distribution over a time span of hours. Therefore, we first labeled the pre-existing pool of the C4-tagged proteins with red fluorescence using ReAsH. After a subsequent chase period of 8 or 20 hours, to allow the synthesis of new C4-tagged proteins, the cells were incubated with FlAsH to label the newly-synthesized proteins with green fluorescence. Pulse-chase labeling of mRNAQ97 exon1-C4 (Fig. 2A) or Ub-Q99-C4 (Fig. 2E) showed a sequential sequestration of these polyQ fragments, as the newly synthesized mRNAQ97 exon1-C4 or polyQ-C4 peptides were recruited to the outer layers of the IBs whereas the initial polyQ fragments were exclusively found in the core of the IB, both after an 8 and 20 hours chase period. The difference in distribution was not due to uneven penetration of FlAsH and ReAsH, as both dyes efficiently label the core of Q99-C4 aggregates [15]. In contrast, C4-tagged proteasomes (PSMB4-C4) showed a different distribution pattern in IBs that were initiated by co-expression of untagged mRNAQ97 exon1 (Fig. 2C) or polyQ peptides (Fig. 2F). After an 8 hours chase period, there was a partial overlap between the pre-existing and newly-synthesized proteasomes. After a 20 hours chase period, a complete overlap in localization of both proteasome pools was observed in ring-like structures. Indicating that the initial ReAsH-labeled pool exchanged with the newly-synthesized FlAsH-labeled pool of proteasomes. These findings indicate that proteasomes are dynamic within IBs. Fluorescence intensity plots of cross sections of IBs show the difference in localization of the initial pool (red) and the chased pool (green) of mRNAQ97 exon1-C4 or proteasome subunit PSMB4-C4 in the IB (Fig. 2B and Fig. 2D, respectively). The difference in recruitment between proteasomes and mHtt was also shown biochemically. Proteasomes were present only in the SDS-soluble fraction of cells expressing mRNAQ97 exon1, whereas polyQ proteins were present in both the SDS-soluble and SDS-insoluble fraction (Supplementary Fig. S2). Together, these findings indicate that proteasomes are not irreversibly trapped in mHtt IBs, but are dynamically recruited albeit with relatively low exchange rates.

Proteasomes recruited into IBs are still catalytically active and accessible

The dynamic recruitment of proteasomes into IBs and the direct interaction between proteasomes and polyQ fragments does not exclude that proteasomes may become catalytically impaired due to clogging by polyQ fragments, as suggested before [5, 6]. To examine whether proteasomes are active and for substrates in IBs, we added activity based probe 1 (ABP1) (BodipyFl-Ahx,LrV5) [16] to living cells. The ABP covalently labels all three catalytic subunits of proteasomes if the active sites are accessible, and allows their detection via fluorescence. When cells expressing mRNAQ97 exon1-C4 or Ub-Q99-C4 were incubated with the ABP1, proteasomes in both the smaller and larger IBs were labeled (Fig. 3A). The activity labeling also overlapped with proteasomes that were visualized via RFP-tagged PSMB4, indicating that there were no proteasomes present in the dense core that were not labeled and thus catalytically inactive (Fig. 3B). To further examine whether the activity-labeled proteasomes are also accessible to substrates, we preformed zymography to identify only those proteasome complexes that are labeled by the activity probe (Fig. 3C). All proteasome complexes were labeled by ABP1 when cell lysates were incubated with the probe prior to native gel electrophoresis (left panel). However, uncapped 20S proteasomes were not labeled with ABP1 when the probe was added after separation of the different proteasome complexes by electrophoresis (right panel). This indicates that proteasome labeling with ABP1 only occurs when proteasomes are in complex with either 19S or PA28 activating caps. Since the probe...
needs access to the 20S core via these activator caps, activity labeling in IBs can therefore only occur when 20S proteasomes are activated and thus accessible for protein substrates, representing functional proteasomes. In addition, subsequent analysis by SDS gel electrophoresis under reducing conditions demonstrated that all three proteasomal catalytic subunits remained equally active in cells with mHtt(Q97)exon1-C4 or Ub-Q99-C4 initiated IBs (Supplementary Fig. S3).

