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MODULATING PROTEASOME ACTIVITY IN HUNTINGTON’S DISEASE

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**ABSTRACT**

Huntington’s disease (HD) is a neurodegenerative disease which is caused by an expansion of the polyglutamine (polyQ) tract in the huntingtin protein. Fragments of the huntingtin protein containing the expanded polyQ tract accumulate into aggregates, thereby recruiting other proteins including components of the ubiquitin proteasome system. The role of proteasomes in HD is controversial. Proteasomes may be unable to degrade the polyQ tracts and become impaired while degrading these polyQ fragments. In addition, induction of immunoproteasomes has been reported in HD and may enable cells to cope with the hazardous protein fragments. In this study, we show that proteasomes are able to cleave within polyQ tracts and that degradation of polyQ peptides can be improved by activation of the proteasome. Increasing proteasomal activity by introduction of the PA28αβ activator improved degradation of expanded polyQ peptides, but these activated proteasomal complexes appeared unable to target the folded huntingtin protein. Alteration of the catalytic activity of proteasomes by introducing immunosubunits in the 20S proteasome increased degradation of mutant huntingtin in vitro but not in cells. Interestingly, modulation proteasomal activity by selective inhibition of individual catalytic sites suggests a specific role for β1 and β5 in mutant huntingtin degradation.

**INTRODUCTION**

Huntington’s disease (HD) is a progressive neurodegenerative disease, resulting in movement disorders and psychological changes [1, 2]. The disease belongs to a group of polyglutamine (polyQ) disorders that are caused by a polyQ expansion in the disease-related protein [3, 4]. When the polyQ repeat, which is located at the N-terminus of the huntingtin (Htt) protein, exceeds the threshold of approximately 37 glutamines, the disease is initiated. This is hallmark by the occurrence of nuclear aggregates or inclusion bodies (IB) in neurons of affected brain regions in patients and transgenic mouse models [5-8]. These aggregates are initiated by accumulation of the aggregation-prone N-terminus of mutant huntingtin (mHtt) fragments, which in turn also recruit other cellular proteins [8, 9]. Whether large aggregates, smaller oligomers or monomeric polyQ-expanded Htt fragments represent the most toxic species is still under debate [10-13]. Nowadays, it is even thought that large aggregates (or inclusion bodies) may be protective, and that the intermediate oligomers are particular toxic to cells [14-16]. Therefore, clearance of these fragments in an early stage, prior to oligomerization, would be beneficial in order to prevent aggregation and toxicity. Intracellular protein clearance can be accomplished by two major degradation systems in cells, the ubiquitin proteasome system (UPS) and autophagy. While it has been shown that induction of autophagy leads to amelioration of the HD phenotype, the autophagic machinery is absent in the nucleus and thus will not be able to target nuclear mHtt fragments or aggregates [17, 18]. This emphasizes the importance of studying the role of the UPS in HD, which is present in both the cytoplasm and the nucleus of the cell.

The role of proteasomes in HD disease progression has been controversial. Various studies in cell culture and patient material reported UPS impairment [19-21], with proteasomes being sequestered into aggregates [9, 22, 23]. Meanwhile, other studies showed a temporal UPS impairment prior to IB formation only, which may be due to accumulation of polyQ fragments [24, 25]. We recently observed that proteasomes were not irreversibly trapped in IBs but are dynamically recruited, and remain accessible for substrates (manuscript submitted). Furthermore, proteasomes were shown to be unable to cleave within polyQ sequences, which led to the hypothesis that the polyQ fragments derived from inefficient cleavage can physically impair proteasomes [23, 26]. However, both in vivo and in vitro studies have shown that polyQ peptides and polyQ proteins can actually be degraded by proteasomes [27-29]. Taken together, it is evident that proteasomes play an important role during development of HD and although proteasomes seem to be able to degrade polyQ proteins, it is not sufficient for efficient clearance. This raises the question whether modification of proteasomal activity is beneficial for degrading mHtt fragments more efficiently and to diminish the disease phenotype.

The UPS operates via recognition of proteins which are marked for degradation by poly-ubiquitination [30, 31]. Consequently, poly-ubiquitinated proteins are unfolded by the 19S activator which guides the substrates into the 20S barrel [32]. Together these complexes are called the 26S proteasomes. The 20S proteasome exists of a barrel-shaped core that contains four stacked rings [33-36]. The outer two rings, called the α-rings, are important for opening and closing of the barrel. The inner two β-rings are responsible for protein cleavage since each ring contains 3 catalytic subunits. Each of these subunits have their own specificity, with caspase activity of β1, trypsin-like activity of β2 and chymotrypsin-like activity of β5. One way to modulate the activity of the proteasome is by exchanging these constitutive catalytic subunits by immunosubunits [37-40]. Two of the genes, coding for immunosubunits β1i and β5i, are present on the MHC class II locus and are transcribed after interferon(IFN)γ stimulation [41]. The third immunosubunit β2i is not located on the MHC class II locus but is also induced after IFNγ stimulation [42-44]. After an inflammatory response, these subunits are incorporated into de novo synthesized immunoproteasomes (20S) that are transiently expressed [45, 46]. Besides immunosubunits, IFNγ also induces subunits of the alternative proteasome activator (PA)28αβ. The 20S core of the proteasome is a latent complex and needs to be activated in order to degrade substrates [47]. The main pool of 20S proteasomes is activated by the 19S activator in an ATP-dependent fashion. Alternatively, the 20S core can also be activated by PA28αβ in order to increase peptidase activity [48-50]. The PA28α is formed by PA28a and PA28β subunits which form a hetero heptameric ring structure that binds to the α-rings of the 20S core, thereby opening the normally closed conformation of the core and increasing peptidase activity. This type of proteasome activation is ATP independent, and unlike the 19S complex, PA28 cannot unfold proteins.

