

# Misfolding of Proteins with a Polyglutamine Expansion Is Facilitated by Proteasomal Chaperones\*<sup>§</sup>

Received for publication, August 13, 2008, and in revised form, September 30, 2008. Published, JBC Papers in Press, November 5, 2008, DOI 10.1074/jbc.M806256200

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Deposition of misfolded proteins with a polyglutamine expansion is a hallmark of Huntington disease and other neurodegenerative disorders. Impairment of the proteolytic function of the proteasome has been reported to be both a cause and a consequence of polyglutamine accumulation. Here we found that the proteasomal chaperones that unfold proteins to be degraded by the proteasome but also have non-proteolytic functions colocalized with huntingtin inclusions both in primary neurons and in Huntington disease patients and formed a complex independently of the proteolytic particle. Overexpression of Rpt4 or Rpt6 facilitated aggregation of mutant huntingtin and ataxin-3 without affecting proteasomal degradation. Conversely, reducing Rpt6 or Rpt4 levels decreased the number of inclusions in primary neurons, indicating that endogenous Rpt4 and Rpt6 facilitate inclusion formation. *In vitro* reconstitution experiments revealed that purified 19S particles promote mutant huntingtin aggregation. When fused to the ornithine decarboxylase destabilizing sequence, proteins with expanded polyglutamine were efficiently degraded and did not aggregate. We propose that aggregation of proteins with expanded polyglutamine is not a consequence of a proteolytic failure of the 20S proteasome. Rather, aggregation is elicited by chaperone subunits of the 19S particle independently of proteolysis.

Huntington disease (HD)<sup>4</sup> is an autosomal dominantly inherited disease caused by the expansion of a polyglutamine (poly(Q)) stretch in the amino-terminal region of huntingtin

(Htt) (1). Proteolysis of Htt is an early event in the pathogenesis of HD generating amino-terminal products encompassing the poly(Q) expansion that accumulate in neurons where they form nuclear and cytoplasmic aggregates and somehow cause neurodegeneration (2–4). The observation that poly(Q) are ubiquitinated has suggested that deficient clearance of mutant Htt by the proteasome causes their accumulation (5). In support of this idea, inhibitors of the proteolytic activity of the proteasome augment aggregation of proteins with a poly(Q) expansion (6–8). Yet conflicting studies question whether or not the proteasome degrades extended poly(Q) stretches (9–13).

The proteasome is a barrel-shaped proteolytic complex composed of the 20S catalytic core particle (CP) and 11S or 19S regulatory particles (RP) flanking one or both ends of the CP (14). The 19S recognizes polyubiquitinated substrates and removes the polyubiquitin chains, and the six ATPases (Rpt1–6) of the 19S particle unfold protein substrates, delivering unfolded and degradation competent proteins to the narrow catalytic chamber of the CP (15). The 26S proteasome is considered to be the most prominent species and is composed of one 20S and one 19S particle. Alternative RPs have been isolated (16), and the assembly of one or two of the various RPs with the CP generates a dynamic repertoire of proteasome complexes, exchanging RPs (17). During proteolysis, ATP hydrolysis dissociates 19S and 20S particles, further highlighting the plasticity of proteasome complexes (18). The 19S ATPases also function non-proteolytically in transcription, DNA repair, and chromatin remodeling (19–25). Altogether, these studies reveal that the proteasome is not a static complex. Rather, individual proteasome components play important roles in a variety of cellular processes.

Deposition of proteins of aberrant conformation is the generic feature of many neurodegenerative diseases, including Alzheimer disease, Parkinson disease, prion disorders, and polyglutamine expansion disorders. In affected neurons the disease-specific proteins accumulate in an amyloid or amyloid-like state characterized by a common cross- $\beta$  structure in which  $\beta$ -strands run perpendicular to the axis of the fibril. The common structure of the pathogenic conformer of the disease-related proteins is in sharp contrast to the fact that the amyloidogenic proteins do not exhibit any sequence similarities or common structural motifs in their native state (26). Thus, a structural transition must occur to convert the different native structures into the common cross- $\beta$ -sheet structure (27–29). This transition between the folded native and the amyloidogenic conformation is prevented by a large thermodynamic

\* This work was initiated with support from the Fondation pour La Recherche sur le Cerveau and the Fondation NRJ-Institut de France. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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§ The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Figs. 1–3.

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<sup>4</sup> The abbreviations used are: HD, Huntington disease; Htt, huntingtin; ATP $\gamma$ S, adenosine 5'-O-(thiotriphosphate); SCA-3, spinocerebellar ataxia type 3; CP, core particle; RP, regulatory particles; GFP, green fluorescent protein; GST, glutathione S-transferase; ODC, ornithine decarboxylase; siRNA, small interfering RNA.

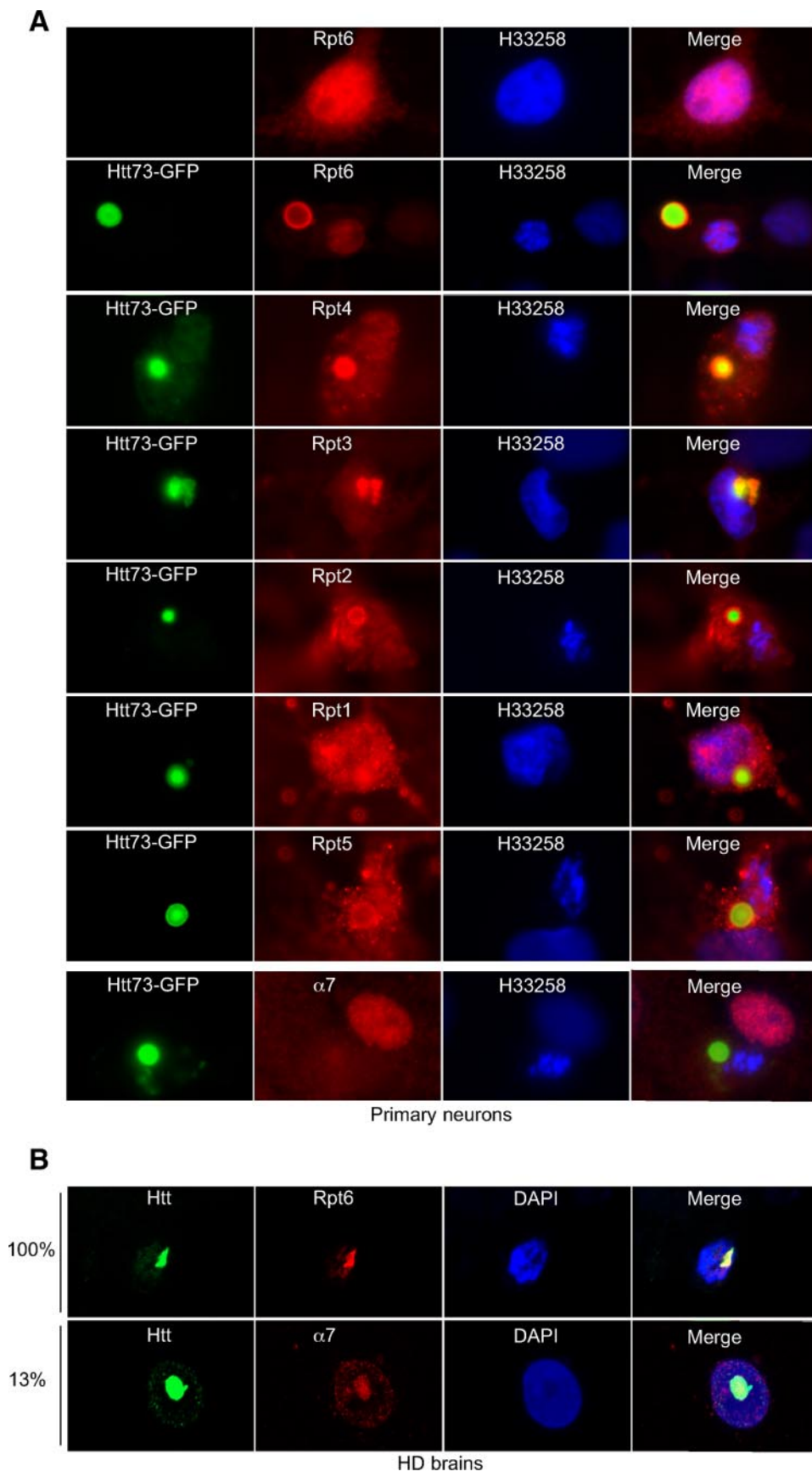
## Misfolding of Poly(Q) Proteins Is Facilitated by 19S ATPases

barrier (30). Thus, it is unlikely that such a transition occurs spontaneously under physiological conditions. The aggregation of poly(Q) has been well described *in vitro* using small synthetic peptides and occurs by nucleated growth polymerization (2, 31). However, in inclusions of HD patients, Htt amino-terminal fragments contain sequences additional to the poly(Q) stretch (32), including a proline-rich region, which strongly antagonizes aggregation (33–36). Thus, to elicit Htt aggregation, some trigger ought to be required to alleviate the inhibition of the proline-rich region and to convert the soluble protein into an aggregate. *In vitro* the rate-limiting and thermodynamically unfavorable step in aggregation of pure poly(Q) peptides is the nucleation reaction, consisting of the structural transition of a monomer into an amyloidogenic conformation. Nucleation of poly(Q) aggregates is viewed as an unfavorable folding reaction (31). What triggers poly(Q) nucleation in neurons of HD patients is unknown, but in cells conformational rearrangements are assisted by chaperones (37). Here we report the finding that Rpt6 (PSMC5) and Rpt4 (PSMC6) facilitate the conversion of soluble mutant Htt amino-terminal fragments to their aggregated state.

