

Global Impairment of the Ubiquitin-Proteasome System by Nuclear or Cytoplasmic Protein Aggregates Precedes Inclusion Body Formation

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Summary

The highly conserved ubiquitin-proteasome system (UPS) controls the stability of most nuclear and cytoplasmic proteins and is therefore essential for virtually all aspects of cellular function. We have previously shown that the UPS is impaired in the presence of aggregated proteins that become deposited into cytoplasmic inclusion bodies (IBs). Here, we report that production of protein aggregates specifically targeted to either the nucleus or cytosol leads to global impairment of UPS function in both cellular compartments and is independent of sequestration of aggregates into IBs. The observation of severe UPS impairment in compartments lacking detectable aggregates or aggregation-prone protein, together with the lack of interference of protein aggregates on 26S proteasome function *in vitro*, suggests that UPS impairment is unlikely to be a consequence of direct choking of proteasomes by protein aggregates. These data suggest a common proteotoxic mechanism for nuclear and cytoplasmic protein aggregates in the pathogenesis of neurodegenerative disease.

Introduction

Accumulation of protein aggregates in IB is a nearly universal feature of sporadic and hereditary neurodegenerative disease (Tran and Miller, 1999). Many of the mutations that cause dominantly inherited neurodegenerative diseases have been demonstrated to dramatically increase the propensity of the mutant gene products to self-associate into protein aggregates *in vitro* and *in vivo* (Fink, 1998; Wanker, 2000), supporting the widely considered hypothesis that aggregation underlies the molecular pathogenesis of many neurodegenerative disorders (Taylor et al., 2002). Aggregates are oligomeric assemblies of nonnative protein conformers—“off-pathway” products derived from intermediates in protein folding or unfolding pathways (Temussi et al., 2003). Thus, protein aggregates can be small, soluble oligomers; large, amorphous assemblies; or highly ordered fibrillar “amyloids” (Temussi et al., 2003).

In cells, aggregated proteins are gathered into IB that often contain numerous other proteins including ubiquitin, proteasome subunits, molecular chaperones, intermediate filaments, protein kinases, and transcription factors (Kopito, 2000; Taylor et al., 2002). Sequestration of one or more of these cellular proteins into IBs—and consequent depletion from the cytosol or nucleus—has been proposed as one possible mechanism through which protein aggregates can damage cells (Perutz et al., 1994). Other proposed mechanisms of aggregate proteotoxicity include disruption of microtubule-dependent axonal transport (Gunawardena et al., 2003), perturbation of membrane permeability (Lashuel et al., 2002), and impaired function of the UPS (Bence et al., 2001; Jana et al., 2001).

The UPS performs two essential functions in eukaryotic cells. It serves as a key controller of gene expression through regulated destruction of critical regulatory proteins like cyclins, protein kinases, and transcription factors (Hershko and Ciechanover, 1998). Also, by recognizing and selectively degrading misfolded and damaged proteins, the UPS protects cells against the potentially toxic effects of protein aggregation (Hershko and Ciechanover, 1998). The significance of this latter role in neuroprotection is underscored by the discovery that loss-of-function mutations in genes encoding UPS components can cause neurodegenerative diseases in humans (Mizuno et al., 2001) and rodents (He et al., 2003; Saigoh et al., 1999) and enhance the cytotoxicity of aggregation-prone proteins linked to dominantly inherited neurodegenerative diseases (Cummings et al., 1999b; Fernandez-Funez et al., 2000). We (Bence et al., 2001) and others (Jana et al., 2001; Verhoef et al., 2002) have previously reported that production or accumulation of intracellular protein aggregates in cells profoundly impairs the functional capacity of the UPS. Although defective UPS function is a robust and reproducible response to protein aggregation, the mechanism by which protein aggregation is linked to UPS impairment remains an open and compelling question. One model posits that aggregated or aggregation-prone proteins directly inhibit or “choke” the 26S proteasome—a situation that might result from their engagement by degradation-resistant (Holmberg et al., 2004; Venkatraman et al., 2004) or hard-to-unfold proteins. A second model, not mutually exclusive with the first, is that protein aggregates indirectly interfere with UPS function by inactivating or depleting a UPS activator. For example, it has been suggested that depletion of proteasomes (Cummings et al., 1999a) or other UPS components (Donaldson et al., 2003) by sequestration into IB could account for the observed impairment of UPS function by protein aggregates.

Most disease-linked aggregated proteins accumulate in IBs that are characteristically restricted to either the nucleus or cytoplasm, and the effective toxicity associated with the presence of these IBs appears to be strongly influenced by the cellular compartment in which the aggregates accumulate (Klement et al., 1998; Saudou et al., 1998). In this study, we have developed report-

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ers, based on the green fluorescent protein (GFP), that can be used to monitor the activity of the UPS specifically in the nucleus or the cytoplasm. We have exploited these compartment-specific reporters to investigate the nature of the interaction between protein aggregates and UPS function. Our data reveal that UPS impairment associated with protein aggregation is global; UPS function is disrupted in both cytoplasmic and nuclear compartments irrespective of the compartment to which aggregation-prone proteins are targeted and in which they accumulate. Although UPS components, including ubiquitin, proteasomes, and molecular chaperones, are enriched in IB, this enrichment does not lead to significant depletion in the *trans* compartment and cannot, therefore, account for UPS impairment. Finally, our data reveal that significant UPS impairment occurs prior to the coalescence of aggregated protein into IBs, an observation that supports the hypothesis that sequestration of aggregates into IBs may be a protective, rather than a pathogenic, response.

Results

Nuclear and Cytoplasmic Compartment-Specific UPS Reporters

In order to assess the effect of nuclear and cytoplasmic-localized protein aggregation on the UPS, we constructed compartment-specific variants of our previously described unstable GFP, GFP^u (Bence et al., 2001) (Figure 1). GFP^u constructs were targeted uniquely to the cytoplasm and nucleus by appending the HIV *rev* nuclear export signal (NES) (Fischer et al., 1995) or the SV40 nuclear localization signal (NLS) (Kalderon et al., 1984) to create NES-GFP^u and NLSGFP^u reporters, respectively. To ensure that these reporters exceeded the molecular weight cut-off for passive diffusion across the nuclear pore complex, the constructs included an extra copy of NES or NLSGFP^u fused in tandem to GFP destabilized by the presence of the CL1 degron (Figure 1A). Microscopic imaging of clonal HEK 293 cells stably expressing these reporters established that the reporters were effectively localized to their correct intracellular compartment at steady state (Figure 1B).