In the present study, we explored the effects of altered proteasome activity in mHtt degradation. Proteasomal activation by PA28 increased degradation of polyQ peptides but degradation of mHtt(Q97) did not appear to be improved. However, manipulation of the catalytic activities of the proteasome affected degradation of mHtt, as replacement of the constitutive subunits for immunosubunits showed improved degradation of mHtt in vitro but not in vivo. Inhibition of individual proteasomal catalytic sites resulted in different cleavage patterns of mHtt, and especially inhibition of β5 resulted in decreased levels of mHtt in vitro and in cells.
RESULTS

PolyQ degradation by IFNγ-modulated proteasomes does not improve mHtt degradation

Conditional mouse models expressing polyQ-expanded Htt show the induction of immunoproteasomes, which may be a cellular response to encounter accumulation mHtt fragments [51, 52]. Since there are discrepancies between the various studies concerning the ability of proteasomes to cleave polyQ peptides, we developed a system where endopeptidase activity against polyQ sequences can be detected via generation of fluorescence. While earlier studies used small polyQ peptides that can be targeted by exopeptidases such as puromycin sensitive aminopeptidase (PSA), we were especially interested in the activation of endopeptidases, as these peptides should be able to cleave within larger polyQ sequences [26, 53]. In our approach, we separated proteases from cell lysates on native PAGE and incubated the wet gel slab with quenched Q8-peptides that only become fluorescent after cleavage between glutamine residues (Fig. 1A).

The flanking sequences of these peptides are synthesized from D-amino acids that makes these peptides inaccessible for exopeptidases, and these peptides can thus only be cleaved within the polyQ sequence via endopeptidase activity. Upon protein complex separation and incubation with the quenched polyQ-peptide reporter, a number of fluorescent bands appeared due to polyQ cleavage by protease activities (Fig. 1B). When the cell lysate was incubated with a proteasome activity-based probe (ABP) [54], which labels active proteasome complexes, a similar cleavage pattern appeared (Fig. 1B) which was absent in lysates that were pre-incubated with proteasome inhibitor (Fig. 1B). Merging both fluorescent signals showed identical patterns confirming that cleavage of the quenched Q8-peptides was achieved by proteasomes (Fig. 1B). Only the band representing the latent 20S complex was lacking after incubation with fluorogenic substrates. This data shows that activated proteasomal complexes are able to cleave between glutamines. To investigate whether changes in proteasomal composition due to IFNγ stimulation affect activity towards Q8-peptides, we compared peptide cleavage patterns by non-stimulated cell lysates with IFNγ-stimulated cell lysates (Fig. 1C). Increased fluorescence of PA28-capped and so-called hybrid proteasomes (19S-20S-PA28) indicates improved degradation of Q8-peptides due to the induction of PA28αβ. Blotting the native gel with PA28α antibodies confirmed the increased levels of PA28αβ-capped complexes (Fig. 1C). Subsequent western blot analysis after running the cell lysates on SDS-PAGE confirmed that equal amounts of 20S proteasomes were loaded, as indicated by immunostaining of α2 subunits.

To examine whether the increased capacity to degrade Q8-peptides could be extrapolated to cells, we compared mHtt degradation in IFNγ-stimulated cells with control cells, both transfected with the N-terminal fragment of mHtt, mHtt(Q97)exon1 (Fig. 1D). Importantly, the induction of immunoproteasomes is a relatively rapid process, while the formation of detectable aggregates takes at least 48 h. To study the effect of IFNγ-modulated proteasomes on soluble levels of mHtt and on aggregate formation of mHtt, we stimulated cells for 24, 48 and 72 h with IFNγ. No significant effects on mHtt(Q97)exon1 protein levels were detected, as quantified by immunoblotting against
Modulating proteasome activity in Huntington's disease

Proteasome activation improves mHtt degradation in vitro but not by PA28 capping

IFNγ activates multiple pathways in the cell to induce antiviral responses, and one of the consequences is the production of reactive oxygen species that oxidize and damage the existing protein pool [55, 56]. In addition, IFNγ may induce the synthesis of defective ribosomal intermediate products (DRiPs) [57-59], resulting in an increased pool of oxidized and misfolded proteins that are subsequently targeted for proteasomal degradation [60]. This makes the UPS to become overloaded with substrates, thereby having a limited capacity for mHtt degradation, but these defective proteins are also prone to aggregation and may accelerate mHtt aggregation. To exclude these additional effects by IFNγ on proteasomal dependent degradation and aggregate formation, an in vitro assay was performed to study the effects of proteasome modulation on mHtt degradation. We used H4-tagged mHtt(Q97) exon1 that was isolated from transfected N2A cells as substrate. Once purified, mHtt(Q97)exon1-H4 was incubated with 20S proteasomes that were isolated from human erythrocytes. We recently showed that mHtt(Q97) exon1-H4 is in vitro completely degraded by 20S proteasomes, thereby generating intermediate polyQ containing fragments [61]. To determine how modulation of these proteasomes affect mHtt(Q97)exon1-H4 degradation, we activated purified 20S proteasomes using different activators. One way to activate 20S proteasomes is gate opening by using RPT peptides which represent the C-termini of the 19S subunits RPT2 and RPT5 [62, 63]. Separation of the catalytic subunits on SDS-PAGE after ABP incubation showed increased activation of the catalytic subunits when the RPT peptides were added (Fig. 2A). When mHtt(Q97)exon1-H4 was incubated with RPT-activated proteasomes, RPT2 and RPT5 peptides increased degradation of mHtt(Q97)exon1-H4 as compared to degradation by control 20S proteasomes (Fig. 2A). Addition of epoxomicin to the reaction confirmed that mHtt was degraded by proteasomes and not by other enzymes (Fig.2A). An alternative method to obtain open-gated proteasomes, is the use of low concentrations of SDS [64]. Similarly as observed for RPT peptides, activation by 0.01 % SDS increased degradation of mHtt(Q97)exon1-H4 (Fig. 1B). In contrast, addition of PA28αβ to the in vitro reaction mixture even prevented proteasomes from degrading mHtt(Q97)exon1-H4, both in the presence and the absence of the 19S cap (Fig. 2C). Activity labeling confirmed activation of the proteasomes by PA28αβ, and complex identification on gradient native gel showed the shift to PA28-capped 20S proteasomes that are highly active (Fig. 2D). Addition of PA28αβ to 26S proteasomes did not result in hybrid complex formation, as only PA28αβ-capped 20S complexes were present (Fig. 2D, last two lanes).