### EXPERIMENTAL PROCEDURES

**Cell Culture and Reagents**—293T cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and transfected in 6- or 12- well plates by using the calcium phosphate method leading usually to >70% transfection efficiency. Routinely, 45,000 cells/ml were plated before transfection. For analytical experiments 0.25  $\mu$ g of Htt73 encoding plasmid were transfected together with 0.125, 0.25, and 0.5  $\mu$ g of plasmid encoding 19S subunits except when indicated otherwise. Where indicated, cells were treated with epoxomycin (Calbiochem).

Primary cultures of rat hippocampal neurons were prepared from E18 rats. Transfections were



performed after 8 days of culture in 6-well plates with 0.5  $\mu\text{g}$  of Htt73-GFP plasmid with or without 0.5  $\mu\text{g}$  of Rpt6 plasmid and Lipofectamine 2000 (Invitrogen).

**Plasmid Constructs**—Htt17 encodes the first 163 amino acids of Htt. Htt73 encodes a similar protein with 73 glutamines. Rpt6 was kindly provided by R. Losson and subcloned in the HindIII and XhoI restriction sites of pXJ41. Rpt6<sup>80–406</sup> was generated by PCR and cloned HindIII and XhoI restriction sites of pXJ41. The cDNAs encoding Rpt4 was amplified by reverse transcription-PCR from HeLa cell total RNA flanked with EcoRI and XhoI sites and cloned into pXJ41. To generate GFP fusion, Htt73 was PCR-amplified, flanked with BamHI and KpnI sites, and cloned in the BglII and KpnI sites of pEGFP-N2 (BD Biosciences). To produce GST fusions, Rpt6 and Rpt6<sup>80–406</sup> were subcloned into the BamHI and XhoI restriction sites of pGEX-4T3 vector in-frame with GST (GE Healthcare). The cDNA sequence coding for the last 52 amino acids of ornithine decarboxylase (ODC) was PCR-amplified using pZS Proteasome Sensor (Clontech) as a template, flanked with XhoI and XbaI restriction sites, and cloned in pCDNA1/Amp vector (Invitrogen) containing Htt73 to produce Htt73ODC. In the ODC-encoding sequence, cysteine 441 was mutated in alanine to generate Htt73ODC<sub>C441A</sub>. The cDNA encoding the first 108 amino acids of huntingtin with an expansion of 50 CAG was cloned in pGEX-4T vector in-frame with GST (GE Healthcare) generating GST-Htt50 $\Delta$ , similar to GST-HD51 described in Scherzinger *et al.* (38). Expression of recombinant proteins was performed as described in Scherzinger *et al.* (38). All constructs were verified by DNA sequencing.

**Small Interfering RNAs**—80 pmol of double-stranded RNA oligonucleotides directed against the target rat Rpt6 (Rpt6 siRNA<sub>1</sub>, ggattgacaggaaaattga; Rpt6 siRNA<sub>2</sub>, ggagatcaaa-gaagtgatt) or Rpt4 (Rpt4 siRNA<sub>1</sub>, tggacagattgtaggcgaa; Rpt4 siRNA<sub>2</sub>, tgaggtatctgccgagaga) or control siRNA *Silencer* Negative Control #1 (Ambion) were transfected in primary hippocampal neurons 8 days after their plating in a 6-well plate using Lipofectamine 2000 (Invitrogen). Neurons were fixed 24 h after transfection.

**Immunocytochemistry and Fluorescence Microscopy**—Transfected cells were fixed with 4% paraformaldehyde and labeled with indicated antibodies. Micrographs were taken at 100 $\times$  magnification on a Leica TCS SP2A OBS confocal microscope or Leica DMRB Fluorescence microscope.

**Human HD Tissue**—Frozen brain samples (parietal cortex, Brodmann area number 7) from two individuals with juvenile HD were obtained from the Harvard Brain Tissue Resource Center. The two cases were a 14-year-old, grade 3 female with 97 CAG repeats and a 18-year-old grade 4 female with 75 CAG repeats, previously described in Hoffner *et al.* (39). Sections were fixed with 4% paraformaldehyde and labeled with a polyclonal antibody directed against the first 17 amino acids of human Htt (1:400 dilution) (39),  $\alpha 7$  (PW8110 BIOMOL, 1:1000 dilution), and Rpt6 antibody (SUG-1b8, Euromedex, 1:5000

dilution). 100 inclusions were examined, 50 nuclear and 50 cytoplasmic, in the two tissues. Micrographs were taken at 63 $\times$  magnification combined with digital zooming on a Zeiss confocal microscope (Carl Zeiss, Inc., Thornwood, NY).

**Immunoblotting and Filter Retardation Assay**—Routinely, 70% confluent cells from a well of a 12-well plate were lysed in 140  $\mu\text{l}$  of boiling Laemmli buffer (25 mM Tris-HCl, pH 6.8, 1% SDS, 25 mM dithiothreitol, 7.5% glycerol, 0.05% bromophenol blue) for immunoblot analysis or 140  $\mu\text{l}$  of ice-cold Nonidet P-40 buffer (25 mM Tris-HCl pH 7.5, 1% Nonidet P-40, 300 mM NaCl, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 5  $\mu\text{g}/\text{ml}$  pepstatin, leupeptin, and aprotinin) for fractionation experiments. 18  $\mu\text{l}$  of protein extracts were loaded on 10% SDS-PAGE and transferred to Optitran BA-S 83 reinforced nitrocellulose membrane (Whatman and Schleicher & Schuell). Equal loading of protein extracts analyzed by immunoblot was controlled by Ponceau Red staining (data not shown) and vimentin. Filter retardation assays were performed as described in Wanker *et al.* (40). Membranes were saturated in 5% dried skimmed milk in phosphate-buffered saline and probed with Htt 2b4 antibody unless specified. The appropriate secondary antibody coupled to peroxidase was revealed using the SuperSignal West Pico Chemiluminescent kit (Pierce). Chemiluminescent images were acquired using the Chemi-Smart 5000 (Vilber-Lourmat) allowing quantitative detection of chemiluminescence. Signals of interest were quantified using Bio-1d software (Vilber-Lourmat).

**Antibodies**—For immunocytochemistry, primary antibodies were diluted as follows: 1:1000 dilution for Tau-1 antibody (Chemicon),  $\alpha 7$  (PW8110 BIOMOL), Rpt1 (ab3322, Abcam), Rpt2 (ab3317), Rpt3 (ab22634), and Rpt5 (ab22635); 1:5000 dilution for Rpt6 antibody (SUG-1b8, Euromedex) or Rpt4 (ab22639). For immunoblots, antibodies were used as follows: 1:5000 for Htt 2b4 antibody and 1:1000 for antibodies against proteasome subunits. Vimentin antibody V 6630 (Sigma) was used at a 1:500 dilution, p21 antibody (BD Pharmingen 556431) at a 1:500 dilution, and E2F-1 (Santa Cruz) sc-251 at a 1:200 dilution. GFP antiserum (BD Biosciences) was used as a control antibody.

**Immunoprecipitation**—293T cells were plated 24 h before transfection in 10-cm dishes at a density of 80000 cells/ml. Transfection were performed with 2.5  $\mu\text{g}$  of Htt73 alone or with 5  $\mu\text{g}$  of Rpt6-FLAG-encoding plasmid. 48 h post-transfection, cells were washed with ice-cold phosphate-buffered saline, lysed in 1 ml of 0.2% Nonidet P-40 buffer (0.2% Nonidet P-40, 50 mM Tris-HCl, pH 7.5, 300 mM NaCl, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 5  $\mu\text{g}/\text{ml}$  pepstatin, leupeptin, and aprotinin), left 10 min on ice, and clarified by centrifugation at 16,000  $\times g$  for 10 min. Soluble proteins were immunoprecipitated with 2  $\mu\text{l}$  of the anti-Rpt6, anti-GFP, or anti-Htt monoclonal antibodies together with 10  $\mu\text{l}$  of protein G-Sepharose overnight at 4  $^{\circ}\text{C}$ . Beads were then washed 3 times in 0.2% Nonidet P-40 buffer and resolved by 10% SDS-PAGE under reducing conditions, transferred to nitrocellulose membranes, and revealed with corresponding antibodies.

**FIGURE 1. Rpt subunits co-localize with Htt inclusions.** A, micrographs of primary neurons either mock-transfected or transfected with Htt73-GFP and labeled with Rpt antibodies or  $\alpha 7$  (20S subunit) antibody and with H33258 dye. B, immunohistochemical analysis of cortical sections from juvenile HD patients with indicated antibodies and 4,6-diamidino-2-phenylindole (DAPI). About 200 inclusions from 2 patients were scored. 100% of inclusions were labeled with Rpt6, whereas a light labeling for  $\alpha 7$  was found in 13% of inclusions in both patients. Representative micrographs are shown.