Exposure of NES and NLSGFP^u-expressing cell lines to the proteasome inhibitor MG132 increased the cell's fluorescence intensity, suggesting that the low steady-state fluorescence in these cell lines is the consequence of reporter synthesis balanced by efficient proteasomal degradation (Supplemental Figure S1 available online at <http://www.molecule.org/cgi/content/full/17/3/351/DC1>). Metabolic pulse-chase analysis established that NES and NLSGFP^u are short-lived ($t_{1/2}$ 60 min) proteins, somewhat more stable than the original nonlocalized GFP^u ($t_{1/2}$ 30 min) (Bence et al., 2001) (Supplemental Figures S1A and S1B). Accumulation of both NES and NLSGFP^u was not observed in cells exposed to tunicamycin, nocadazole, or brefeldin A, suggesting that it is a specific response to UPS impairment and not a secondary response to other types of cell stress (data not shown).

To evaluate the utility of NES and NLSGFP^u levels as indicators of compartment-specific UPS activity in living cells, we used flow cytometry to monitor the effect of

proteasome inhibition on GFP^u fluorescence in clonal cell lines stably expressing these constructs (Supplemental Figures S1C and S1D). GFP fluorescence for all three reporters increased in proportion to proteasome inhibitor concentration (Supplemental Figure S1C). The concentration of inhibitor required to produce 50% of the maximum fluorescence increase (EC_{50}) for all three reporters was 600–900 nM—similar to the previously reported value of 845 nM for GFP^u (Bence et al., 2001). Comparison of the fractional increase in GFP^u fluorescence with fractional inhibition of chymotrypsin-like activity of proteasomes in reporter cell lines chronically exposed to lactacystin revealed that both of the compartment-specific reporters are more sensitive to loss of proteasome function, reaching half-maximal fluorescence at 60%–70% proteasome inhibition, compared to ~90% for noncompartmentalized GFP^u (Supplemental Figure S1E). Thus, NES and NLSGFP^u are sensitive and dynamic *in vivo* reporters of UPS activity in cytoplasmic and nuclear compartments, respectively.

Polyglutamine Aggregation Leads to Impaired UPS Activity in the *cis* Compartment

To determine whether aggregation is associated with impairment of cytoplasmic UPS function, we measured GFP fluorescence in NESGFP^u cells expressing huntingtin exon 1 containing either a short, nonpathogenic polyglutamine repeat (HttQ25) or a highly aggregation-prone, pathogenic repeat (HttQ103) (Figure 2A). As expected, no IBs were apparent in cells expressing HttQ25, and NESGFP^u levels were not significantly different from untransfected cells (Figures 2A and 2C). By contrast, juxtannuclear cytoplasmic IB were common in cells expressing HttQ103. In these cells, NESGFP^u fluorescence was substantially elevated by comparison to HttQ25-expressing cells (Figures 2A and 2C), confirming our earlier findings with nonlocalized GFP^u.

To test if aggregation within the nucleus leads to accumulation of nuclear GFP^u, we expressed ataxin-1 plasmids with a short (Atx1Q30) or long (Atx1Q82) polyglutamine repeat in NLSGFP^u cells. Ataxin-1 harbors a strong, intrinsic nuclear localization sequence (Klement et al., 1998); both wild-type (wt) and expanded ataxin-1 were more than 99% restricted to the nuclear compartment in NLSGFP^u cells (Figure 2B). No significant difference was observed between the fluorescence of NLSGFP^u cells expressing Atx1Q30 and control untransfected cells (Figures 2B and 2C). Cells transfected with Atx1Q82 exhibited frequent nuclear IBs; NLSGFP^u levels in these cells were significantly elevated compared to cells expressing nonexpanded ataxin-1 (Figures 2B and 2C). Ataxin-1Q82K772T, which bears an inactivating mutation in the NLS (Klement et al., 1998), formed exclusively cytoplasmic IB and led to a significant increase in NESGFP^u fluorescence (Figure 2C). Similarly, appending the SV40 large T antigen NLS to HttQ103 caused it to form nuclear inclusions and increased the fluorescence of NLSGFP^u to a similar extent to that associated with Atx1Q82 inclusions (Figure 2C).

Polyglutamine Aggregation Leads to Impaired UPS Activity in the *trans* Compartment

To determine whether protein aggregates must be physically present within the same compartment in which