**IBs in mHtt transgenic mice brains contain active proteasomes**

To translate our finding to an in vivo model, we incubated brain sections of R6/2 transgenic mice that express mHtt(Q200) [17] with ABP1 in order to confirm the presence of active proteasomes in neuronal IBs. Both the cerebral cortex and striatum in R6/2 mice have the most mHtt-induced IBs, and immunohistochemistry was used to identify the mHtt-containing IBs in neuronal cells in the cerebral cortex (Fig. 4). Colocalization of N18 (mHtt), NeuN (neuronal nuclei marker) and ABP1 confirmed the presence of active proteasomes within IBs in mouse brains, with 93 % (±6 %, SD) of the nuclear inclusions efficiently labeled with the proteasome activity probe, and 87 % (±5 %, SD) of the cyttoplasmic inclusion bodies. To confirm the specificity of proteasome activity labeling in mouse brain tissue we pre-incubated brain slices with proteasome inhibitor epoxomycin, resulting in a decrease of fluorescence labeling (data not shown).

**DISCUSSION**

When we visualized the dynamics of proteasomes over a time span of hours, an exchange of proteasomes was observed (Fig. 2C). This is in contrast to earlier Fluorescence Recovery After Photo-bleaching (FRAP) experiments in living cells that did not show recovery of fluoresecently-tagged proteasomes within a time span of minutes [5]. This indicates that proteasomes have a slow on-off rate into IBs, which was also suggested by the differences in distribution of fluoresecently-tagged proteasomes in aggregates (Fig. 1A). Our data is in correspondence with the assumed protective role of IB formation, which was shown when aggregating GFP-tagged mHtt fragments in striatal neurons were imaged in time by automated fluorescence microscopy [18]. IB formation improved cell survival when compared to cells that showed only a diffuse mHtt distribution but no aggregates. Similarly, IB formation also coincided with less proteasomal impairment, which was detected using a short-lived UPS reporter [19]. A drop in proteasome activity was only observed just before IBs were formed, indicating that IB formation would indeed be a protective mechanism to sequester toxic mHtt species in the cell that may otherwise impair the UPS.

The observed recruitment of proteasomes into IBs appears to be independent of ubiquitination of the aggregated polyQ fragments. Aggregates induced by mHtt [20] but also by pure polyQ peptides [7] are initially devoid of ubiquitin, while proteasomes are directly recruited when small aggregates are formed. Although we show that the recruited proteasomes remain catalytically active and

![Image](image_url)

**Fig. 2. Proteasomes are dynamically recruited into polyQ initiated IBs.**

(A) Fluorescence pulse-chase experiments show sequestration of mHtt polyQ fragments into IBs. Cells expressing mHtt(Q97) exon1-C4 for 48 h showed IBs that were labeled with ReAsH (red, R). Following a chase of 8 or 20 h, newly-synthesized mHtt(Q97) exon1-C4 was labeled with FlAsH (green, F), which was localized in a new layer around the existing IB induced by previously synthesized mHtt fragments. (B) Cross section of a mHtt(Q97)exon1 initiated IB showing differences in fluorescence pixel intensities of FlAsH and ReAsH labeling after a chase of 20 h. (C) Fluorescence pulse-chase experiments show redistribution of proteasomes in mHtt(Q7)exon1-C4 induced IBs. Cells were transfected with untagged mHtt(Q7)exon1-C4 to induce IBs and the proteasome subunit PSMB4-C4 which was subsequently labeled with ReAsH (red, R). Following a chase of 8 or 20 h, newly-synthesized PSMB4-C4 was labeled with FlAsH (green, F), showing an exchange between old (R) and newly-synthesized (F) proteasomes in yellow. (D) Cross section of C4-tagged proteasomes in the mHtt initiated IBs showing large overlap in fluorescence pixel intensities of FlAsH and ReAsH labeling after a chase of 20 h. (E) Fluorescence pulse-chase experiment shows sequestration of Q99 peptides into IBs. Cells expressing Ub-Q99-C4 for 48 h were labeled with ReAsH (red, R). Following a chase of 8 or 20 h, newly-synthesized Q99 peptides were labeled with FlAsH (green, F), which was localized in a new layer around the existing Q99 peptide in IBs (red, R). (F) Fluorescence pulse-chase experiment shows redistribution of proteasomes in polyQ peptide IBs. Cells containing untagged Q103-peptide IBs co-expressed the proteasomal subunit PSMB4-C4 for 48 h which was labeled with ReAsH (red, R). Following a chase of 8 or 20 h, newly-synthesized PSMB4-C4 was labeled with FlAsH (green, F), showing an exchange between old (R) and newly-synthesized (F) proteasomes with colocalization in yellow. Scale bar = 5 μm.
accessible for substrates, a competition between regular UPS substrates and aggregating mHtt as substrates for the proteolysis network might affect the turn-over of other intracellular proteins, as recently proposed [20]. As discussed earlier, this would explain the increase in half-lives of various short-lived UPS reporters [19].