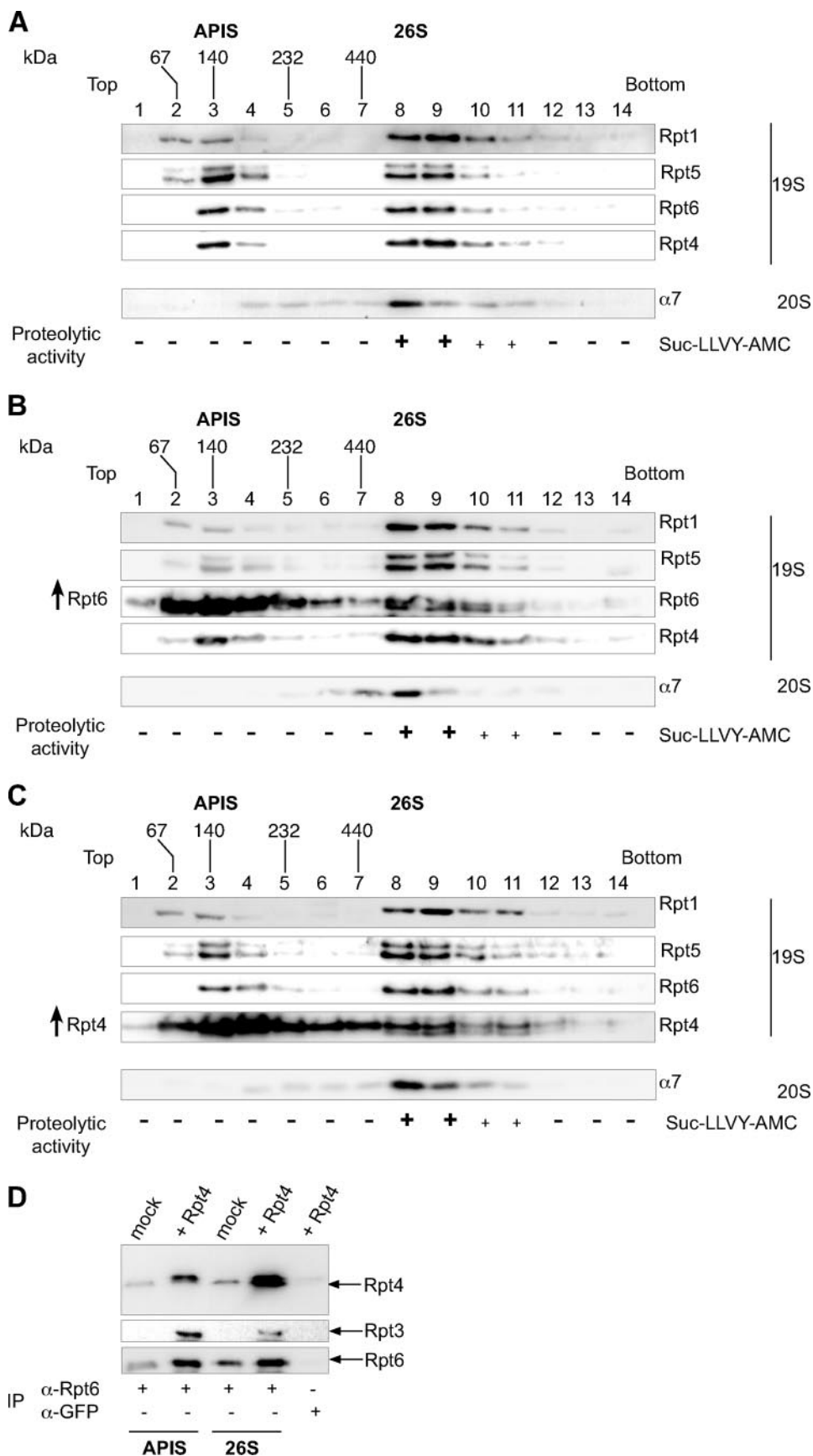
## Misfolding of Poly(Q) Proteins Is Facilitated by 19S ATPases

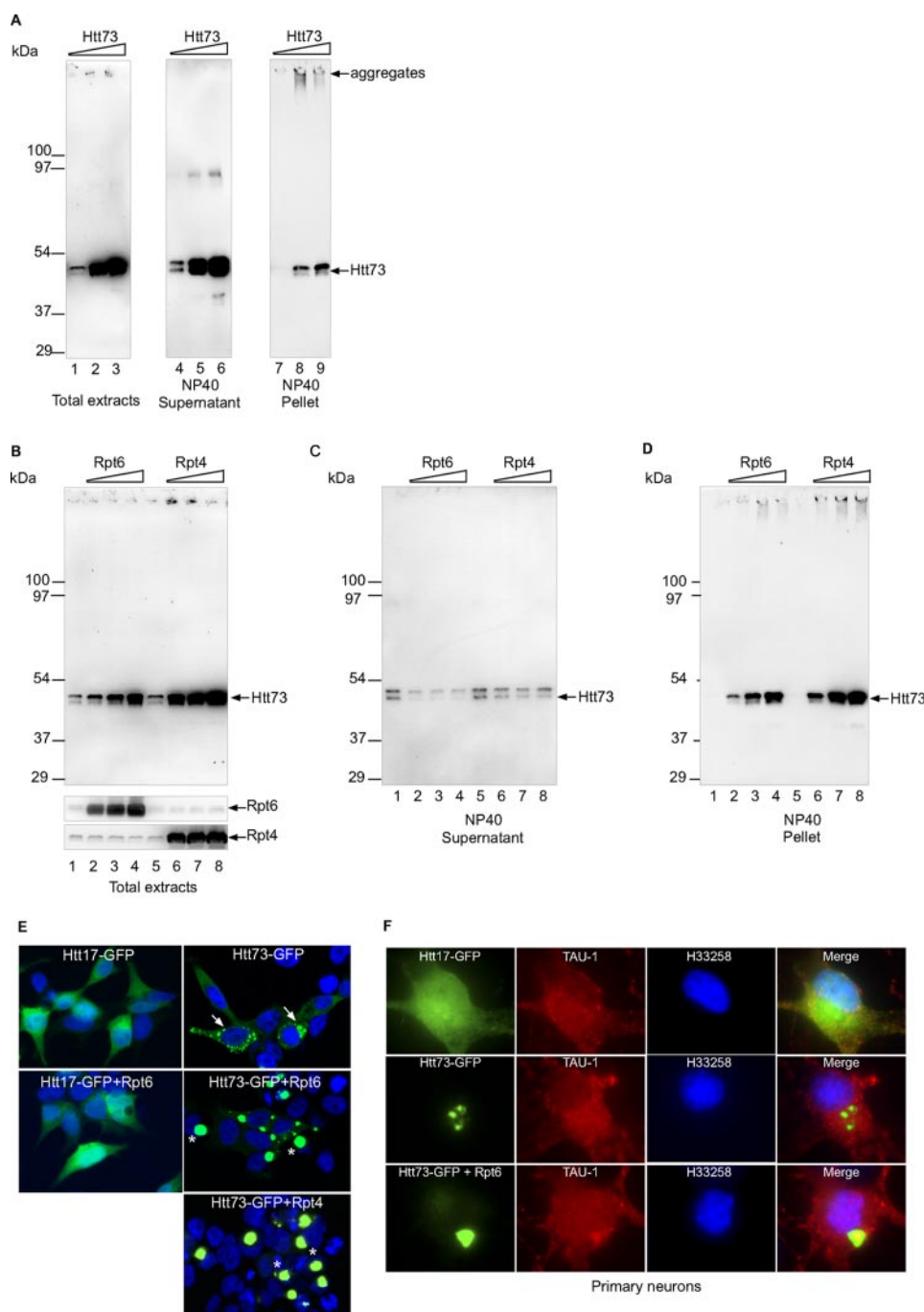
**GST Pulldown Assay**—GST, GST-Rpt6, and GST-Rpt6<sup>80–406</sup> were expressed in *Escherichia coli* strain BL21 (DE3) pLysS and purified by glutathione-Sepharose chromatography. For each pulldown experiment, about 0.1 μg of GST or GST-Rpt6 fusion protein was incubated with Htt73 produced in 293T cells and extracted in 1% Nonidet P-40 buffer. After 3 h of incubation at room temperature, beads were washed 3 times for 5 min in 1% Nonidet P-40 buffer, and complexes were analyzed by SDS-PAGE followed by immunoblots.

**Glycerol-gradient Sedimentation**—225000 cells were plated in a 5-cm dish and transfected with empty vector or the indicated constructs 24 h after plating. Cells were lysed 40 h post-transfection in 200 μl of proteasome lysis buffer (50 mM Tris-HCl, pH 8.0, 1% Triton X-100, 1 mM EDTA, 1.5 mM MgCl<sub>2</sub>, 1 mM dithiothreitol, and 5 mM ATP). Extracts were clarified by centrifugation and fractionated through linear 20–40% glycerol gradients in polyallomer tubes of 11 × 60 mm for 13 h at 40,000 rpm in a SW60 rotor (Beckman Coulter). Each 4-ml gradient was collected into 14 equal fractions of 300 μl. Aliquots of each fraction were subjected to immunoblots and peptidase assay. Note that all the proteasome complexes sedimented below their expected molecular weights, as previously reported (25).

**Proteasome Activity**—Assays for proteasome activity were performed using the substrate *N*-Succinyl-Leu-Leu-Val-Tyr-aminomethylcoumarin as described in Kisselev and Goldberg (41).