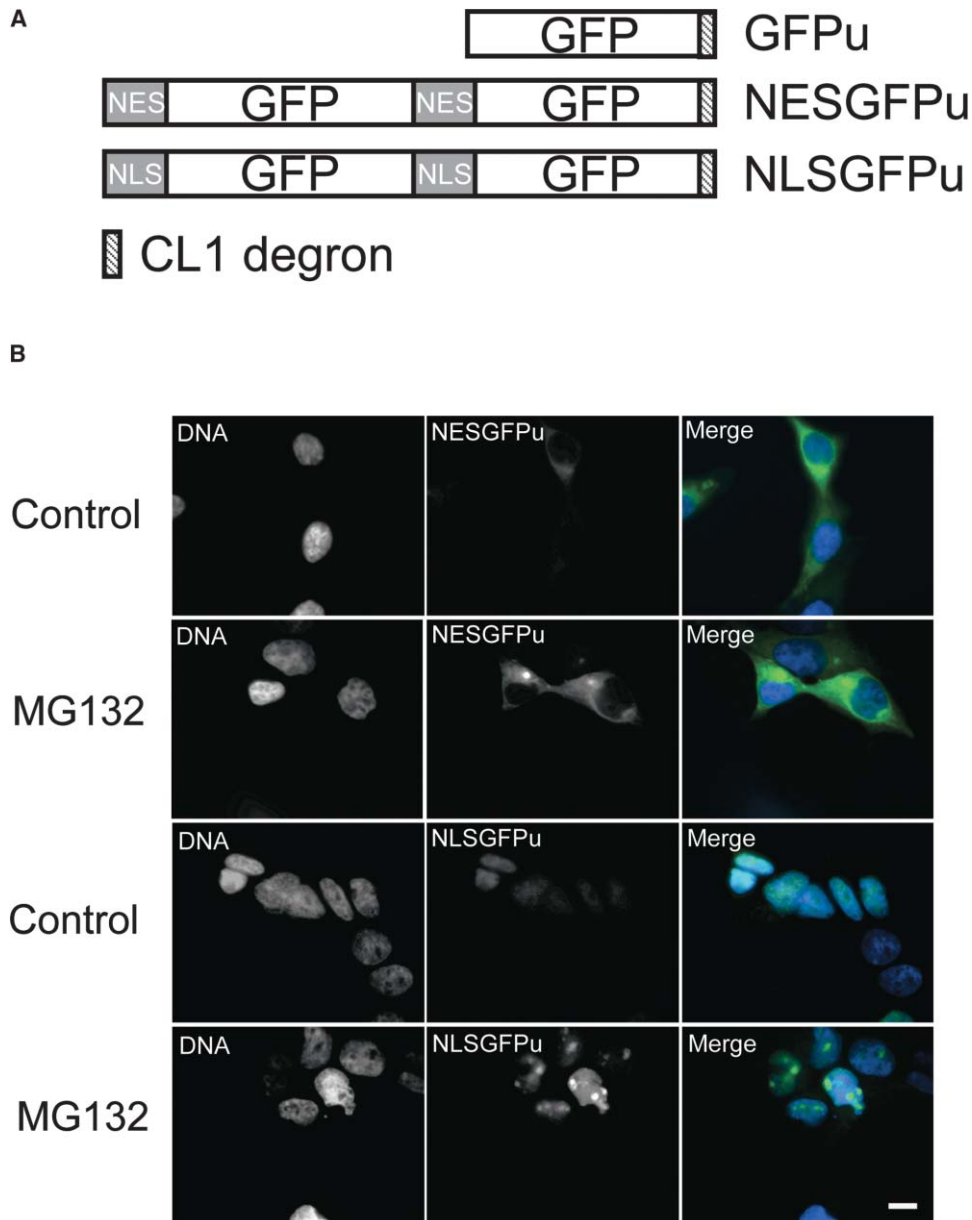


Figure 1. Design and Expression of Compartment-Specific UPS Reporters

(A) Graphical representation of GFP^u variants. NLS and NESGFP^u constructs consist of a tandem repeat of GFP to which a nuclear localization or export sequence, respectively, was appended. Each construct contained the strongly destabilizing CL1 degron at the C terminus.

(B) Localization of compartment-specific UPS reporters. Fluorescence microscopy of clonal HEK293 cells expressing NLSGFP^u or NESGFP^u in cells untreated (control) or exposed to 10 μ M MG132 for 8 hr. Bar, 2 μ m.

UPS impairment occurs, we assessed the effect of expression of compartmentally restricted ataxin-1 and Htt on NES and NLSGFP^u localized to the *trans* compartment (Figure 3). Cells expressing Atx1Q30 displayed a small but significant increase in NESGFP^u levels, consistent with the slight tendency of this protein to aggregate and cause cell toxicity when overexpressed (Fernandez-Funez et al., 2000) (Figures 3A and 3C). NESGFP^u fluorescence was significantly increased in cells containing nuclear ataxin-1 inclusions (Figures 3A and 3C). Similarly, NLSGFP^u fluorescence was increased in cells with cytoplasmic HttQ103 inclusions, but not with HttQ25

expression (Figures 3B and 3C). Thus, protein aggregation can impair nuclear and cytoplasmic UPS function when the aggregates accumulate in *cis* or in *trans*.

Rhodopsin Aggregation Impairs the UPS in the *cis* and *trans* Compartments

To determine whether the effects of protein aggregates on the UPS are specific for polyglutamine-containing proteins, we tested the effect of rhodopsin P23H expression on fluorescence of NLS or NESGFP^u (Figure 4). Rhodopsin P23H is synthesized on ER bound ribosomes and is unable to fold to an export-competent state (Illing