The inability of PA28αβ-capped proteasomes to degrade mHtt suggests that the PA28 cap forms a physical barrier which prevents entrance into the catalytic cavity, which may be due to the folded structure of the mHtt. The proline-rich region at the C-terminus can form a knick in the secondary structure of mHtt, that may prevent entrance into the PA28αβ-capped core [65]. Another obstacle could be the secondary structure of the expanded polyQ sequence, which is supposed to form multiple β-sheets in its folded state [66]. To examine their contribution to the limited degradation by PA28αβ-capped proteasomes, the proline-rich region was removed from mHtt (mHtt(Q97)exon1-ΔPRO) and incubated with isolated proteasomes, as well as wild-type Htt(Q25)exon1-H4 with a shorter sequence of 25 glutamines (Fig. 2E). Unlike mHtt(Q97-exon1-H4, mHtt(Q97)exon1-ΔPRO-H4 degradation by PA28αβ activated proteasomes was as efficient as by non-capped proteasomes (Fig. 2E). Additionally, wild type Htt(Q25)exon1-H4 was rapidly degraded by PA28-capped proteasomes although at slower rate than when compared to non-capped proteasomes (as shown by the longer exposure time). Together, this indicates that the presence of the proline-rich region obstructs entering...
of mHtt-exon1 into the cavity of PA28αβ-capped proteasomes, although the polyQ expansion also contributes to a slower degradation. These results indicate that activation of 20S proteasomes via direct opening of the α-ring improves mHtt degradation. However, activation by the PA28αβ cap allows increased entering of ABP but limits entering of folded mHtt into the proteasome.

**PA28-capped proteasomes improve polyQ peptide degradation but not mHtt degradation in cells**

Previous results showed that mHtt cannot be degraded by PA28αβ-capped proteasomes in vitro. However PA28-capped proteasomes in lysates of IFNγ-stimulated cells had increased activity towards quenched Q8-peptides. Moreover, overexpression of PA28αβ in cells increased cleavage of quenched Q8-peptides two-fold, which is considerably more when compared to IFNγ-induced proteasome activation (Fig. 3A). To analyze whether expanded polyQ peptides that exceed the disease-related threshold of 36-40 glutamines are also better degraded by PA28αβ-capped proteasomes in cells, HEK293 cells were transfected with PA28αβ or the individual PA28 subunits together with pure Q54-peptides using a GFP-Ub-Q54 construct. Upon synthesis of GFP-Ub-Q54, the GFP-Ub is cleaved immediately by C-terminal hydrolases and as a consequence the Q54-peptides are released as pure peptides without a starting methionine [67]. As a control, we cotransfected the chaperone DnaJB6 that keeps polyQ peptides in a soluble form and reduces aggregation [68]. After 72 hours of expression, cells were harvested and lysates were blotted with antibodies against polyQ sequences and β-actin (Fig. 3B). The aggregated fraction was loaded on a filtertrap assay and blotted for polyQ aggregates (Fig. 3B). When PA28αβ was co-expressed, a significant reduction of both soluble and aggregated Q54-peptide levels was observed (Fig. 3B), although expression of PA28α alone also seemed to have an effect on polyQ peptide degradation. This can be explained by the fact that homomeric PA28α is also able to activate proteasomes, although with less affinity than heteromeric complexes [49, 69]. Additionally, it was shown in HEK293 cells that overexpression of PA28α stabilizes endogenous PA28β, thus increasing proteasome activation [70]. Proteasome complex identification on native gel indeed showed a shift towards PA28-activated proteasomes when only PA28α was expressed (Supplementary Fig. 1). To confirm whether the effects were proteasome dependent, we incubated the cells the last 16 hours in the presence of the proteasome inhibitor epoxomicin (Fig. 3C). Inhibition of proteasomes reduced the observed PA28αβ effect on soluble polyQ peptides and aggregation levels, indicating that the soluble Q54-peptides were degraded by proteasomes (Fig. 3C). The limited decrease in aggregation after epoxomicin incubation can be explained by the relatively short incubation time with the inhibitor compared to the long expression time of PA28αβ.

While degradation of polyQ peptides is apparently improved by PA28αβ both in vitro and in cells, isolated mHtt was not degraded by PA28-capped proteasomes (Fig. 2E). This may be due to the requirement to unfold mHtt prior to proteasomal digestion which may be facilitated by the proteostasis network in cells. When HEK293 cells were cotransfected with mHtt(Q97) and PA28αβ, PA28α or PA28β, and lysates were blotted to demonstrate polyQ levels, effect of PA28 on both soluble and insoluble levels was not observed (Fig. 3D). Together, this data shows that proteasome activation by PA28αβ improves polyQ peptide degradation in cells but does not improve mHtt turnover. This may well be due to the inability of folded mHtt to enter the proteasome.