**Aggregation Assays**—GST-Htt50Δ (0.5 μM) was incubated with or without purified 19S proteasome (70 nM; Boston Biochem) in the presence of 10 units of thrombin (Amersham Biosciences) at 37 °C with 300 rpm shaking in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10% glycerol, 20 mM MgCl<sub>2</sub> with or without 5 mM ATP or 15 mM ATPγS. Reactions were stopped at the indicated times by adding an





**FIGURE 3. Htt73 misfolding is facilitated by proteasomal chaperones.** *A*, lysates of cells expressing increasing amounts of Htt73 were either boiled in 1% SDS or fractionated into NP40 supernatant and pellet and analyzed by immunoblotting with Htt antibody. *B–D*, Htt73 was co-expressed with increasing amounts of Rpt4 or Rpt6, and whole cell lysates or supernatants and pellets were analyzed by immunoblotting with Htt, Rpt6, or Rpt4 antibodies. *E*, micrographs of cells expressing Htt17-GFP alone or together with Rpt6. Confocal micrographs of cells expressing Htt73-GFP either alone or together with Rpt6 or Rpt4 are shown. Nuclei were stained with H33258. *Arrows* show cells with small inclusions, and *asterisks* show cells with large inclusions. *F*, micrographs of primary neurons expressing Htt17-GFP and Htt73-GFP alone or together with Rpt6. Neuronal identity was confirmed by staining with TAU-1 antibody, and nuclei are revealed with H33258.

equal volume of Laemmli buffer and boiling for 5 min. Aliquots corresponding to 50 ng of GST fusion protein were analyzed by SDS-PAGE stained with Coomassie Brilliant Blue, and filter retardation assays revealed with Htt antibody.

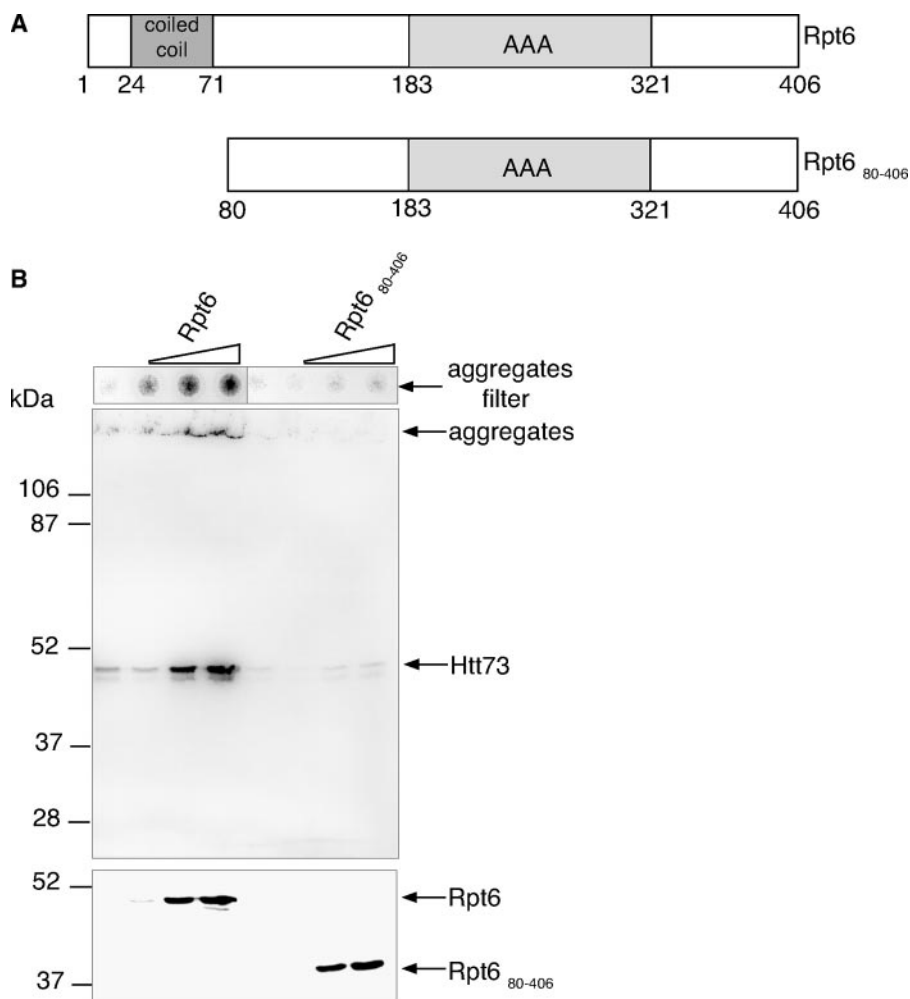
**RESULTS**

Aggregation of peptides with a long poly(Q) stretch can occur spontaneously *in vitro*, but in yeast aggregation of the amino-terminal fragment of Htt requires the presence of the chaperone Hsp104 (42). Although chaperones are widely conserved throughout evolution, there is no mammalian orthologue of Hsp104. The typical feature of Hsp104 and the bacterial ATPase ClpB proteins is the presence of two AAA domains and a middle region or M-domain, adopting a coiled-coil structure essential for the Hsp104/ClpB protein remodeling function (43, 44). AAA ATPases in available databases were examined aiming to identify human proteins containing both a coiled-coil and an AAA domain. We found that only the ATPase subunits of the 19S proteasome, Rpt6, Rpt4, Rpt3, Rpt2, and Rpt1, exhibit these specific features. Interestingly, an immunohistochemical study of brain tissue from patients with spinocerebellar ataxia type 3 (SCA-3) has revealed that poly(Q) inclusions co-localize with subunits of the 19S proteasome but rarely of the 20S catalytic particle (45). These observations together with the unfoldase function of the 19S ATPases in protein degradation prompted us to investigate whether Rpts contribute to inclusion formation.

To determine whether co-localization of regulatory subunits of the proteasome with poly(Q) inclusions was a generic feature of distinct

**FIGURE 2. Overexpressed and endogenous Rpts are subunits of two distinct complexes, the 26 S proteasome and APIS.** *A–C*, lysates of mock-transfected cells or cells overexpressing Rpt6-FLAG or Rpt4-FLAG were analyzed by glycerol gradients sedimentation. An aliquot of each fraction was analyzed by immunoblots and for peptidase activity with *N*-Succinyl-Leu-Leu-Val-Tyr-aminomethylcoumarin as indicated below the immunoblots (41). *APIS*, ATPase proteins independent of 20S (22). *D*, fractions 2–4 (*APIS*) and 8–9 (26 S) of glycerol gradients similar to those shown in (*A* and *C*) were pooled, immunoprecipitated, and revealed with indicated antibodies. Horseradish peroxidase-conjugated goat anti-mouse  $\kappa$  were used to reveal the immunoblots, as Rpts co-migrate with the heavy chain of the immunoglobulins.

## Misfolding of Poly(Q) Proteins Is Facilitated by 19S ATPases



**FIGURE 4. The coiled-coil region is essential for the activity of Rpt6 on Htt73.** *A*, schematic representation of Rpt6 domain organization and coiled-coil mutant analyzed in this study. *B*, lysates of cells expressing Htt73 alone or together with increasing amounts of Rpt6 wild type or Rpt6<sup>80-456</sup> deletion mutant were analyzed by immunoblots and filter retardation assays.

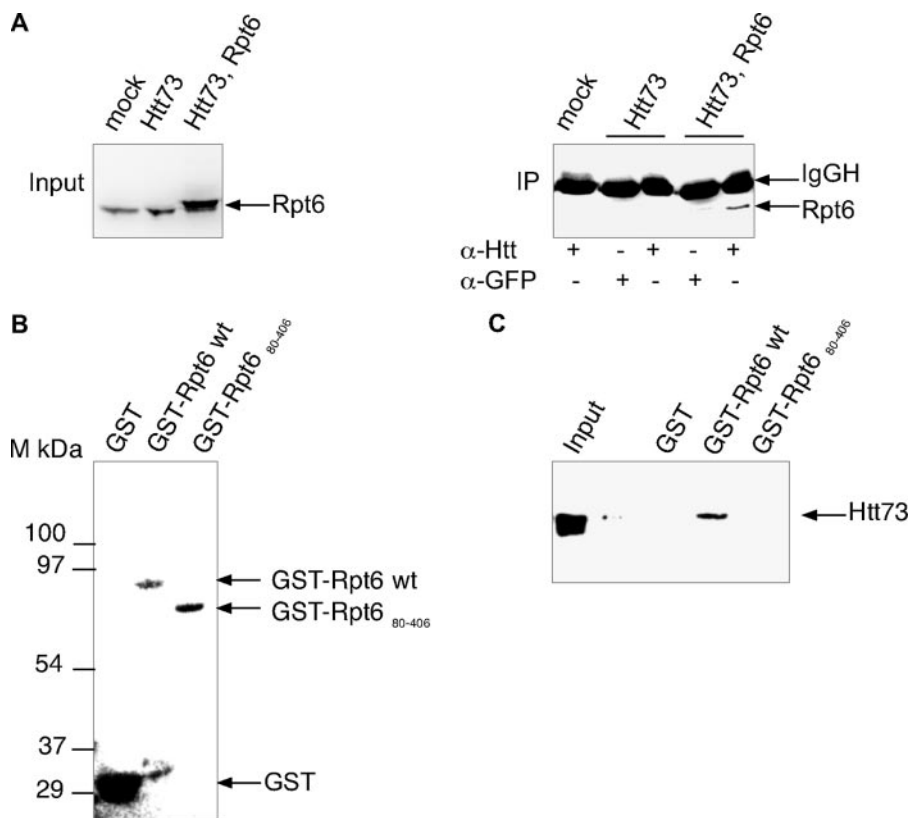
poly(Q) disorders, we analyzed the localization of several proteasome subunits in nerve cells containing mutant Htt inclusions. In primary neurons, Rpt6 staining was extremely intense around poly(Q) aggregates, in contrast to its diffuse and mostly nuclear localization in untransfected cells (Fig. 1A). Similarly, Rpt4 and Rpt3 co-localize with poly(Q) inclusions. The other 19S ATPases were also enriched around poly(Q) inclusions (Fig. 1A). In contrast, the 20S proteasome was not enriched around the poly(Q) inclusions, as revealed by immunostaining with antibodies directed against the 20S subunit,  $\alpha 7$ . These observations reveal that Rpt subunits co-localize with mutant Htt inclusions in neuronal cells independently of the 20S proteasome. To determine whether these observations were relevant to HD, we stained cortical sections of post-mortem HD brains with Htt and Rpt6 or  $\alpha 7$  (20S) antibodies. We performed this analysis on brain tissue from two juvenile HD patients to examine a large number of cytoplasmic and nuclear inclusions. Like primary neurons expressing mutant Htt, all the inclusions from both HD patients were intensely labeled with Rpt6 regardless of their subcellular localization (Fig. 1B and data not shown). In contrast, only a small fraction of Htt inclusions (13%) exhibited a light staining for the 20S subunit (Fig. 1B).