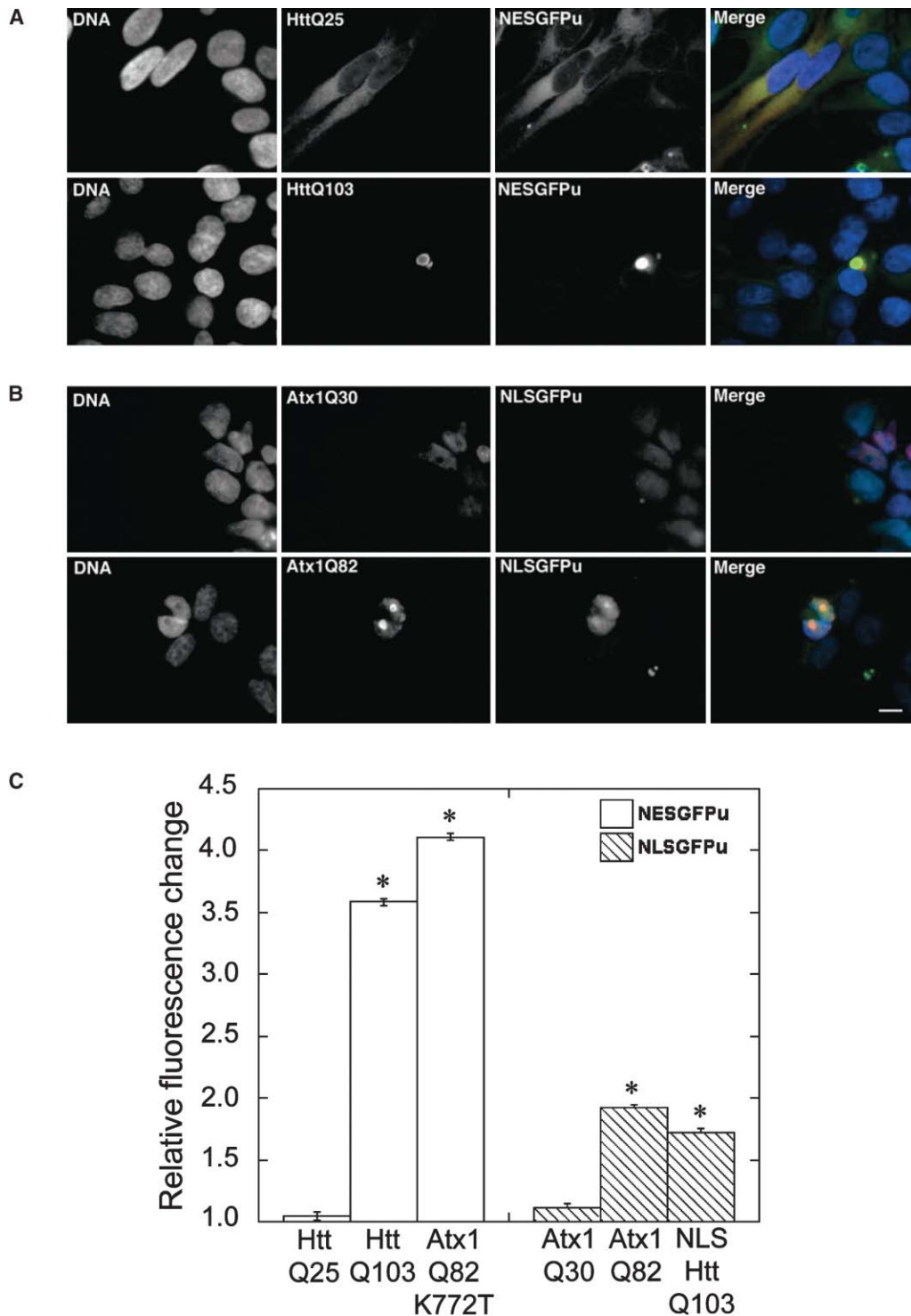


Figure 2. Protein Aggregation in the *cis* Compartment Impairs UPS Function

(A) Representative images of NESGFP^u-expressing cells transiently transfected with C-terminal *myc* epitope-tagged huntingtin exon 1-expressing plasmids with a polyglutamine repeat length of either 25 (HttQ25) or 103 (HttQ103).

(B) Representative images of NLSGFP^u-expressing cells transiently transfected with N-terminal FLAG epitope-tagged ataxin-1-expressing plasmids with a polyglutamine repeat length of 30 (Atx1Q30) or 82 (Atx1Q82). Images in (A) and (B) were acquired for nuclear staining (blue), Htt or Atx1 (red), and the compartment-specific GFP^u (green).

(C) Quantification of NESGFP^u and NLSGFP^u fluorescence in *cis*. Cells expressing nonaggregating protein (HttQ25 and Atx1Q30) or IB (HttQ103, Atx1Q82, NLSHttQ103, or Atx1Q82K772T) were identified by immunofluorescence microscopy to the appropriate epitope tag, and the corresponding total cellular NESGFP^u or NLSGFP^u fluorescence was measured with a cooled CCD camera. The mean fluorescence value for a given population ($n = 300\text{--}400$) was calculated and compared to the mean fluorescence value of the corresponding NESGFP^u or NLSGFP^u populations not expressing Htt or Atx1 constructs. The mean fluorescence values relative to HttQ25 or Atx1Q30 were compared by the Student's *t*-test. * $p < 0.001$.

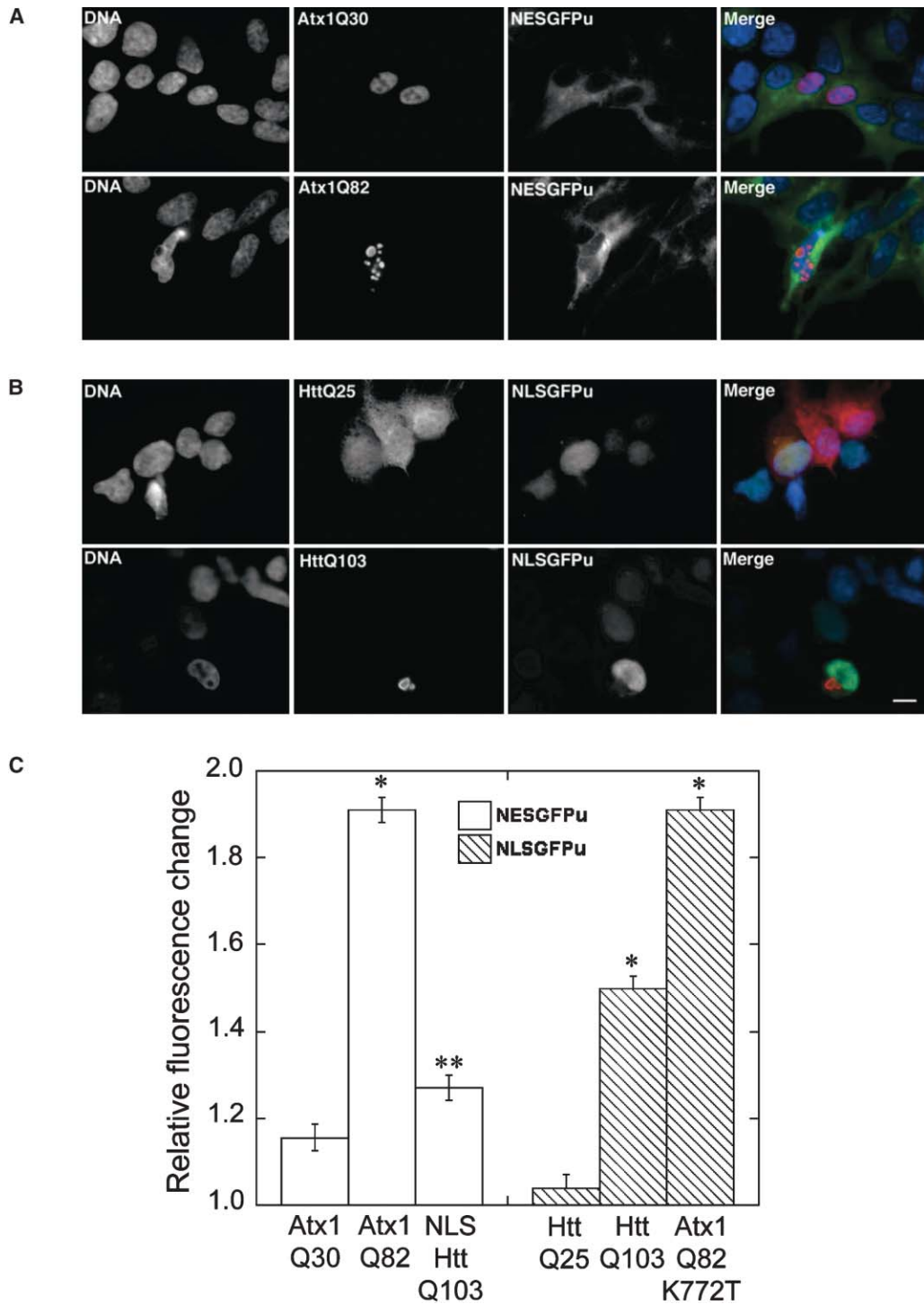


Figure 3. Protein Aggregation in the *trans* Compartment Impairs UPS Function

(A) Representative images of NESGFP^u-expressing cells transiently transfected with N-terminal FLAG epitope-tagged ataxin-1-expressing plasmids with a polyglutamine repeat length of 30 (Atx1Q30) or 82 (Atx1Q82).

(B) Representative images of NLSGFP^u-expressing cells transiently transfected with a C-terminal *myc* epitope-tagged huntingtin exon 1-expressing plasmids with a polyglutamine repeat length of either 25 (HttQ25), or 103 (HttQ103). Images in A and B were acquired for nuclear staining (blue), Htt or Atx1 (red), and the compartment-specific GFP^u (green).