Our observation that proteasomes which are recruited into IBs remain active and accessible seems in contrast to earlier reports that suggest that proteasomes can become impaired due to unsuccessful attempts to degrade polyQ proteins. When examining polyQ protein degradation by proteasomes in vitro and in living cells, it was suggested that proteasomes may actually be unable to degrade the polyQ repeat present in the proteins. The undigested, expanded polyQ peptide derived from a degraded mHtt fragment may be unable to diffuse out of the proteasome, resulting in proteasomal impairment. A stable interaction between fluorescently-tagged proteasomes and fluorescent mHtt fragments was indeed observed by FRET microscopy [5], although this could also be due to a reversible interaction when proteasomes act on mHtt in IBs, as shown by FLIM microscopy (Fig. 1C). Importantly, our fluorescent pulse-chase experiments show that these interactions are reversible since there is an exchange of proteasomes at the periphery if IBs, in contrast to mHtt and polyQ peptides that are irreversibly trapped in IBs.

PolyQ fragments that enter the proteasome appear to be efficiently degraded, since less aggregation is observed when polyQ proteins like mHtt are targeted towards the proteasome [21]. This indicates that the proteasome can indeed deal with polyQ proteins, which is in line with our results that proteasomes recruited into IBs remain catalytically active and accessible for substrates. The dynamic recruitment of active proteasomes to IBs may also occur in other protein-folding diseases such as Parkinson’s disease and ALS. Therefore, up-regulation of the UPS may be a potential therapeutic target to slow down disease progression.
**MATERIALS AND METHODS**

**DNA Constructs.** mHtt(Q97)exon1 was generated by replacing the C-terminal GFP sequence of polyQ-expanded mHttexon1-GFP (kindly provided by Prof. RR. Kopito, Stanford University, USA) for a tetracysteine C4-tag [FLNCCPGCCCMEE] and a stop codon [14]. Ub-Q103 was generated as described previously [7]. Ub-Q99-C4 was generated by introducing a C4-tag after GFP-Ub-Q99 using two annealed C4-primers, forward 5′-GATCTGTTTCTAATGTGTTCACTGGTTTGATATGCCCTAACT-3′ and reverse 5′-CTAGATTAAAGGGTCTACATCAACAGGGGAACTTACAACTA-3′. PSMB4-mRFP was kindly provided by Prof. NP. Dantuma (Karolinski Institute, Stockholm, Sweden), and the PSMB4-GFP and PSMB4-C4 constructs were generated by replacing the mRFP for an eGFP or C4-tag.

**Cell culture and transfection.** HeLa, Mel JuSo (melanoma) and HEK293 cells were cultured in DMEM supplemented with 10 % fetal calf serum at 37°C in a 5 % CO₂ atmosphere. HEK293 cells were transfected with jetPEI (Polyplus transfection), HeLa and Mel Juso cells were transiently transfected with HD Fugene as described by the manufacturer (Roche Applied Sciences, Mannheim, Germany). For confocal microscopy imaging cells were grown on 2 cm coverslips (Menzel Glaser, Braunschweig, Germany) in 6-well plates.

**Biarsenical labeling and confocal imaging.** At 48 h after transfection, HeLa and Mel JuSo cells were stained as described by Martin et al. [14]. Briefly, 1 mM ReAsh which was pre-incubated in 10 mM 1,2-ethanedithiol (EDT, Sigma-Aldrich, Sigma-Aldrich, Steinheim, Germany) in dimethyl sulfoxide (DMSO) for 10 min. Subsequently, cells were washed using PBS ( Gibco/Invitrogen, Breda, The Netherlands) and incubated for 45 min at 37°C with 1 μM ReAsh in Optimem ( Gibco/Invitrogen, Breda, The Netherlands), followed by 4 washes at RT in wash medium (complete DMEM medium with 1 mM EDT). Mel Juso cells were fixed with 2 % paraformaldehyde (EM, Hatfield, PA, USA) in 1x PBS (Gibco/Invitrogen, Breda, The Netherlands) prior to labeling and HeLa cells were fixed after labeling. Samples were examined using a Leica TCS SP2 confocal microscope equipped with an Ar/Kr laser and 40x or 63x objective (Leica Microsystems, Mannheim, Germany).