These results indicate that the chaperone subunits co-localize with HD inclusions largely independently of the 20S subunits.

Seeking further evidence for the existence of Rpt subunits independently of the proteasome, we analyzed the distribution of proteasome subunits from cell lysates by glycerol gradient fractionation. 19S and 20S subunits as well as the proteolytic activity of the proteasome were localized in high molecular weight fractions 8 and 9, indicating that these fractions contained the 26S proteasome (Fig. 2A). In addition to the 26S proteasome, 19S subunits were also found in lower molecular weight fractions devoid of 20S subunits and proteolytic activity (Fig. 2A, fractions 2–4). This sedimentation profile is reminiscent of the gel filtration profile of the free 19S-like complex (25), also called APIS complex (ATPase proteins independent of 20S) (22). Thus, in mammalian cells Rpts are subunits of 2 distinct complexes, the 26S proteasome and APIS or free 19S-like complex.

The observation that inclusions of expanded poly(Q)-containing proteins co-localize with Rpts independently of the proteasome (Fig. 1 and (45)) suggested that APIS could be involved in inclusion formation. We, therefore, attempted to

increase the abundance of this complex by overexpressing some of its subunits. The sedimentation profile of overexpressed Rpt4 and Rpt6 was similar to the sedimentation profile of the endogenous subunits. In fractions 8 and 9 of the glycerol gradients, Rpt6 antibody revealed 2 bands corresponding to the endogenous protein and the overexpressed FLAG-tagged Rpt6, indicating that overexpressed Rpt6 was a subunit of the 26S proteasome complex (Fig. 2B). A large fraction of overexpressed Rpt6 was detected in fractions 2–4 of the gradient together with the APIS complex. Similarly, a fraction of overexpressed Rpt4 entered the 26S proteasome, whereas most of the protein was found in the smaller APIS complex (Fig. 2C). Note that overexpression of Rpt4 or Rpt6 did not alter the sedimentation of the endogenous proteasome nor its proteolytic activity (Fig. 2, A–C, and data not shown). To confirm these observations, immunoprecipitations were carried out with Rpt6 antibodies on glycerol gradient fractions containing either APIS (fractions 2–4) or the 26S complex (fractions 8–9). Both endogenous and overexpressed Rpt4 were detected in Rpt6 immunoprecipitates performed on fractions 2–4 as well as fractions 8–9 of the glycerol gradients (Fig. 2D). Rpt3 was also detected in both complexes but only when Rpt4 was overex-



**FIGURE 5. Rpt6, but not Rpt6 lacking the coiled-coil region, binds to Htt73.** *A*, lysates of cells transfected with empty vector and Htt73 alone or together with Rpt6 analyzed by immunoblot (*left panel*) or immunoprecipitated (IP) with Htt or GFP antibody followed by Rpt6 immunoblot (*right panel*). *B*, GST, GST-Rpt6, and GST-Rpt6<sup>80-406</sup> fusion proteins were expressed in *E. coli*, purified on glutathione-Sepharose beads, separated by SDS-PAGE, transferred onto nitrocellulose, and stained with Ponceau Red. *C*, Htt73 containing cell lysates (Input) were incubated on beads containing GST, GST-Rpt6, or GST-Rpt6<sup>80-406</sup> and washed, and complexes bound to beads were revealed with Htt antibody.

pressed. The amounts of Rpt3 immunoprecipitated in the endogenous complexes were probably below the detection limit of the Rpt3 antibody. Altogether, these findings establish that endogenous and overexpressed Rpt4 and Rpt6 are engaged in two complexes, the 26S proteasome and APIS. Most of the overexpressed Rpt6 and Rpt4 co-sedimented with APIS and recruit other subunits of the complex. We, thus, used this experimental setting to analyze whether Rpts modulated Htt misfolding and aggregation.

Nucleation is the rate-limiting step of poly(Q) aggregation *in vitro* (31). To determine whether similar features could be observed in cells, we expressed large amounts of Htt73 and performed biochemical fractionation. We found that a 10–25-fold increase in the SDS-soluble protein leads to a very modest increase of Nonidet P-40-insoluble, presumably misfolded Htt73 (Fig. 3A). Thus, cells can accumulate a large amount of Nonidet P-40-soluble Htt73, suggesting that aggregation requires not only a high protein concentration but also additional events. Because the function of Rpts is to unfold proteins and because they co-localize with poly(Q) inclusions, we asked whether these unfoldases might assist the conversion from soluble to aggregated mutant Htt. Increasing the levels of Rpt6 or Rpt4 led to a remarkable and dose-dependent increase of Htt73 when low amounts of Htt73 were co-transfected with Rpt6 or Rpt4 (Fig. 3, B–D,

lanes 1, 4, and 8, compared with Fig. 3A, lanes 3, 6, and 9). Biochemical fractionation studies revealed that nearly all the SDS-soluble Htt73 that accumulated upon Rpt6 or Rpt4 overexpression was Nonidet P-40-insoluble, suggesting that it was in a pre-aggregated misfolded state (Fig. 3, B–D). In contrast, when large amounts of Htt73 were transfected alone, cells contained very high levels of soluble Htt73 (Fig. 3A). These experiments revealed that Rpt6 and Rpt4 convert soluble Htt into an insoluble species. To further characterize the effect of Rpt6 and Rpt4 on Htt73, Htt73 fused to GFP was overexpressed either alone or together with Rpt6 or Rpt4 (Fig. 3E). Although cells expressing Htt73-GFP alone contained either diffuse fluorescence or small fluorescent foci, cells co-expressing Htt73-GFP and Rpt6 or Rpt4 contained large inclusions (Fig. 3E). This was also observed in primary neuronal cells expressing Htt73-GFP alone or together with Rpt6, indicating that Rpt6 enhances misfolding and aggregation of Htt with a poly(Q) expansion regardless of cell type (Fig.

3F). Htt17-GFP expressed alone or together with Rpt6 never formed inclusions (Fig. 3E and data not shown). Northern blot analyses were carried out with cells co-expressing Rpt6 with Htt73 and revealed that the effects of Rpt6 overexpression on Htt accumulation were not due to changes at the level of Htt mRNA (supplemental Fig. 1). Previous studies have indicated that Rpt subunits of the 19S have distinct functions (46). To determine whether the effect on mutant Htt aggregation was a general property of all Rpts or was restricted to Rpt6 and Rpt4, Htt 73 was overexpressed with the other Rpts. Increasing the levels of Rpt6 or Rpt4 led to a remarkable and dose-dependent increase of aggregated Htt73, as revealed by immunoblots and filter retardation assays (supplemental Fig. 2). Overexpression of Rpt3 modestly enhanced aggregation of Htt73, in contrast to Rpt2, Rpt1, and Rpt5, which had no significant effect (supplemental Fig. 2). This indicated that overexpression of an individual Rpt was not sufficient to trigger Htt aggregation but, rather, that the effects of Rpt6 and Rpt4 on Htt73 were specific. Because cellular protein concentration varies with cell density and equal cell numbers were seeded in each experiment, the fact that the levels of the cellular protein vimentin did not vary in the different conditions revealed that none of the transfected constructs elicited cell death (supplemental Fig. 2). This is in agreement with previously studies that estab-

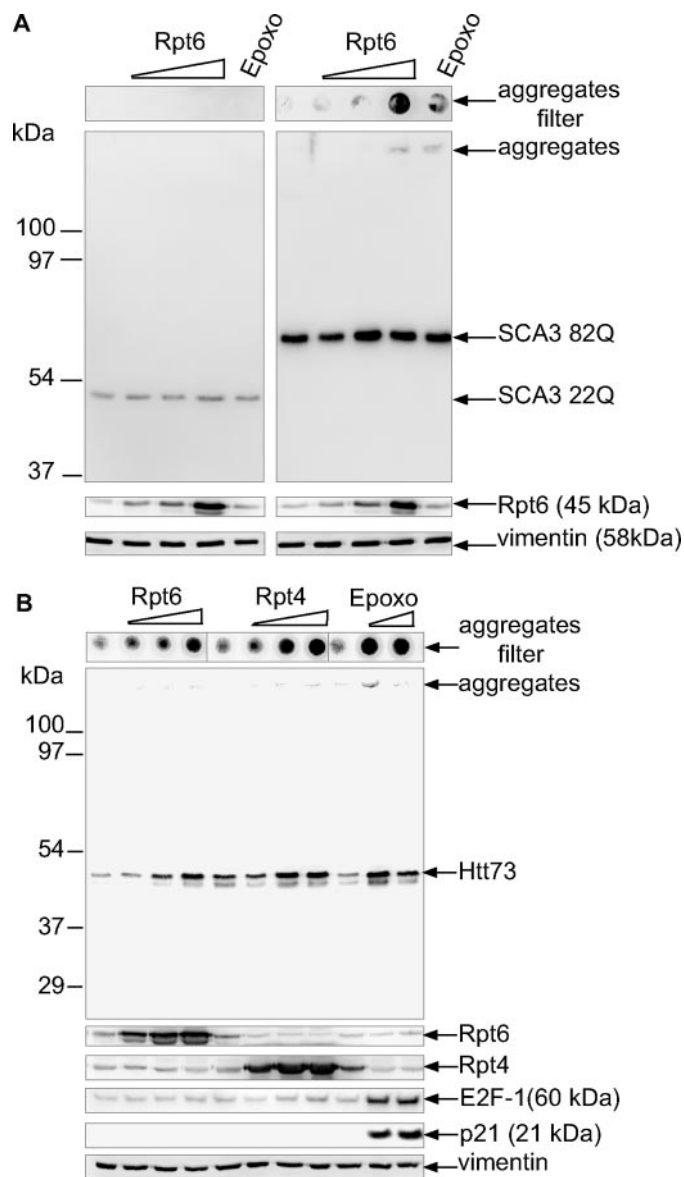
## Misfolding of Poly(Q) Proteins Is Facilitated by 19S ATPases