**Immunoproteasomes improve mHtt degradation in vitro but not in cells**

In addition to manipulation of the accessibility and activation of 20S proteasomes via protein activators, the proteasomal catalytic activities themselves can also be modulated by exchanging the three catalytic subunits β1, β2 and β5 for the immunosubunits β1i, β2i and β5i, respectively. To evaluate the direct effect of these alterations in catalytic sites on mHtt degradation, we compared
in vitro degradation of mHtt(Q97)exon1-H4 by constitutive proteasomes and immunoproteasomes that were isolated from human spleen (Fig. 4A). Interestingly, not only was mHtt more rapidly degraded by immunoproteasomes since a faster decrease of input levels was observed, but also the appearance of additional proteolytic fragments suggests different rates of cleavage activities (Fig. 4A, fragments are indicated by asterisks). This effect was solely due to altered catalytic activities since equal levels of 20S proteasomes were determined by α2 antibodies and the absence of PA28α excluded a potential effect of PA28αβ on degradation velocity, as shown with the use of PA28α antibodies (Fig. 4A). The differences in degradation patterns were reproduced with different badges of proteasomes isolated from different donors (data not shown), indicating that replacement of constitutive subunits for immunosubunits improves degradation of mHtt(Q97)exon1-H4.

To investigate whether degradation of mHtt(Q97)exon1 is also improved in cells, HeLa cells that stably expressed the immunosubunits β1i, β2i and β5i were used to optimize incorporation in the 20S core [71]. To determine the rate of incorporation, 2D electrophoresis was used to separate all constitutive subunits and immunosubunits. Lysates of both control HeLa cells and HeLa cells that stably expressed all three immunosubunits were incubated with ABP for labeling of the active catalytic subunits. Subsequently, proteins were separated in two dimensions to identify all 6 catalytically-active subunits. Fluorescence analysis showed almost complete replacement of the constitutive subunits by immunosubunits (Fig. 4B). Immunoblotting for LMP2 and α2 confirmed the presence of the immunosubunit in the stable cell line only, whereas α2 was present at a similar levels (Fig. 4B).

To analyze whether the exchange by cellular immunoproteasomes improves quenched Q8-peptide degradation, cell lysates were subjected to zymography (Fig. 4C). Quantification of the fluorescent signal emitted by the quenched peptide, however, did not reveal a significant difference between control and immuno HeLa cell line (Fig. 4C). To further examine whether mHtt degradation is affected by immunoproteasomes in cells, mHtt(Q97)exon1 and GFP were expressed for 24 hours in HEK293 cells. GFP was cotransfected as an internal control, to normalize for transfection differences between the cell lines. Cell lysates were subjected to western blotting and filtertrap assays, and mHtt levels were normalized to GFP levels (Fig. 4D). After 24 hours expression, aggregates were not yet detected and analysis of the soluble fraction did not reveal differences between both cell lines. Taken together, this shows that immunoproteasomes better deal with mHtt in vitro, but that polyQ peptide degradation and mHtt degradation in cells is not affected.

### Contribution of individual proteasome subunits in mHtt degradation

Since mHtt fragments and also polyQ peptides are degraded by proteasomes, we next aimed to determine the importance of each individual catalytic subunit in mHtt degradation, using inhibitors that are directed against a specific catalytic site [72-74]. To examine whether a particular site is mainly responsible for cleaving between glutamines, the quenched polyQ peptides were added to
isolated proteasomes that were pretreated with the selective inhibitors (Fig. 5A). Prior to loading on a native gel, constitutive proteasomes were incubated with PA28αβ and the inhibitors. Activation by PA28αβ was necessary to increase the degradation signal to levels that were detectable by fluorescence scanning. After electrophoresis, the gel was incubated with the quenched Q8–peptides and fluorescence was imaged (Fig. 5A). Largest decrease in fluorescence signal was observed when β5 activity was inhibited, indicating that the chymotryptic activity is mainly responsible for polyQ cleavage, as has been suggested before [27].

To explore the effects of specific inhibition on mHtt degradation, the inhibitors were used in the in vitro degradation of mHtt(Q97)exon1-H4 (Fig. 5B). ABP labeling of proteasomes demonstrates complete abrogated β1 activity whereas little activity of β2 and β5 remained after addition of the corresponding inhibitors. Due to cross reactivity between the β5 inhibitor and β2, we were not able to increase inhibitor concentrations. Nevertheless, proteasomes were still capable to degrade mHtt(Q97)exon1-H4 after inhibition of β2 or β5, as degradation products were generated (Fig. 4B). When compared to non-inhibited proteasomes, β5-inhibited proteasomes showed faster loss of mHtt input levels (Fig. 5C). In contrast, inhibition of β1 activity prevented the generation of the smaller cleavage product (Fig. 5B, indicated by arrow heads). Additionally, immunostaining of the C-terminal His-sequence showed that the lower fragment was not stained, suggesting that the β1 cleavage site is in the C-terminus of the mHtt protein. Next, we examined for potential alterations in the mHtt cleavage pattern and degradation velocity after β1 or β5 inhibition in cells, respectively. HEK293 cells were transfected with mHtt(Q97)exon1 for 24 hours and inhibitors were added 16 hours before harvesting. Compared to the DMSO control, β2 inhibition did not affect mHtt levels, while β1 inhibition slightly increased the soluble levels (Fig. 5E). When β5 was inhibited with two different concentrations (at 5 μM β2 activity was affected as well), decreased in soluble mHtt(Q97) exon1 levels were detected (Fig. 5E). To determine whether this decrease was due to more efficient degradation or a shift towards the insoluble fraction, we immunostained mHtt aggregates with 1C2 antibodies in a filtertrap assay (Fig. 5F). Since only at high concentrations of β5 inhibition (which also affected β1 and β2 activity) caused a significant increase in aggregate formation, our results suggest improved mHtt(Q97)exon1 degradation only when the β5 activity is impaired. Together, these results suggest important but opposing functions for β1 and β5 in mHtt degradation. While β5 seems to be the main activity able to cleave within polyQ sequences, inhibition of this activity also seemed to accelerate mHtt degradation.