(C) Quantification of NESGFP^u and NLSGFP^u fluorescence in *trans*. Quantification and computational methods as in Figure 2C. **p* < 0.001, ***p* < 0.05.

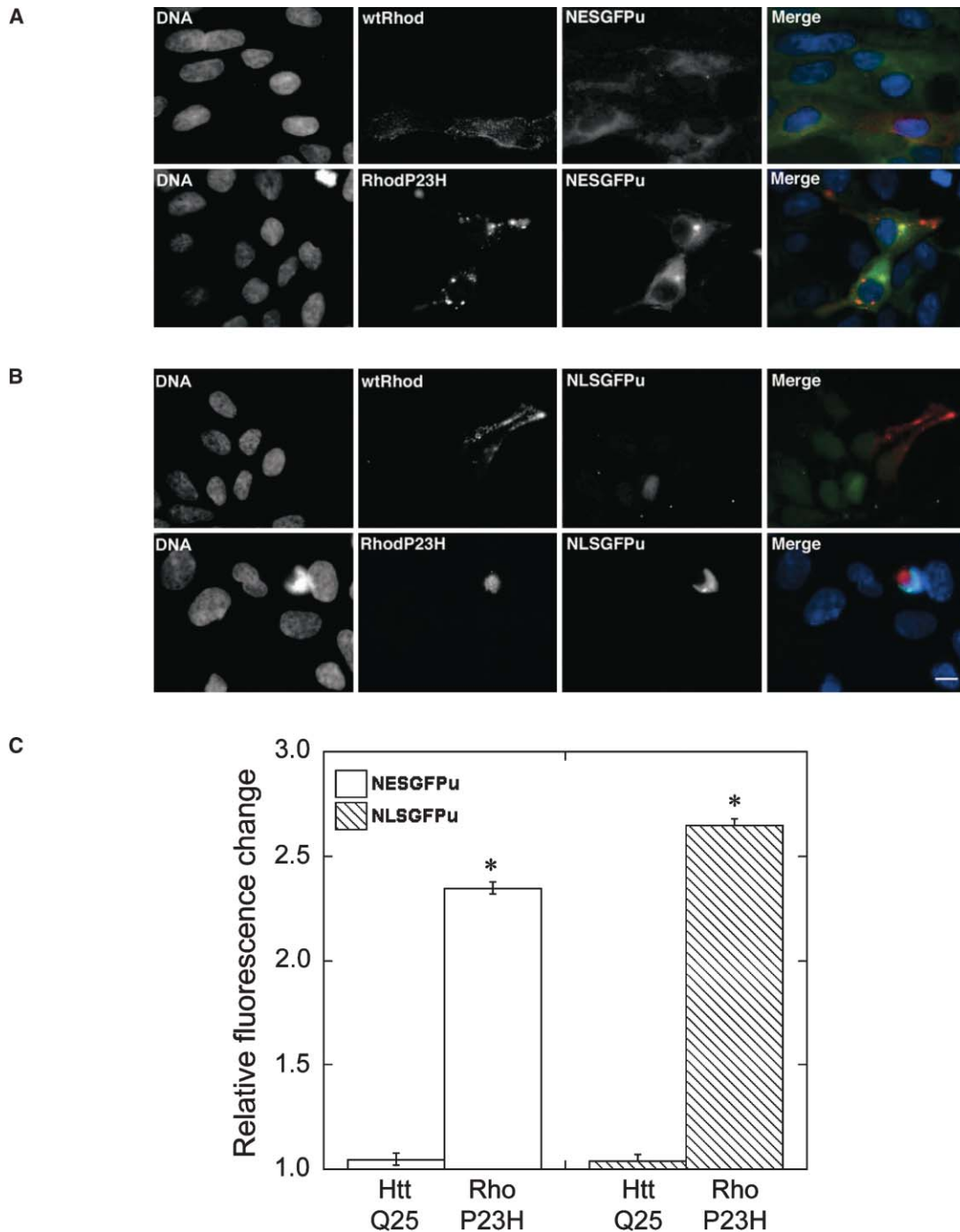


Figure 4. Rhodopsin P23H Aggregation Impairs the UPS in *cis* and *trans* Compartments

HEK293 cells stably expressing NESGFP^u (A) or NLSGFP^u (B) were transfected with wt or P23H rhodopsin. Rhodopsin-expressing cells were identified by immunofluorescence staining using the 1D4 rhodopsin-specific mAb (red) and imaged for NESGFP^u or NLSGFP^u (green) or nuclear staining (blue).

(C) Quantification and computational methods as in Figure 2C. * $p < 0.001$.

et al., 2002; Sung et al., 1993). This mutant form of the protein is subsequently recognized and degraded by UPS-dependent, ER-associated degradation and is highly prone to self-associate into aggregates that are effectively sequestered into cytoplasmic IBs (Illing et al., 2002; Saliba et al., 2002). Wt rhodopsin is efficiently

folded and expressed at the cell surface in NESGFP^u (Figure 4A) or NLSGFP^u (Figure 4B) cells, whereas rhodopsin P23H was localized to strictly cytoplasmic punctate foci (Figure 4A) or large inclusions (Figure 4B). Expression of wt rhodopsin at similar levels did not lead to IB formation and failed to elicit a detectable increase

in either NESGFP^u or NLSGFP^u fluorescence (Figures 4A and 4B, top). By contrast, rhodopsin P23H expression resulted in a marked increase in the fluorescence of both reporters (Figures 4A–4C). These data establish that global UPS impairment by aggregates in the *cis* or *trans* compartment is not restricted to polyglutamine proteins.

Inclusion Body Formation Is Not Required for *cis* or *trans* UPS Impairment

The preceding data reveal a correlation between the presence of inclusions and increased fluorescence of NES and NLSGFP^u, suggesting a tight linkage between protein aggregation and UPS impairment—irrespective of whether or not the aggregates accumulate in the same or different compartment from the reporter. In principle, this effect could be a direct consequence of IB formation per se or an effect of the protein aggregates prior to sequestration into IBs. These two states can be readily distinguished by microscopy—highly aggregation-prone proteins like HttQ103 can be observed either to be diffusely distributed, with no detectable IBs, or more than 95% sequestered into IBs (Figures 2A and 3B). We found that cells expressing diffusely localized HttQ103 in the absence of detectable IB exhibited significantly elevated levels of NESGFP^u fluorescence compared to neighboring HttQ103-negative cells (Figure 5A, top) or HttQ25-expressing cells (data not shown). Similarly, both NES and NLSGFP^u levels were elevated in cells expressing diffuse Atx1Q82 in the absence of IBs (Figures 5A and 5B). Thus, UPS impairment results from an intrinsic property of the aggregation-prone protein and not from its sequestration into IBs.