lished that mutant Htt is not toxic 2 days post-expression (47, 48). We next sought to determine which domains were important for the activity of Rpt6. The amino terminus of Rpt6 contains a coiled-coil domain, a region which was found important for the chaperone function of other AAAs (43). Therefore, the amino-terminal 79 amino acids of Rpt6 containing the coiled-coil region were deleted, generating a protein fragment, Rpt6<sup>80–406</sup>. Overexpression of increasing doses of Rpt6 together with Htt73 augmented the levels of Htt73, detected on immunoblots, by 1.3-, 3-, and 7-fold and the levels of aggregated Htt73, detected on filter retardation assays by 2-, 5-, and 7-fold. These -fold increases correspond to the mean values of four independent experiments. In contrast, Rpt6 lacking the coiled-coil region, expressed at a level similar to the full-length protein, was devoid of any activity on Htt73 (Fig. 4). This result reveals that the coiled-coil region of Rpt6 is essential for its function on Htt aggregation.

The results presented so far indicate that Rpt6 and Rpt4 assist mutant Htt misfolding and aggregation. We next tested whether this effect involved a direct interaction between Rpt6 and Htt73, as suggested by co-localization studies (Fig. 1). Coimmunoprecipitation experiments were carried out using cells expressing Htt73 alone or together with Rpt6. Htt coprecipitated Rpt6, whereas a control antibody did not (Fig. 5A). To determine whether Rpt6 and Htt73 interact directly, recombinant Rpt6, either wild type or lacking the coiled-coil region, was produced in *E. coli* as a fusion with GST and immobilized on glutathione beads. Nonidet P-40 lysates containing Htt73 produced in 293T cells were then incubated with GST and GST-Rpt6. Htt73 was selectively retained by GST-Rpt6 (Fig. 5B). Deleting Rpt6 coiled-coil region abolished its ability to bind Htt. These interaction data suggest that the effect of Rpt6 on Htt aggregation is mediated through a direct interaction and requires the coiled-coil region.

Because a fraction of Rpts were found independently of the proteasome in SCA-3 (45), we tested whether overexpressing Rpt6 affected accumulation of ataxin-3, a protein unrelated to Htt, which contains a poly(Q) expansion in SCA-3. Rpt6 overexpression dramatically enhanced mutant ataxin-3 aggregation but had no effect on wild-type ataxin (Fig. 6A). Previous studies have reported that proteasome inhibition increases accumulation and aggregation of Htt (6–8). Therefore, we next analyzed whether Rpt6 or Rpt4 overexpression generally impaired proteasomal degradation, thereby enhancing accumulation of expanded poly(Q). Epoxomycin, which blocks the catalytic activity of the 20S proteasome, provoked accumulation of both SDS-soluble and aggregated Htt73 or aggregated mutant ataxin-3 (Fig. 6. A and B). Similarly to epoxomycin treatment, Rpt6 or Rpt4 overexpression enhanced Htt73 accumulation and aggregation (Fig. 6B). In contrast, we found that overexpressing these 19S subunits did not stabilize the short-lived proteins E2F-1 and p21 (Fig. 6B), whereas inhibition of the proteolytic activity of the proteasome markedly stabilize these two short-lived proteins (Fig. 6B). These results indicate that Rpt6 or Rpt4 overexpression does not impair proteasomal degradation.

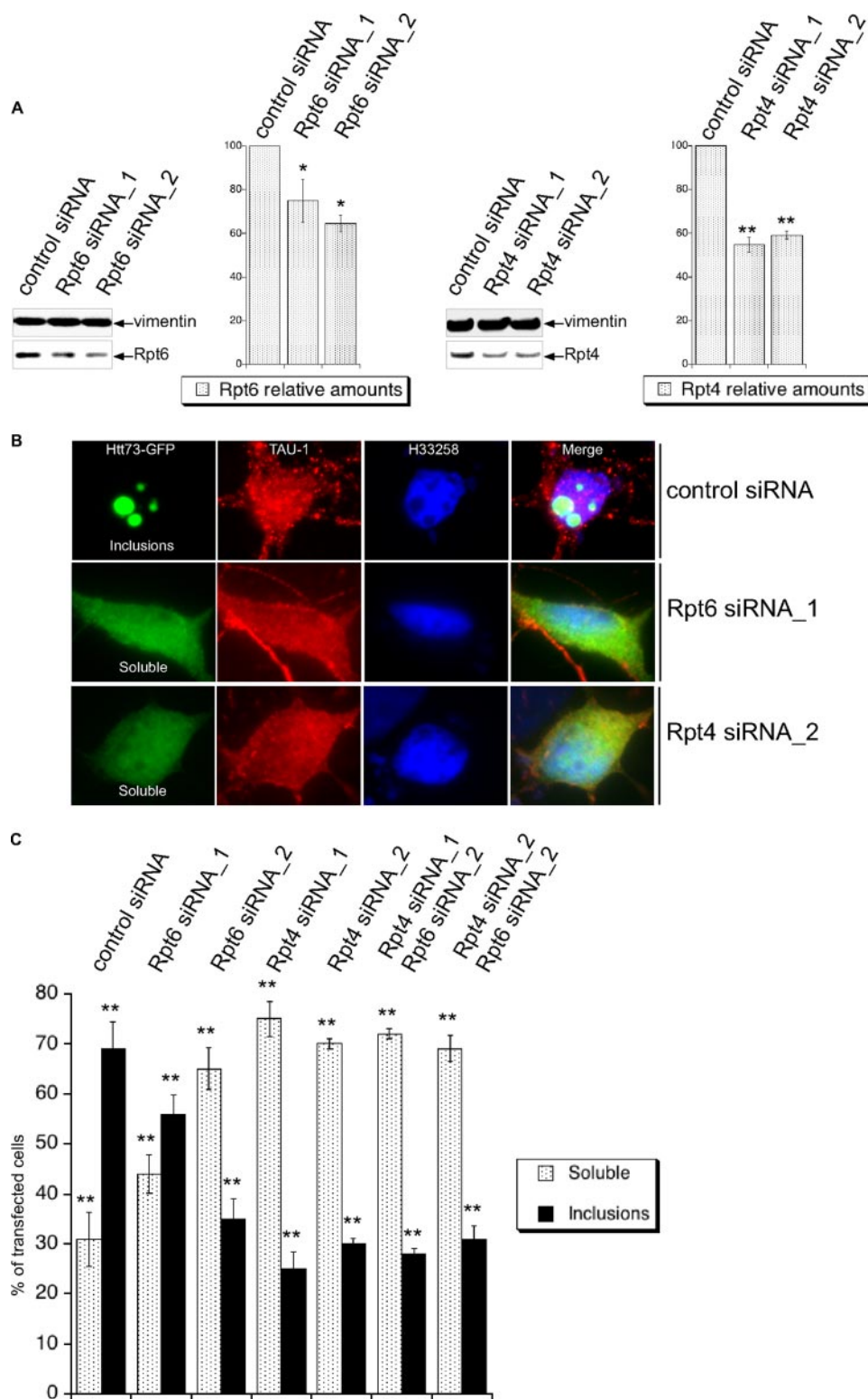
Having shown that increasing the levels of Rpt4 and Rpt6 promotes mutant Htt misfolding, we next tested whether we



**FIGURE 6. Rpt6 and Rpt4 enhance misfolding of proteins containing expanded poly(Q) independently of proteolysis.** A, immunoblots and filter retardation assays of cells overexpressing SCA3 22Q or 82Q alone or together with Rpt6 and either left untreated or treated with 1  $\mu$ M epoxomycin (*Epoxo*) for 14 h. B, 293 T cells were transfected with Htt73 alone or together with Rpt6 and Rpt4 encoding plasmids and either left untreated or treated with 0.5 or 1  $\mu$ M epoxomycin for 14 h and analyzed by immunoblots and filter retardation assays.

could interfere with the endogenous proteins in primary neurons. Two siRNAs targeting Rpt6 were found to significantly reduce the levels of Rpt6 by 25 and 40%, respectively. Similarly, two siRNAs designed against Rpt4 reduced the levels of the protein by  $\sim$ 50% (Fig. 7A). We found that reducing the levels of Rpt6 or Rpt4 substantially reduced the number of cells containing poly(Q) inclusions when we examined cells expressing mutant Htt together with siRNA targeted against Rpt6 or Rpt4 (Fig. 7, B and C). 70% of neurons expressing mutant Htt contained inclusions, and 30% of cells exhibited diffuse fluorescence. This ratio was inverted in cells transfected with Rpt4 or Rpt6 siRNAs (Fig. 7C). The number of cells expressing mutant Htt was very similar when the levels of Rpt4 and Rpt6 were