DISCUSSION

The role of proteasomes in HD has been an issue of a long-time discussion. Most studies examining proteasome activity in HD-related models have shown impairment of proteasomal functions, which is explained by the observed sequestration of proteasomes into aggregates and the incompetence of proteasomes to properly degrade mHtt proteins [19, 22, 23, 26]. The inability of proteasomes to cleave within polyQ sequences is considered to cause release of polyQ fragments or peptides by the proteasomes or even clogging of proteasomes by nondegradable polyQ fragments. More

Fig. 5. Proteasomal inhibition by active-site specific inhibitors.

(A) β5 is responsible for polyQ cleavage. Isolated 20S proteasomes were pre-incubated with PA28αβ caps and indicated inhibitors. Epoxomycin (E) was used as a general inhibitor and the specific inhibitors against the β1, β2 or β5 catalytic site. Proteasomes were subjected to native gel electrophoresis and incubated with Q8–peptide (upper panel). Equal loading and activation was confirmed by blotting against α2 and PA28 (lower panels). Inhibition of β5 activity resulted in least fluorescence. (B) mHtt(Q97)exon1-H4 degradation is affected by specific proteasome inhibition. mHtt(Q97)exon1-H4 and 20S proteasomes were incubated with DMSO, Epoxomycin (E) or the three specific inhibitors. Proteasomes were incubated with ABP before loading on SDS gel and scanned for fluorescence (upper panel). Absence or decrease in the corresponding subunit activity band demonstrated the efficiency of the inhibitor. Blotting for polyQ sequences by the 3B5H10 antibody and for the C-terminus of mHtt using a His-antibody showed different cleavage fragments (arrowhead). Equal proteasomes levels were confirmed by blotting for α2. (C) Quantification of mHtt(Q97)exon1-H4 levels in in vitro experiments (mean ± SEM; two tailed unpaired t-test; **p<0.001, ***p<0.01, *p<0.05). (D) Soluble and (E) aggregated pool of mHtt in cells after specific inhibition. HEK293 cells were transfected with mHtt(Q97)exon1 cells were harvested after 24 h and β1, β2 and β5 inhibitors were added for 16 h. Cell lysates were incubated with ABP to determine inhibition efficiency (upper panel) and subjected for Western blotting and filtertrap assay. mHtt levels were determined by 1C2 antibodies against polyQ sequence (middle panel, arrowhead), β-actin levels were determined as loading control (lower panel). Blotting for polyQ sequences by the 3B5H10 antibody and for the C-terminus of mHtt using a His-antibody showed different cleavage fragments (arrowhead). Equal proteasomes levels were confirmed by blotting for α2. (C) Quantification of mHtt(Q97)exon1-H4 levels in in vitro experiments (mean ± SEM; two tailed unpaired t-test; **p<0.001, ***p<0.01, *p<0.05). (D) Soluble and (E) aggregated pool of mHtt in cells after specific inhibition. HEK293 cells were transfected with mHtt(Q97)exon1 cells were harvested after 24 h and β1, β2 and β5 inhibitors were added for 16 h. Cell lysates were incubated with ABP to determine inhibition efficiency (upper panel) and subjected for Western blotting and filtertrap assay. mHtt levels were determined by 1C2 antibodies against polyQ sequence (middle panel, arrowhead), β-actin levels were determined as loading control (lower panel).
recent in vivo studies indicated that proteasomal impairment is only a temporal effect prior to the formation of large inclusion bodies, which rescues the cell by sequestering the hazardous, degradation-resistant proteins [24, 25]. Yet, we recently showed that the proteasome is able to degrade mHtt [61], that the activity of proteasomes is not impaired and that proteasomes are not irreversibly sequestered into aggregates (manuscript submitted). These findings raised the question whether the proteasome is not only a contributor to the disease due to reduced activity but may actually be a potential target for intervention, especially since reduction of mHtt levels has been shown to be an effective way of restricting the disease phenotype [75-78]. Additionally, since HD is an age-related disease, reduced activity of proteasomes in aging cells may be linked to the onset of the [79, 80]. Independent of the underlying cause of reduced proteasomal activity, amelioration of the proteasomal system to improve mHtt degradation is an interesting therapeutic option.

**Activation of the proteasome**

When we examined whether proteasomes can degrade polyQ sequences, we showed that the proteasome cleaves within polyQ peptides, mainly by its β5 catalytic site. Thusfar, only the aminopeptidase PSA and to a lesser extent tripeptidyl peptidase II (TPPII) have been identified as peptidases that are able to cleave small polyQ peptides by nibbling off glutamine residues from the N-terminal side. PSA is not able to target expanded polyQ peptides as it can only degrade peptides up to 30 amino acids, and lacks endopeptidase activity to cleave within polyQ sequences [81]. Increased in polyQ peptide degradation was observed when cells were stimulated with IFNγ, which was due to the activation of proteasomes by PA28αβ. However, IFNγ stimulation of cells did not improve degradation of polyQ-expanded proteins or polyQ peptides (data not shown) and even appeared to induce increased aggregation. This could be explained by the broad cascade of anti-inflammatory response events triggered by IFNγ, including increased generation of both oxidized proteins and DRIPs that are aggregation prone and compete with mHtt for degradation by the induced immunoproteasome.