UPS Impairment Is Not the Result of Steady-State Sequestration of UPS Components

Previous studies have reported that proteasome core and regulatory particles, molecular chaperones, and ubiquitin are all enriched in IBs in both the nucleus and cytosol, suggesting that one way aggregates might impair UPS function is by recruiting or sequestering limiting components of the UPS (Taylor et al., 2002). Although these cellular components are indeed enriched in IB relative to the surrounding cytoplasm, the extent to which they are actually depleted from other cellular compartments has not been hitherto evaluated. To assess the extent to which the compartmental distribution of these cellular components is altered by the presence of protein aggregates, we used quantitative image analysis to assess the extent of redistribution of proteasome subunits and molecular chaperones (Figure 6). The distribution of the 20S core particle of the proteasome was measured by quantification of intrinsic fluorescence of LMP2-GFP in HT1080 fibrosarcoma cells stably expressing this construct (Reits et al., 1997) and by immunofluorescence in HEK 293 cells transiently transfected with nuclear or cytoplasmically localized polyglutamine proteins. No significant alteration of the cytoplasmic/nuclear distribution of proteasome subunits was detected in cells containing visible polyglutamine nuclear or cytoplasmic inclusions (Figures 6B and 6C). Although immunofluorescence is often not a quantitative technique, we have employed it in this study to assess only

the ratio of antigen distribution between nuclear and cytoplasmic compartments in the same cell. Control experiments using GFP-tagged proteins confirmed the validity of this ratiometric approach (data not shown). Similar results for the distribution of 19S and 11S regulatory particles as well as for ubiquitin and Hsp70 were obtained from immunofluorescence labeling. Although many of the tested components of the UPS were visibly enriched in the IBs (Figures 6A and 6B), the actual fraction of UPS components in the cytoplasm or the nucleus was unaltered. These data suggest that recruitment of proteasome subunits and other UPS-associated proteins to IBs is not accompanied by a significant change in their overall cellular distributions.

Proteasomes Are Not Inhibited by Protein Aggregates In Vitro

Because the vast majority of expanded Htt and Atx1 is sequestered in IBs, whereas proteasomal subunits are not significantly sequestered in IBs, it is unlikely that simple steady-state depletion of these factors could account for the observed UPS impairment. However, because some chaperones (Kim et al., 2002; Stenoien et al., 2002) and proteasome subunits (Stenoien et al., 2002) associate dynamically with IBs, it is possible that indigestible aggregates could inhibit UPS function by directly binding to and “choking” proteasomes. To test this possibility, we assessed the effect of in vitro-generated polyglutamine aggregates on the degradation of ubiquitin-dependent and ubiquitin-independent substrates by purified 26S proteasomes (Figure 7). A fragment of ¹²⁵I-labeled cyclin B containing the destruction box necessary for its proper ubiquitylation (cyclin N100) was ubiquitylated in vitro (Chen and Fang, 2001). Autoradiography of the reaction mixture resolved by SDS-PAGE revealed that the substrate was fully converted to a ladder of high molecular weight species (Figure 7A), which were identified as ubiquitin conjugates because the ladder could be resolved back to monomeric cyclin N100 by treatment with a recombinant ubiquitin-specific protease, Ubp41 (Baek et al., 1997) (data not shown). Ubiquitylated cyclin N100 was efficiently and rapidly degraded in a dose-dependent fashion by purified rat liver 26S proteasomes (Figure 7B). This degradation was ATP dependent and could be inhibited by addition of proteasome inhibitors MG132 or lactacystin (data not shown).

To evaluate the effect of polyglutamine-containing proteins on proteasome-mediated degradation of Ub_n-cyclin N100, we generated a pair of constructs consisting of the N-terminal exon of Htt containing either 18 or 51 Gln residues fused to glutathione S-transferase (GST), with an intervening tobacco etch virus (TEV) protease cleavage site and a C-terminal S epitope tag. As described by Scherzinger and colleagues (1999), the presence of the GST tag maintains both Q18 and Q51 in a soluble form. Upon cleavage of the GST moiety with TEV protease, the Q51, but not the Q18, fragment rapidly formed high molecular weight aggregates, as judged by a filter retardation assay (Wanker et al., 1999) (Figure 7C). Neither the soluble GST-conjugated Htt fragments nor the released polypeptides had any discernable effect on the degradation of Ub_n-cyclin N100, even when