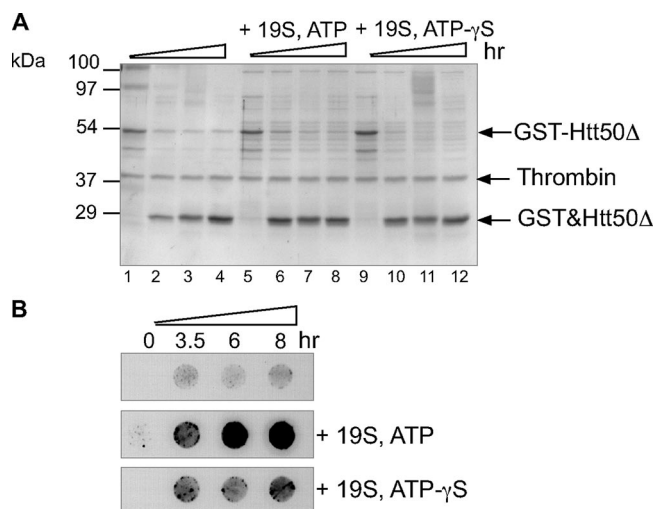
**FIGURE 7. Endogenous Rpt6 and Rpt4 are required for inclusion formation in primary neurons.** *A*, immunoblots showing Rpt6, Rpt4, and vimentin in extracts of primary neurons transfected with control, Rpt6, or Rpt4 siRNAs. Ratio of Rpt6 and Rpt4 proteins, normalized to vimentin, in protein extracts of primary neurons transfected with control, Rpt6, or Rpt4 siRNAs. Quantifications were performed on three independent experiments. *B*, primary neurons were transfected with control, Rpt6 siRNA\_1, or Rpt6 siRNA\_2 together with Htt73-GFP, fixed 24 h post-transfection, and labeled with TAU-1 antibody. Micrographs of neurons containing diffuse fluorescence or inclusions are shown. *C*, ratio of primary neurons transfected with control, Rpt6, or Rpt4 siRNAs together with Htt73-GFP, expressed as percentage of transfected cells. Data were scored from 10 independent experiments corresponding to more than 4000 cells in total. \*\*,  $p < 0.01$ ; \*,  $p < 0.02$ .

reduced and in control cells, indicating that the siRNAs were not toxic under our experimental conditions (data not shown). This analysis demonstrates that reducing the levels of Rpt4 or Rpt6 in primary neurons significantly decrease inclusion formation.

Overexpressed Rpt6 and Rpt4 recruited other Rpts (Fig. 2D), suggesting that their stimulatory activity on Htt misfolding was mediated by a complex containing Rpts. To directly examine this hypothesis, we next tested whether purified 19S proteasome could modulate Htt aggregation *in vitro*. Aggregation of mutant Htt was monitored by filter retardation assay as described in Scherzinger *et al.* (38). Cleavage of GST-Htt released the GST moiety and initiated Htt aggregation (Fig. 8, A and B). The addition of substoichiometric amounts of purified 19S proteasome and ATP strongly enhanced aggregation of mutant Htt (Fig. 8B). The stimulatory effect of 19S proteasome on mutant Htt aggregation *in vitro* was significantly decreased by ATP- $\gamma$ S, a non-hydrolysable ATP analogue (Fig. 8B). The addition of 19S proteasome with or without ATP- $\gamma$ S did not affect the cleavage of GST-Htt (Fig. 8A). This suggests that the stimulatory activity of the 19S proteasome on Htt aggregation is mediated by its unfoldase function.

Altogether, the results presented here reveal that ATPases of the 19S proteasome function non-proteolytically in facilitating misfolding and aggregation of proteins with a poly(Q) expansion. Because Htt inclusions are ubiquitinated, Htt is likely to be addressed to the proteasome by a ubiquitin-dependent pathway (3, 4). We next investigated what would be its fate if targeted to the proteasome by the degradation pathway utilized by the evanescent protein ODC (49). The 37 carboxyl-terminal amino acids of ODC have been found to be an efficient and autonomous destabilizing sequence when fused to heterologous proteins (49). We tested

## Misfolding of Poly(Q) Proteins Is Facilitated by 19S ATPases



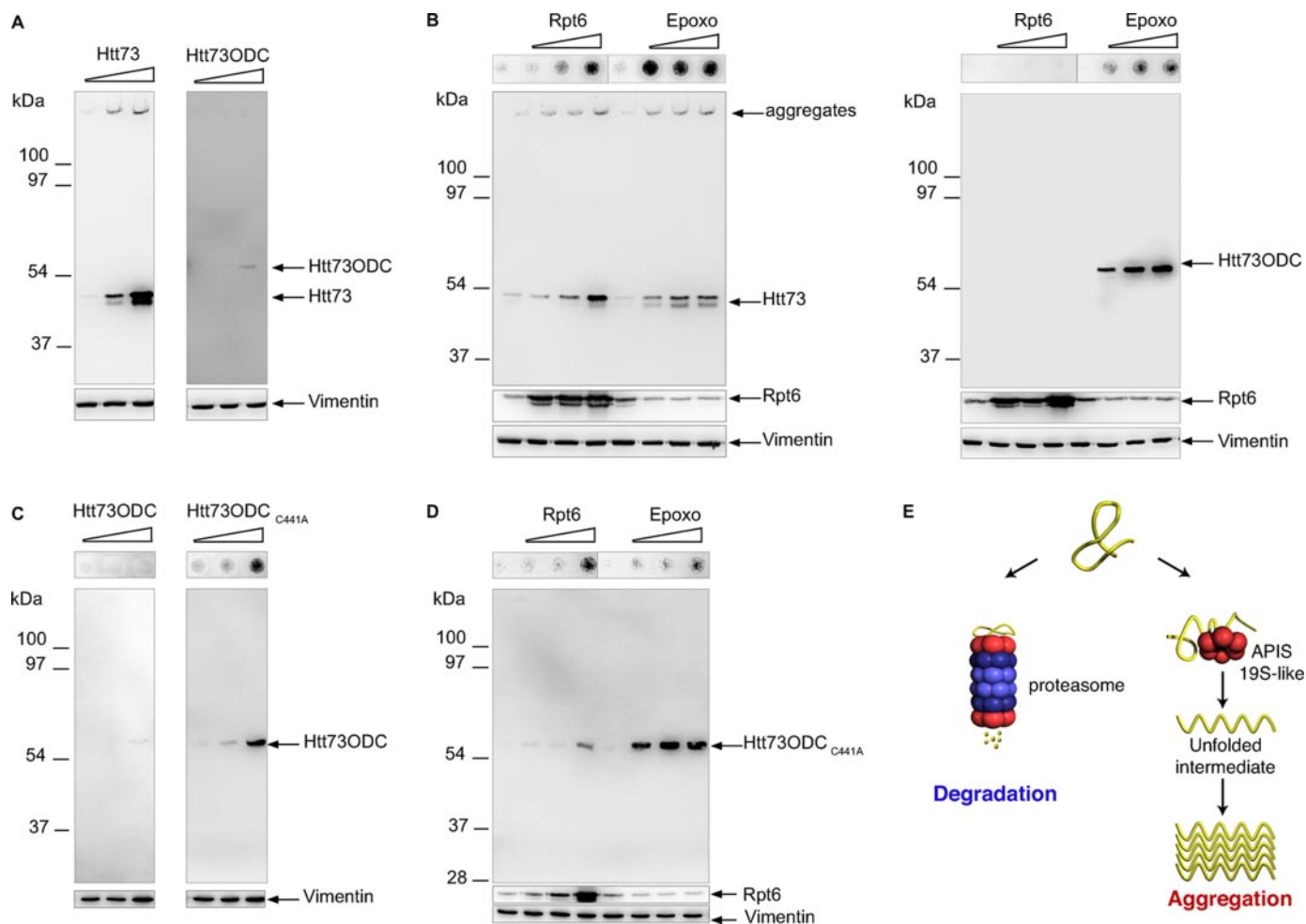
**FIGURE 8. The 19S enhances mutant Htt aggregation *in vitro*.** *A*, time course of proteolysis of GST-Htt50ΔC with or without 19S and ATP $\gamma$ S. 0.5  $\mu$ M GST-Htt50ΔC was cleaved with 10 units of thrombin in the absence or presence of 70 nM 19S and 15 mM ATP $\gamma$ S. Aliquots of the reaction were analyzed after 3.5, 6, or 8 h at 37 °C by SDS-PAGE stained with Coomassie Brilliant Blue. Note that GST and Htt50Δ co-migrate. *B*, samples shown in *A* were analyzed by filter retardation assays and revealed with Htt antibodies.

whether fusing ODC destabilizing sequence to Htt73 could enhance its degradation. Htt73 and Htt73ODC were expressed in 293T cells. Although both soluble and aggregated Htt73 were readily detected by immunoblots and filter retardation assays, Htt73ODC was hardly detectable, even when large amounts of DNA encoding this protein were transfected, suggesting that the protein was very rapidly and efficiently degraded (Fig. 9A). We confirmed that Htt73ODC was fully degraded and did not escape our detection (supplemental Fig. 3). When the proteolytic capacity of the proteasome was compromised, Htt73ODC was stabilized, confirming that Htt73ODC is a proteasome substrate (Fig. 9B, right panel). However, Rpt6 overexpression had virtually no effect on Htt73ODC (Fig. 9B, right panel). The fact that Htt73ODC is insensitive to Rpt6 overexpression but is stabilized by inhibition of the catalytic activity of the proteasome further supports the conclusion that Rpt6 overexpression does not perturb proteasomal degradation. When the ability of the proteasome to degrade proteins is compromised, small but detectable amounts of Htt73ODC aggregates were found by means of their retention on a cellulose acetate filter (Fig. 9B). These observations suggest that, when the catalytic activity of the proteasome is compromised, a small amount of Htt73ODC recovers its ability to aggregate. To obtain further insight into the mechanism by which the ODC carboxyl terminus destabilizes mutant Htt, we mutated cysteine 441 to alanine, as this point mutation has been shown to stabilize ODC (49). In contrast to Htt73ODC, Htt73ODC<sub>C441A</sub> was readily detectable, and its aggregation increased upon Rpt6 overexpression (Fig. 9, C and D). The finding that the ODC destabilizing sequence converts Htt73 into an evanescent protein indicates that the proteolytic capacity of the proteasome is not the limiting factor for mutant Htt degradation.