Indeed, Seifert et al. have shown a direct effect between upregulation of oxidized protein levels and DRIPs, and their clearance via immunoproteasomes [60]. To study the effects of proteasome modifications independently of other factors, we performed in vitro experiments using isolated proteasomes, immunoproteasomes and isolated PA28 caps to examine direct effects of altered proteasome activity on mHtt(Q97)exon1 degradation. Activation of the proteasome via opening of the 20S barrel by S5D or RPT peptides, to mimic gate opening via the 19S cap, resulted in increased degradation of mHtt(Q97)exon1. However, whereas activation by PA28αβ resulted in more activity labeling by ABPs when compared to the other activators, degradation of mHtt(Q97)exon1 did not occur. Removal of the proline-rich region allowed degradation by PA28-capped proteasomes, suggesting that the folded mHtt fragment simply cannot enter PA28-capped proteasomes. Unfolding by the 19S cap may therefore be needed to enable entrance into the proteasomes. Alternatively, degradation of mHtt by PA28αβ-capped proteasomes may be improved by chaperones. Whereas various chaperones have been identified that prevent mHtt aggregation [82-88], no direct link between mHtt solubilization by chaperones and subsequent targeting for degradation by the proteasome has been established.

Yet, the expanded polyQ stretch is considered as the obstacle in mHtt degradation, but we show here that proteasome activation by PA28αβ improves degradation of polyQ peptides with a disease-related length. This is in agreement with earlier studies that showed proteasomal cleavage of short polyQ-peptides in the presence of the PA28γ (K188E) activator [27]. Additionally, a role of polyQ peptides in oxidative stress has been assigned to PA28αβ [70]. These findings indicate that PA28αβ improves clearance of misfolded and aggregation-prone polyQ peptides, but is unable to deal with folded mHtt. When mHtt can be unfolded first by the 19S cap, PA28αβ may become more relevant in mHtt processing, either by forming hybrid proteasomes or by acting downstream of the 26S proteasome to target released polyQ products. However, in this scenario it is crucial that mHtt is efficiently targeted for 19S recognition.

Another interesting feature for modulation of proteasomal activity is the proposed interplay between PA28 and HSP90. HSP90 is important for the maintenance of intracellular homeostasis by supporting protein folding as a molecular chaperone [89, 90]. HSP90 is also involved in activation and capping of proteasomes and is, in association with the 20S proteasome, responsible for degradation of partly-unfolded oxidized proteins [91, 92]. Since PA28 has been identified as a crucial mediator in the refolding mechanism of HSP90, PA28-mediated refolding of protein substrates by HSP90 and degradation by the proteasome may be linked [93, 94]. This hypothesis is supported by the molecular-coupling hypothesis that suggests that specific inserts at the exterior of the PA28 cap function as binding domains for chaperones, thus targeting unfolded proteins to the proteasome [95]. In addition, it was recently reported that HSP90 is indeed involved in the solubilization of mHtt [88].

**Modulation of proteasomal activity**

When isolated mHtt is degraded by proteasomes in vitro, it appeared that immunoproteasomes can degrade mHtt with highest efficiency. Replacing all constitutive catalytic sites by immunoproteasome catalytic sites results in an general upregulation of trypsin-like and chymotrypsin-like activity [38, 40]. However, focusing on de activity changes by individual subunits, the largest difference is caused by the replacement of β1 for β1i. Replacement by β1i reduces caspase activity, and increases chymotrypsin-like activity [96]. Experiments with specific inhibitors showed that β5, which has chymotrypsin-like activity, is mainly responsible for cleavage between glutamines, thus supporting the conclusion that mHtt is more efficiently degraded by immunoproteasomes. However, when we extrapolated these in vitro findings to cells, we did not find significant differences, either in polyQ peptide cleavage or mHtt degradation. The inability of immunoproteasomes to accelerate mHtt degradation in living cells may be due to inefficient targeting of mHtt to (immuno)proteasomes, which in agreement with the findings that cytoplasmic mHtt is to a large extent cleared by autophagy [61]. This, however, does not explain why immunoproteasomes do not degrade Q8-peptides faster in zymography assays.
The results that we obtained with subunit-selective proteasome inhibitors seem contradictory with the data showing that β5 is responsible for quenched Q8-peptide cleavage. Yet, our in vitro data indicates increased clearance of mHtt after β5 inhibition, which may be caused by the (relative) increase of the remaining proteasomal activities, thereby altering protein turnover and generating different products [97]. Indeed, we observed that inhibition of β5 induces increased ABP labeling of the β1 site of isolated proteasomes (Fig. 5B). Additionally, we show by specific inhibition of β1 that this site is important for the generation of a specific cleavage site in the C-terminus and in cells it causes a small increase in soluble mHtt levels. This may suggest that an improved function of β1, maybe in concert with β2, improves mHtt degradation.

On the basis of this data we conclude that modification of the catalytic activity in the core of the 20S proteasome is effective with respect to mHtt degradation. Remarkably, it seems that not polyQ degradation is improved but that cleavage in the flanking sequences is improving mHtt turnover. This assumption is supported by the observations that immunoproteasomes do not improve Q8-peptide degradation in zymography in gels and that inhibition of β5, which is the major site for polyQ degradation, is not a limiting factor in mHtt processing. Exploring the relevance of cleavage sites in the flanking sequences and identification of the catalytic subunit responsible for this cleavage site may improve mHtt clearance.