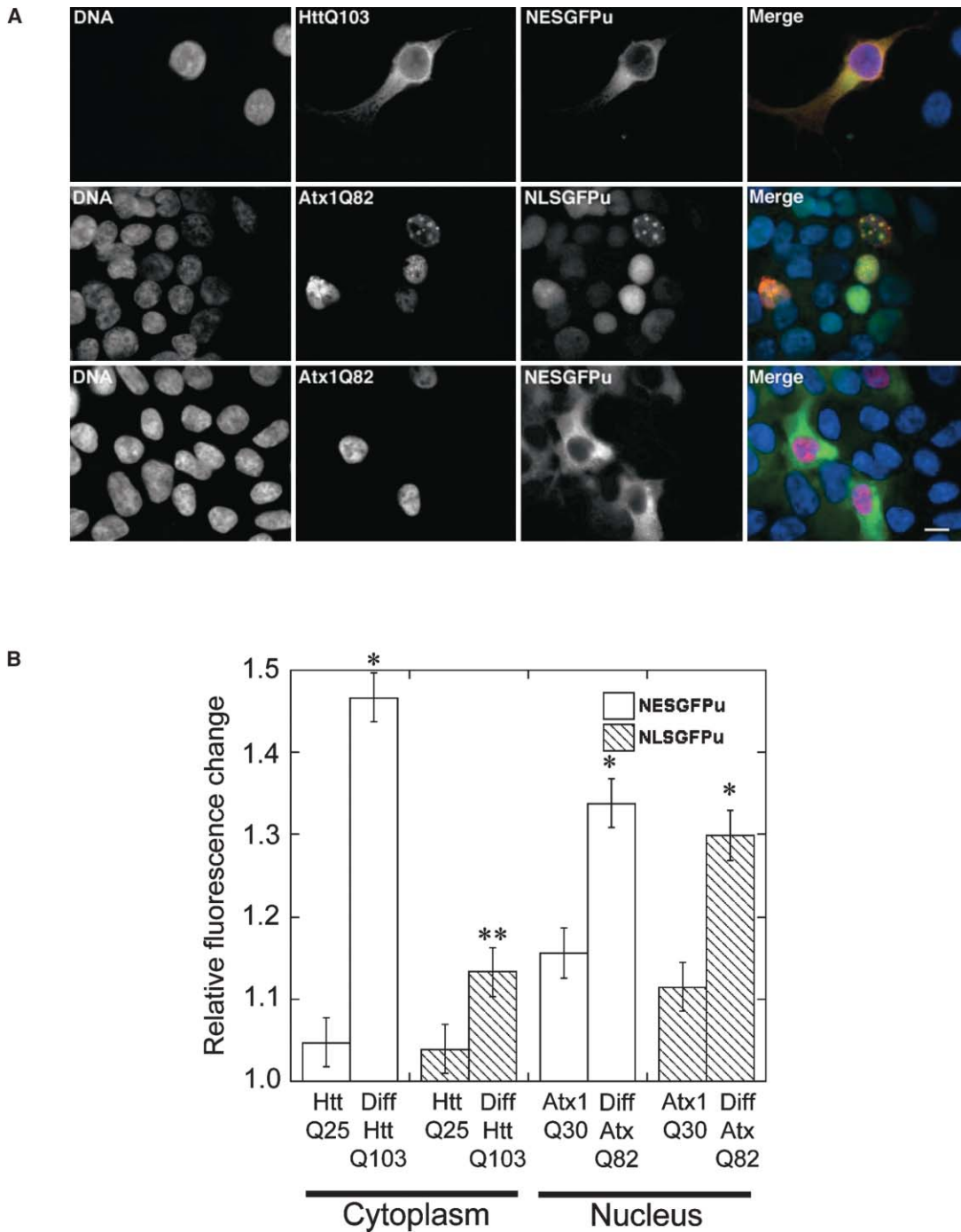


Figure 5. Inclusion Body Formation Is Not Essential for UPS Impairment

(A) Cells with diffuse localization of aggregation-prone polyglutamine proteins (HttQ103 and Atx1Q82, red) were identified by immunofluorescence staining and imaged for corresponding levels of NESGFP^u or NLSGFP^u fluorescence (green).

(B) Data were quantified and analyzed as in Figure 2C, except that only cells without IB were included in the analysis. **p* < 0.001, ***p* < 0.05.

present at a 100-fold molar excess (Figure 7E). We did not observe a reduction in proteasome activity even when the TEV cleavage reaction was conducted in the presence of preaggregated “seed” composed of aggregated polyglutamine peptide (see below), a procedure known to enhance aggregation kinetics (Chen et al., 2001) (Figure 7E).

In order to further characterize the effects of aggregated polyglutamine-containing proteins on proteasome function, we assessed the effect of small soluble oligomeric polyglutamine aggregates as well as highly aggregated fibrillar species (Chen et al., 2001; Poirier et al., 2002) (Supplemental Figure S3) on the chymotrypsin-like peptidase activity of 26S proteasomes by using the

fluorogenic substrate Suc-LLVY-AMC. We did not observe any inhibition of substrate degradation in the presence of any class of Q44 peptide aggregates, even when they were present at 100-fold molar excess over proteasomes (Figure 7D).

UPS Impairment Is Not the Result of Simple Substrate Competition or Sequestration of the Fluorescent UPS Reporters

Because our results are largely based on the usage of fluorescent UPS reporters, we sought to address two major concerns to validate our findings. First, the simplest possible mechanism for our observation of global UPS impairment is that overexpressed UPS substrates directly compete for a limiting factor required for proteasome-dependent proteolysis. To test this possibility, we examined the effect of maximally overexpressing ubiquitin-dependent and ubiquitin-independent proteasome substrates on NESGFP^u fluorescence (Supplemental Figure S2). The L158P mutant of von Hippel-Lindau (VHL) protein is unable to fold and is efficiently degraded in a ubiquitin- and proteasome-dependent fashion with $t_{1/2} \sim 3$ hr (M. Scott and J. Frydman, personal communication). We also examined the effect of overexpressing ornithine decarboxylase (ODC), a proteasome substrate that is degraded in a ubiquitin-independent fashion (Coffino, 2001). Despite the rapid turnover rate of ODC and L158P-VHL, their overexpression had no effect on NESGFP^u fluorescence levels (Supplemental Figure S2). Thus, although many misfolded proteins are efficient proteasome substrates, UPS impairment appears to be a property unique to aggregation-prone proteins. The simplest interpretation of these data is that UPS impairment is not the consequence of simple substrate competition. Second, in some cases we observed colocalization of the nuclear or cytoplasmic reporter with the IBs (Figures 2A and 2B), suggesting a possible sequestration of our reporters into IB. However, the fraction of NES or NLSGFP^u sequestered into inclusions is less than 17% of the total, and we never observed colocalization of either reporter with IBs in the *trans* compartment (Figure 3) or with rhodopsin P23H (Figure 4). Finally, control experiments reveal that, although some NESGFP^u was sequestered in IBs after exposure of cells to a reversible proteasome inhibitor, all of the reporter's fluorescence declined monotonically with kinetics commensurate with the experimentally determined half-life upon inhibitor washout (data not shown). Thus, the limited colocalization of NES or NLSGFP^u in IBs does not necessarily reflect a pool of aggregated stable reporter. Perhaps a fraction of GFP^u is recruited to IBs by an HDAC6-dependent mechanism that appears to couple ubiquitylated proteins to retrograde transport by the microtubule motor cytoplasmic dynein (Kawaguchi et al., 2003).

Discussion

Accumulation of intracellular protein aggregates in intracellular IBs has long been recognized to be a pathognomonic feature of neurodegenerative disease. However, the cellular mechanisms by which these abnormal protein structures are linked to underlying cellular pathol-

ogy have remained elusive. One important clue is the discovery that the function of the UPS, which plays a central role in the elimination of potentially toxic misfolded proteins, is impaired by protein aggregates. We have investigated nucleocytoplasmic compartmentalization of pathogenic protein aggregates to help elucidate the manner by which aggregated proteins interact—and interfere—with the UPS. Our data establish that UPS impairment by protein aggregates is global and that the capacity of the entire cellular UPS is compromised by the presence of aggregates that are tightly restricted to either the nuclear or cytoplasmic compartment. These data have important implications for understanding why aggregates are toxic to cells and the mechanism of UPS dysfunction in neurodegenerative disease.

