The role of the proteasome in Huntington's disease

Krom, S.

Citation for published version (APA):
Krom, S. (2013). The role of the proteasome in Huntington’s disease.
DYNAMIC RECRUITMENT OF ACTIVE PROTEASOMES INTO POLYGLUTAMINE INITIATED INCLUSION BODIES

S. Schipper-Krom¹
K. Juenemann¹
A. H. Jansen¹
A. Wiemhoefer¹
R. van den Nieuwendijk²
D. Smiths³

M. A. Hink⁴
G. Bates²
H. Overkleeft²
H. Ova⁵
E. A. J. Reits¹

¹ Department of Cell Biology and Histology, Academic Medical Center, University of Amsterdam, Meibergdreef 15, 1105 AZ The Netherlands
² Department of Bio-Organic Synthesis, Institute of Chemistry, University of Leiden, Einsteinweg 55, 2333 CC Leiden, The Netherlands
³ Department of Medical and Molecular genetics, King’s College London School of Medicine, Guy’s Hospital, Great Maze Pond, SE1 9RT, London, United Kingdom
⁴ Section Molecular Cytology, Swammerdam Institute for Life Sciences, University of Amsterdam, Sciencepark 904, 1090 GE Amsterdam, The Netherlands
⁵ Department of Cell Biology II, Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands

Revised version submitted to FEBS letters
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 agglomeration [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 proteosomal 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 biarsenical 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(Q97)exon1-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.
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 mHtt(Q97)exon1-C4 (Fig. 2A) or Ub-Q99-C4 (Fig. 2E) showed a sequential sequestration of these polyQ fragments, as the newly synthesized mHtt(Q97) 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 mHtt(Q97)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 mHtt(Q97)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 mHtt(Q97)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 accessible for substrates in IBs, we added activity based probe 1 (ABP1) (BodypiFL-Ahx,LV5) [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 mHtt(Q97)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 205 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
IBs in mHtt transgenic mouse 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 cytoplasmic inclusion bodies. To confirm the specificity of proteasome activity labeling in mouse brain tissue we pre-incubated brain slices with proteasome inhibitor epoxomicin, 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 fluorescently-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 fluorescently-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 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 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).
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 undegisted, 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 (FLNCCGCCMPE) 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’-GATCCTTCTAAATGTTGCTCGGTGTTGATAGAACCTAAAT-3’ and reverse 5’-CTAGATTAAGGGTCTACAAACAGGAAACATTAAAGAACA-3’. PSMB4-mRFP was kindly provided by Prof. NP. Dantuma (Karolinska 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 transient 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.

Biosensical 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-ethanediethiol (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 (EMS, Hatfield, PA, USA) in 1x PBS (GIBCO/Invitrogen, Breda, The Netherlands) prior to labeling and HeLa cells were pelleted after labeling. Samples were examined using a Leica TCS SP2 confocal microscope equipped with Ar/Kr laser and 40x or 63x objective (Leica Microsystems, Berlin, Germany) and analysed in SymPhoTime 5.13 software (Picoquant, Berlin, Germany). For each ROI the fluorescence decay curve was fitted using a mono-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 fits and the normalized Chi² value.

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 10 % 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). 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).

Confocal FLIM. We used EGFP and the red biosensical fluorophore ReAsh as a FRET pair. Data was acquired using an Olympus IX81 inverted microscope with a FITC and confocal detection head, controlled by FV2.3 software. Donor fluorophores were excited using a 440 nm 20 MHz pulsing laser diode (Picoquant, Berlin, Germany), as controlled by a Sepiall laser driver unit (Picoquant, Berlin, Germany) and attenuated 10 times by a neutral density filter. The light was guided via a D405/480/560/635 primary dichroic mirror (Chroma, Brattleboro, VT, USA) through a water immersed 60x UPlanAp- objective (NA 1.2) into the sample. Cellular samples were grown on 24 mm round coverslips (Menzel Glaser, Braunschweig, Germany), fixed in 2 % paraformaldehyde (EMS, Hatfield, PA, USA) and stored in PBS (GIBCO/Invitrogen, Breda, The Netherlands). The emission light was guided via a size-adjustable pinhole, set at 120 μm, through the Olympus detection box to the fibre output channel. The optical fibre was coupled to a custom-made detection box (Picoquant, Berlin, Germany) containing MPD avalanche photodiodes. The light was guided into one of the MPDs and filtered by a 525/45 emission filter (Chroma, Brattleboro, VT, USA). The photon arrival times were recorded by a Picoharp 300 time-correlated single-photon counting system (Picoquant, Berlin, Germany) and analysed in SymPhoTime 5.13 software (Picoquant, Berlin, Germany). For each ROI the fluorescence decay curve was fitted using a mono-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 fits 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).

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. The chase, newly synthesized proteins were labeled with University of Copenhagen).
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) for electrophoresis and subsequently scanned for fluorescence on a Typhoon imager (GE Healthsciences) using the 520 BP 40 filter. The gel slab was incubated for 20 min in 20 ml Overlay buffer (20 mM Tris pH 7.5, 5 mM MgCl₂, 1 mM ATP and 0.25 μM ABP1) and washed for 3 h in Destain buffer (5 % acetic acid, 20 % MeOH). The gel slabs were transferred to PVDF membrane (Millipore, Bedford, MA, USA) and western blotting was performed 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 in 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 

**Acknowledgements**

We like to thank Alicia Sanz Sanz, Andrea Lehmann and Dr. Frédéric Ebstein for their contributions in the experiments, Dr. R. Hartmann-Petersen for providing the MCP236 antibody, prof. PM. Kloeotzel for fruitful discussions, and Prof. CIF. van Noorden for critically reading the manuscript.
Dynamic recruitment of active proteasomes into polyglutamine initiated inclusion bodies

Dynamic recruitment of active proteasomes into polyglutamine initiated inclusion bodies

REFERENCES