## DISCUSSION

In this study we report on the finding that in cells aggregation of Htt amino-terminal fragments with a pathogenic poly(Q) expansion is dependent on a remodeling event mediated by Rpt6 and Rpt4. Proteasomal chaperones are subunits of 2 complexes, the 26S proteasome and the previously reported APIS or 19S-like complex (this study and Refs. 22 and 25). Although the function of the proteasome in protein degradation is well recognized, there is also growing evidence indicating that 19S ATPases, in particular Rpt6, function independently of degradation (50). In HD patients, inclusions are often found in the nucleus, where a non-proteolytic function of the 19S ATPases has been extensively reported. A remodeling activity of Rpts independently of degradation provides a unifying mechanism to reconcile their diverse functions. Conditions provoking the dissociation of the 19S from the 20S have been reported previously. The 19S RP disassembles during the catalytic cycle (18). Binding of alternative RP such as PA28 to the CP also provokes dissociation of the ATPases (17). Intriguingly, pharmacological inhibition of the catalytic function of the proteasome stimulates binding of PA28 to the proteasome (17), suggesting that free 19S particles may be released by this treatment. What precisely modulates the relative abundance of 26S proteasome and APIS (or free 19S-like complex) remains to be determined. However, it is noteworthy that aging was reported to promote proteasome disassembly in flies (51).

In neurons of HD (this study) and SCA-3 (45) brains as well as in primary neurons expressing mutant Htt and Rpts, but not 20S subunits, co-localize with poly(Q) inclusions, indicating that Rpts are not associated with the catalytic particles in poly(Q) inclusions. This suggests that Rpts may be involved in inclusion formation independently of the proteolytic function of the proteasome. In support of this hypothesis, we found that overexpression of Rpt6, Rpt4, and more modestly Rpt3 enhance mutant Htt and SCA-3 aggregation. Because the majority of overexpressed Rpt4 and Rpt6 co-sediment with APIS, we propose that Rpt4 and Rpt6 remodel proteins with a poly(Q) expansion and thereby elicit their aggregation independently of degradation. Overexpressing Rpt6 or Rpt4 has no effect on the levels of vimentin or the short-lived E2F-1, p21, and Htt7ODC, showing unambiguously that Rpt6 or Rpt4 overexpression does not impair proteasome degradation. More importantly, reducing the levels of Rpt6 or Rpt4 in primary neurons decreases inclusion formation. Together, these results indicate that endogenous Rpt6 and Rpt4 enhance Htt misfolding, and this effect is likely mediated through an unfoldase function of these proteins. *In vitro* reconstitution experiments support this model; substoichiometric amounts of 19S complex promote mutant Htt aggregation, and this activity is markedly attenuated by ATP $\gamma$ S. Htt supplied in the *in vitro* aggregation assay is not ubiquitinated, suggesting that the 19S does not require ubiquitination of Htt to recognize and remodel Htt. Thus, ubiquitination does not seem to be required for the aggregation process *per se*. However, it is possible that in cells ubiquitination of Htt plays a role in targeting Htt to the proteasome. This is in agreement with previous *in vitro* studies on the chaperone function of the proteasome (52). When the domain composi-



**FIGURE 9. The degradation signal of ODC converts Htt73 into a highly unstable protein.** *A*, lysates of cells transfected with increasing amounts of Htt73 or Htt73ODC encoding plasmids analyzed by immunoblots. *B*, cells were transfected with Htt73 (*left panel*) or Htt73ODC (*right panel*) alone or together with increasing amounts of Rpt6 encoding plasmid and left untreated or treated with 0.25, 0.5 and 1  $\mu\text{M}$  epoxomycin (*Epoxo*) for 14 h. Lysates were analyzed by immunoblots or by filter retardation assays. *C*, cells transfected with increasing amounts of Htt73ODC or Htt73ODC<sub>C441A</sub> were analyzed by immunoblots. *D*, cells were transfected with Htt73ODC<sub>C441A</sub> alone or together with increasing amounts of Rpt6 encoding plasmid and left untreated or treated with 0.25, 0.5, and 1  $\mu\text{M}$  epoxomycin for 14 h. Lysates were analyzed by immunoblots or by filter retardation assays revealed with Htt antibody. *E*, possible routes followed by Htt: degradation by a proteasome complex (this study and Ref. 13) or misfolding and aggregation by APIS or 19 S-like complex.

tion of Rpts was carefully examined, we found that the three ATPases enhancing poly(Q) aggregation, Rpt6, Rpt4, and Rpt3, possess a coiled-coil domain of nearly 50 amino acids. In contrast, the 19 S ATPases unable to enhance poly(Q) aggregation have either no coiled-coil region (Rpt5) or only a short one (Rpt1, Rpt2) according to domain predictions with the web-based tool *SMART*. The importance of the equivalent M-region of the bacterial ATPase ClpB and the yeast Hsp104 suggested that this finding might not be fortuitous. We, thus, deleted the coiled-coil region in Rpt6 and found that although Rpt6<sup>80–406</sup> was expressed at levels similar to the full-length protein, this mutant lacking the coiled-coil domain was inactive toward poly(Q) aggregation (Fig. 4). In agreement with this result, Rpt6 lacking the coiled-coil region was no longer able to bind mutant Htt (Fig. 5). Because Htt misfolding is an early event in HD, our findings suggest that in the disease process, the formation of misfolded and presumably toxic species may be assisted by Rpt6 and Rpt4. A recent study has revealed that tau inclusions, the pathological hallmark of Alzheimer disease, Pick disease, and other tauopathies, are immunoreactive for Rpt3, raising the

question of whether the findings reported here for Htt and SCA-3 may be a generic feature of many neurodegenerative diseases (53).

Monomeric pure poly(Q) peptides are in a random coil conformation (54), but the addition of the polyproline domain present in the carboxyl terminus of the poly(Q) stretch in Htt imposes structural constrain to the protein, which adopts a proline type II helical conformation (36). This explains why the proline-rich region of Htt antagonizes the toxicity of the poly(Q) expansion (34, 35). Thus, a remodeling event ought to be required to alleviate the inhibitory function of the proline-rich region and to elicit the conformational transition into the  $\beta$ -sheet conformation. In agreement with biophysical data, we found that in cells large amounts of Htt fragments with expanded poly(Q) can be expressed in a soluble state (this study and Ref. 55). This implies that aggregation is not just a consequence of an increase in concentration but is dependent in the cellular milieu on additional events. The data presented here indicate that proteasomal chaperones facilitate the transition from soluble to aggregated proteins with a poly(Q) expansion.

## Misfolding of Poly(Q) Proteins Is Facilitated by 19S ATPases

Because inhibition of the proteolytic activity of the proteasome increases Htt aggregation, it has been proposed that aggregates form as a consequence of an impairment of the proteolytic function of the proteasome. However, two conflicting studies question whether purified proteasomes can degrade poly(Q) peptides *in vitro* (9, 13). Here we show that the addition of wild type but not mutant ODC destabilizing sequence to Htt with a poly(Q) expansion profoundly destabilizes this aggregation-prone protein. It is noteworthy that under certain conditions ODC can be degraded by the 20S proteasome (56). Although the mechanism by which wild-type but not mutant ODC carboxyl terminus destabilizes Htt remains to be determined, it is likely that Htt-ODC is degraded by the same ubiquitin-independent pathway as ODC. Thus, the route by which Htt is targeted to the proteasome determines its fate (Fig. 9E). Furthermore, the fact that the addition of the ODC destabilizing sequence converts mutant Htt into a short-lived protein shows unambiguously that, in cells, the proteolytic capacity of the 20S proteasome is not a factor limiting mutant Htt degradation. We propose that misfolding and aggregation of proteins with poly(Q) expansion arise as a consequence of a remodeling event elicited by a non-proteolytic function of proteasomal chaperones.

In conclusion, the data presented here reveal that aggregation of proteins with poly(Q) expansion requires a transition from a benign to an aggregation-prone conformation, a process which in cells is facilitated by proteasomal chaperones. Aggregation of proteins with a poly(Q) expansion is, thus, reminiscent of a typical folding reaction, requiring the assistance of chaperones.

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*Acknowledgments*—We are extremely grateful to Aude Derevier for preparing neuronal hippocampal cells, to Harm Kampinga for the SCA22 and SCA82 constructs, to Xavier Jacq for stimulating discussions, and to Michel Goedert and Nigel Unwin for inspiring comments on the manuscript.

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