Conclusion

In the present study, we show that both isolated proteasomes and proteasomes in cell lysates can cleave within polyQ sequences. Proteasomal degradation of short polyQ peptides and polyQ peptides of disease related length can be increased by the PA28αβ-capped proteasomes, both in vitro and in cells. Modulation of the activity by replacement of the catalytic subunits for immunosubunits or by remodeling activity by using inhibitors did not improve polyQ degradation but enhanced mHtt degradation in vitro, suggesting that the cleavage sites in the flanking sequences of N-terminal mHtt(Q97)exon1 are also relevant for mHtt degradation by the proteasome. However, induction of immunoproteasomes in cells by constant expression of immunosubunits did not improve mHtt degradation. Insufficient targeting towards proteasomal degradation may be the underlying cause of this observation. Inhibition of the β5 activity did show a decrease in mHtt levels in cells, however activation of the autophagy machinery due to proteasomal inhibition has to be excluded.

Finally, while we focussed on proteasomal degradation of mHtt, recent reports also suggest a role for immunoproteasomes in maintenance of cellular homeostasis [60, 70, 98, 99]. Prior to inclusion body formation, proteasomes may become temporarily impaired due to an overload of the proteostasis network, leading to misfolding and accumulation of other proteasomal substrates that may start to aggregate [24, 25]. It will be interesting to study the indirect effects of proteasome modulation in HD, as the burden of aggregation-prone substrates in the cell could be counter-acted by immunoproteasomes which are presumably more efficient in degrading ubiquinated conjugates than constitutive proteasomes. Induction of immunoproteasomes in HD patients has already been established, though as a secondary response to inflammation [51, 52]. Earlier activation of the UPS could decrease general protein accumulations and thereby reducing the disease phenotype.

MATERIALS AND METHODS

Constructs. The constructs mHtt(Q97)exon1-H4 and mHtt(Q97)exon1-ΔPRO-H4 were generated by cloning the mHtt(Q7)exon1 (kindly provided by Prof. RR Kopito, Stanford University, USA) and mHtt(Q97)exon1-ΔPRO (kindly provided by J. Steffen, University of California, USA) sequence with a 5’ ‘Xhol and 3’ BamHI site into a vector encoding a C-terminal H4 tag (His-HA-HA-His, kindly provided by J. Steffen, University of California, USA). GFP-Ub and GFP-Ub-Q54 were generated as described previously [67].PA28α and PA28β (kindly provided by Prof. PM Kloetzel, Charité Universitätsmedizin Berlin, Germany) were cloned into a pcDNA3 vector using EcoRI. DNAJ86 was generated as described before [68].

Cell culture and transfection. HEK293 and the stable cell line HeLa A2 (clone 33) and clone 33/2 (HeLa A2 +β1i, β2i and β5i) (kindly provided by Prof. PM Kloetzel, Charité Universitätsmedizin Berlin, Germany) were cultured in DMEM supplemented with 10% fetal calf serum at 37°C in a 5% CO2 atmosphere [71]. HEK293 cells were transfected with jetPEI as described by the manufacturer (Polyplus transfection). The stable cell lines were transfected with X-treme Gene HD DNA transfection reagent as described by the manufacturer (Rochdiagnostics, Mannheim, Germany). HeLa A2 (clone 33) cells were kept on selection in 2 μg/ml puromycin and 300 μg/ml hygromycin. Cells were stimulated with 100 U/ml IFNγ for 72 h unless indicated otherwise. Proteasomes were inhibited with 250 nM epoxomicin, 5 μM β1 inhibitor (MV8111) [72], 10 μM β2 inhibitor (PR671A) [74] and 2.5 or 5 μM β5 inhibitor (PR523A) [73] for 16 h (Selective inhibitors were kindly provided by Prof. HS Overkleeft, Leiden Institute of Chemistry, The Netherlands).

Visualizing peptide degradation in gel. HEK293 cells were harvested in TSDG buffer (10 mM Tris pH 7.5, 25 mM KC1, 10 mM NaCl, 1.1 mM MgCl2, 0.1 mM EDTA and 8% glycerol) and lysed by 3 freeze/thaw cycles in liquid nitrogen. After centrifugation (15 min, 20,817 g) the protein concentration of the supernatant was determined by Bradford protein assay (Serva, Heidelberg, Germany). For proteasome labeling 0.5 μM ABP was added to 40μg lysate [54]. For proteasome inhibition 0.5 μM Epoxomicin was added or similar amounts of DMSO in control lysates and incubated for 1 h at 37°C. After incubation 4x Native sample buffer (20 mM Tris pH 8.0, 50 μl glycerol, 0.1% bromophenol blue) was added. The samples were loaded on a 3-12% NativePAGE Novex Bis-Tris Gels (Invitrogen, Life Technologies Europe BV, Bleiswijk, Netherlands) and separated by electrophoresis (3 h at 150V). When isolated proteasomes were used, 4 μg of 20S proteasomes were pre-incubated for 1 h with proteasome inhibitors; 10 μM epoxomicin, 2 μM β2 inhibitor (PR671A) and 1 μM β1 (MV8111) and β5 inhibitor (PR523A). 6 μg of purified PA28αβ caps (kindly provided by Prof. M. Rechsteiner, University of Utah School of Medicine, USA), were added 30 min prior to loading. To detect peptide cleavage in gel, the wet gel slab was incubated directly after electrophoresis for 20 minutes in 20 μl
Overlay buffer (20 mM Tris pH 7.5, 5 mM MgCl₂, 1 mM ATP) with 400 μM of the quenched peptides. Fluorescent intensities were measured on a Typhoon imager (GE Healthcare) using the 580 BP 30 filter. For Western blotting, native gels were transferred to a PVDF membrane (Millipore, Bedford, MA, USA) in transfer buffer (25 mM Tris pH 7.5, 192 mM Glycine, 20 % MeOH) using theCriterion blotter (Biorad, Hercules, CA, USA). α2 proteins were detected by the MCP236 antibody (1:1000, kindly provided by Prof. Rasmus Hartmann-Petersen, Biologisk Institut, University of Copenhagen, Copenhagen), PA28α were directed against RVQPAQAKVDVFRED (1:3000, kindly provided by Prof. M. Groettrup, University of Konstanz, Germany) [100]) and antibody detection was done by the Odyssey detection system (Licor Biosciences, Lincoln, NE, USA).