**Native gel analysis.** HEK293 cells were harvested in TSDG buffer (10 mM Tris pH7.5, 25 mM KCl, 10 mM NaCl, 1.1 mM MgCl₂, 0.1 mM EDTA and 8 % glycerol) and lysed by 3 freeze/thaw cycles in 10 mM NaCl, 1.1 mM MgCl₂, 0.1 mM EDTA and 8 % glycerol) and lysed by 3 freeze/thaw cycles in 1 mM EDT). Mel Juso cells were fixed with 2 % paraformaldehyde (EMS, Hatfield, PA, USA) in 1x PBS (Gibco/Invitrogen, Breda, The Netherlands) prior to labeling and HeLa cells were fixed after labeling. Samples were examined using a Leica TCS SP2 confocal microscope equipped with an Ar/Kr laser and 40x or 63x objective (Leica Microsystems, Mannheim, Germany).

**Soluble and insoluble fraction analysis.** HEK293 cells were lysed in 1.5 % SDS buffer (70 mM Tris pH 6.8, 1.5 % SDS, 20 % glycerol) and sonificated by the Soniprep150 (Sanyo, Leicester, UK), 50 mM EDT was added and samples were boiled for 10 min prior to 1 h centrifugation at 20,817 x g at RT. The pelleted fraction, representing the insoluble fraction, was incubated with 10 μl 100 % formic acid, incubated at 37°C for 30 min and lyophilized for o/n in a speedvac (Eppendorf, Hamburg, Germany). 1.5 % SDS buffer was added to the pellet, following with 10 μl 100 % formic acid, incubated at 37°C for 30 min and lyophilized for o/n in a speedvac (Eppendorf, Hamburg, Germany). For each ROI the fluorescence decay curve was fitted using a multi-exponential decay fit, including the estimated instrument response function (IRF) and a background term. For data analysis only fluorescence decays having more than 10,000 registered photons in the peak were considered and the quality of the fit was judged by visual inspection of the fitsresiduals and the normalized Chi² value.

**Pulse-Chase experiments.** HeLa cells were labeled with ReAsh as describes above to stain the pre-existing pool of C4-tagged proteins. After washing the cells were incubated at 37°C for 8 or 20 h in 20 % fetal calf serum supplemented DMEM. After the chase, newly synthesized proteins were labeled with University of Copenhagen), and detection was done by the Odyssey detection system (LICOR Biosciences, Lincoln, NE, USA).
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Proteasome activity labeling in living cells. Mel Ju5o cells were incubated for 1 h at 37°C in serum free medium supplemented with 0.25 μM ABP1 (BodyFiL-Ahx3LVS) [16]. Cells were subsequently washed 3 times in PBS (GIBCO/Invitrogen, Breda, The Netherlands) and fixed in 2 % paraformaldehyde (EMS, Hatfield, PA, USA) prior to ReAsH staining.

Activity labeling in gel. HEK293 cells were harvested in TSDG buffer (10 mM Tris pH 7.5, 25 mM KCl, 10 mM NaCl, 1.1 mM MgCl2, 0.1 mM EDTA and 8 % glycerol), lysed by three freeze/thaw cycles in liquid nitrogen and protein levels were determined by a Bradford assay (Serva, Heidelberg, Germany). 40 μg of cell lysates were incubated with 0.5 μM ABP1, 0.5 μM Epoxomicin or similar amounts of DMSO for 1 h at 37°C. Samples were loaded on a 4-12 % Criterion XT Precast Bis-Tris gel (Biorad, Hercules, CA, USA) and subsequently stained with 0.1 % w/v Coomassie Brilliant Blue R-250 (Sigma, St Louis, MO, USA) or western blotting was preformed as described above.

Activity labeling in vivo. Freshly frozen brains of 15-week-old R6/2 mice (mHtt(Q200)) and littermate controls were used (kindly provided by prof. G. Bates, London). 10 μm thick brain slices were mounted on 1 mm thick PEN membrane slides (Zeiss, Oberkochen, Germany), dried for 10 minutes at room temperature and stored at -20°C until further use. Sections were incubated for 10 minutes with 1 μM ABP1 in 10 mM PBS. After 3x washing with PBS, sections were fixed with 4 % paraformaldehyde (EMS, Hatfield, PA, USA). Next, sections were pre-incubated in permeabilization buffer (1 % BSA, 2 % FCS and 0.4 % Triton-X100) for 1 h and incubated with antibodies against the N-terminal region of mHtt-exon1 (sc-8767, 1:500, Santa Cruz, Santa Cruz, CA, USA) and the neuronal nuclear protein NeuN (ABP1 in 10 mM PBS. After 3x washing with PBS, sections were fixed with 4 % paraformaldehyde (EMS, Hatfield, PA, USA) and subsequently stained with 0.1 % w/v Coomassie Brilliant Blue R-250 (Sigma, St Louis, MO, USA) or western blotting was preformed as described above.

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