**Western blot analysis and filtertrap assay.** Cells were harvested in lysis buffer (50 mM Tris/HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 % Triton-X100, supplemented with complete mini protease inhibitor cocktail (Roche)). After centrifugation (15 min, 20000 x g) the concentration of the supernatant was determined by a Bradford protein assay (Serva, Heidelberg, Germany). 30 μl supernatant was boiled for 10 min at 99°C with 6x sample loading buffer (350 mM Tris/HCl pH 6.8, 10 % SDS, 30 % glycerol, 6 % β-mercaptoethanol, bromphenol blue) added. Proteins were fractionated on 12.5 % SDS-PAGE gels and transferred to a PVDF membrane (Millipore, Bedford, MA, USA). Western blot membranes were blocked with 5 % milk, incubated with primary antibodies anti-polyQ 1CZ (1:1000, Millipore, MAB1574), anti-polyQ (1:1000, Sigma-Aldrich 385H10), polyclonal rabbit anti-GFP (1:1000, kindly provided by Prof. J. Neefjes, NKI, The Netherlands), anti-β-actin (1:1000, Santa Cruz, SC-130656), anti-α2 (1:1000, MCP236, kindly provided by Prof. Rasmus Hartmann-Petersen, Biologisk Institut, University of Copenhagen, Copenhagen), anti-PA28α (1:3000, kindly provided by Prof. M Groettrup, University of Konstanz, Germany) [100], anti-α (1:1000, Abcam, ab3328), anti-β2i (1:2000, kindly provided by Prof. M Groettrup, University of Konstanz, Germany) [100] and subsequently incubated with secondary antibodies IRDye 680 or IRDye 800 (1:10000; Li-COR Biosciences). Infrared signal was detected using the Odyssey imaging system (Licor). Filtertrap assay was performed with the pellet obtained after centrifugation of the cell lysate. Pellet with aggregates was resuspended in benzonase buffer (1 mM MgCl₂, 150 mM NaCl, 10 mM Tris/HCl pH 8.0) and blocked with 5 % milk for further treatment like western blot membranes.

**In vitro degradation assays.** Htt(Q97)exon1-H4 was purified as described before [61]. 100 ng purified Htt(Q97)exon1-H4 protein was incubated with 0.3 μg mammalian open-gated 20S proteasomes (kindly provided by Prof. B. Dahlmann, Charité Universitätsmedizin Berlin, Germany) in 1x 20S buffer (10 mM Tris/HCl pH 7.4, 30 mM NaCl, 1 mM MgCl₂, 400 μM fresh DTT) in a total volume of 20 μl treated with or without 1 μM Epoxomicin at 37°C for 8 h. After the incubation period, proteasomes were incubated with 0.5μM ABP for an additional 0.5 h. Reactions were stopped by adding 6x sample loading buffer (350 mM Tris/HCl pH 6.8, 10 % SDS, 30 % glycerol, 6 % β-mercaptoethanol, bromphenol blue) followed by 3 min at 99°C. For native experiments 4x sample native sample buffer was added and samples were directly frozen. For activation of the 20S proteasomes 50 μM RPT peptides, 0.01 % SDS or 3 μg isolated PA28 caps were added to the reaction (kindly provided by Prof. M. Rechsteiner, University of Utah School of Medicine, USA). ATP Regeneration solution (Enzo Lifesciences, NY, USA) was added to reactions conducted with the 26S proteasome.

**Activity labeling in 2D.** A confluent 10 cm plate of HeLa cells was harvested in 500 μl proteasome-activity buffer (50 mM Tris pH 7.5, 50 mM Sucrose, 50 mM MgCl₂, 1 mM DTT, 1 mM ATP) followed by lysis by 3 cycles of freezing/thawing in liquid nitrogen. Protein concentration were determined using Bradford (Serva, Heidelberg, Germany), 500 μg final protein concentration was obtained and incubate with 0.5 μM ABP for 1 h at 37°C. TCA precipitation was performed to reduce the sample volume and the protein pellet was dissolved in 125 μl Urea buffer (7.7 M Urea, 2.2 M Thiourea, 4 % CHAPS, 30 mM Tris pH 9.8 with 0.5 % hydroxyethyl-disulfid (destreak reagent, GE Healthcare) and 2 % IPG buffer(pH 3-10 NL, GE Healthcare) freshly added. Sample was loaded on an Immobiline drystrip (pH 3-10 NL, GE Healthcare) and incubated o/n at room temperature. IEF was performed on a Protean IEF Cell (Biorad, Hercules, CA, USA) using the following program; 0.1min 50 V, 30 min 200 V; 30 min 200 V, 30 min 400 V, 30 min 600 V, 60 min 3500 V, 240 min 3500 V, 10 min 200 V. After focusing, the strips were incubated for 0.5 h in equilibration buffer (50 mM Tris pH 8.8, 6 M Urea, 30 % (v/v) Glyceral, 20 % (v/v) SDS, BPB) with 10 mg/ml fresh DTT. Subsequently, the strips were directly transferred in equilibrium buffer with 25 mg/ml IAA and incubated for 0.5 h. The strips were recovered, loaded on top of a 12 % SDS-PAGE gel and fixed in agarose sealing solution (15 % v/v glyceral, 1 % agarose, 1x Leammli electrophoresis buffer, BBP). Electrophoresis was performed at 30 mA per gel. Fluorescent detection was done on a Trio Thynphoon (GE Healthcare) 580 BP 30 filter to detect the ABP.
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