Relationship of Aggregate Location to Toxicity

A common pathological feature linking many neurodegenerative diseases is the presence of intranuclear IBs. The finding that nuclear targeting enhances the toxicity of aggregates (Peters et al., 1999), combined with studies suggesting that nuclear exclusion suppresses huntingtin (Peters et al., 1999; Saudou et al., 1998) and ataxin-1 (Klement et al., 1998) toxicity, strongly implies that nuclear delivery of aggregates is important for pathogenesis. The data presented here, along with work from other labs (Hackam et al., 1999; Peters et al., 1999), suggest that protein aggregation is inherently toxic irrespective of subcellular location. It is possible that the confined region of the nuclear compartment increases the effective concentration of aggregation-prone proteins thereby accelerating aggregation rate and time to pathology. It is also likely that multiple aberrant interactions arising from protein aggregation—including specific inactivation of transcription factors together with global UPS impairment—dictate overall toxicity.

Mechanism of UPS Impairment by Protein Aggregation

Two possible models could account for the impairment of UPS function by protein aggregation. The simplest mechanism is that protein aggregates directly inhibit or sequester 26S proteasomes. Because proteasomal proteolysis is highly processive (Akopian et al., 1997), aggregates could be potent inhibitors by virtue of being both undegradable and slowly released. This “proteasome choking” model predicts that in cells exhibiting near complete loss of UPS function, a substantial fraction of total cellular proteasomes should be associated with protein aggregates. Thus, aggregates could simply sequester proteasomes away from cellular sites where they are required, as suggested from the observation of proteasome subunits in IBs in brains from human (Schmidt et al., 2002) and animal models (Cummings et al., 1998) of neurodegenerative disease. Although casual inspection of immunofluorescent images of proteasome subunit distribution in aggregate-containing cells indicates a clear enrichment of proteasome subunits in IBs, our data demonstrate that this enrichment is not accompanied by significant depletion of proteasomes from nuclear or cytoplasmic pools. In IBs containing cells, the vast majority (>99%) of expanded Htt is present

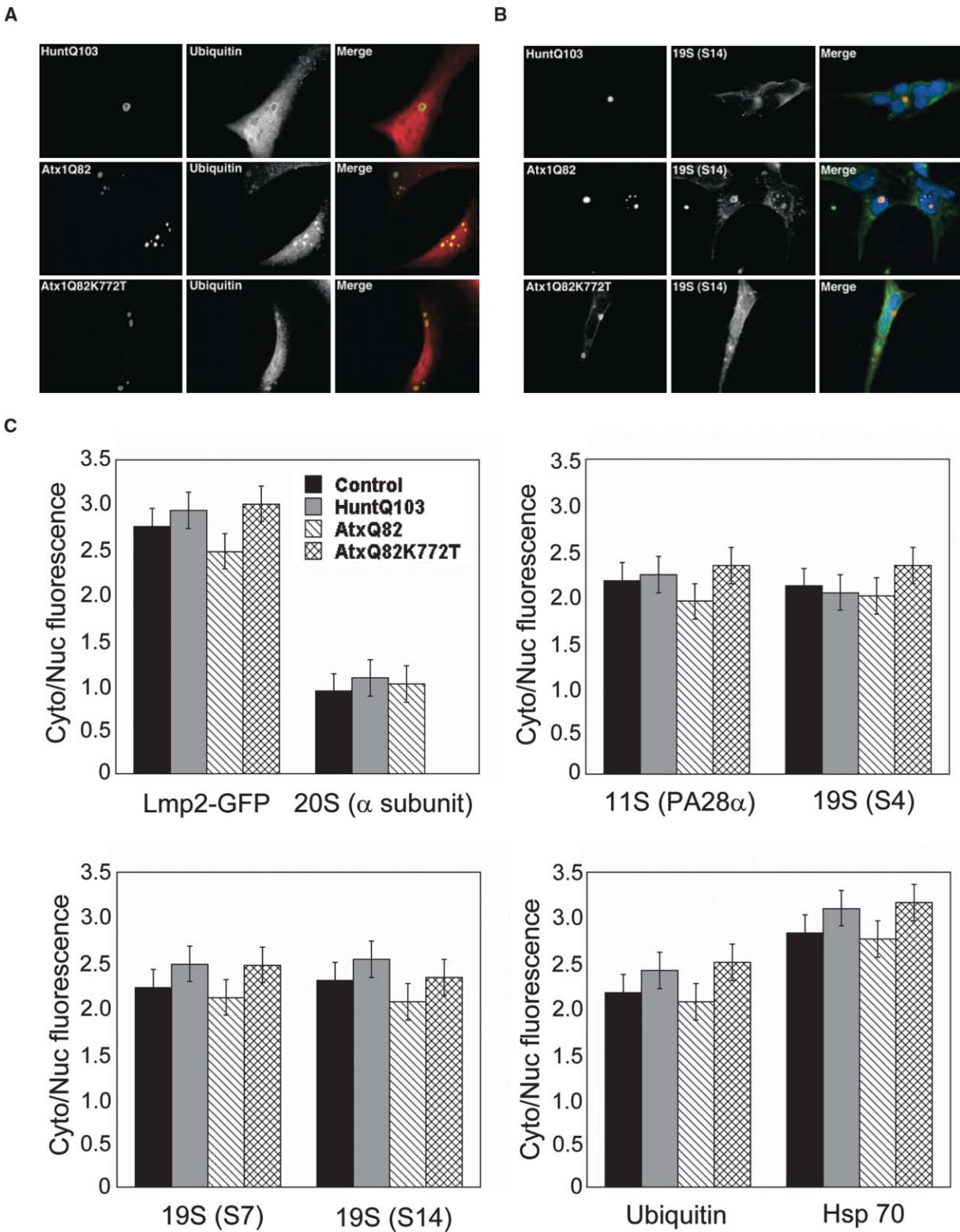


Figure 6. Inclusion Body Formation Does Not Cause Significant Redistribution of Key UPS Components

Representative immunofluorescence images of cells expressing the indicated aggregation-prone polyglutamine constructs. Cells were stained with antibodies to the respective epitope tags (HttQ103, Atx1Q82, and Atx1Q82K772T) and with antibodies against ubiquitin ([A], red) and 19S subunit S14 ([B], green).

(C) Quantification of cytoplasmic:nuclear immunofluorescence intensity ratios for the indicated antigens in untransfected cells (black bar), HttQ103-expressing cells (gray bar), Atx1Q82-expressing cells (hatched bar), or Atx1Q82K772T-expressing cells (double-hatched bar). (n = 40–50 cells).

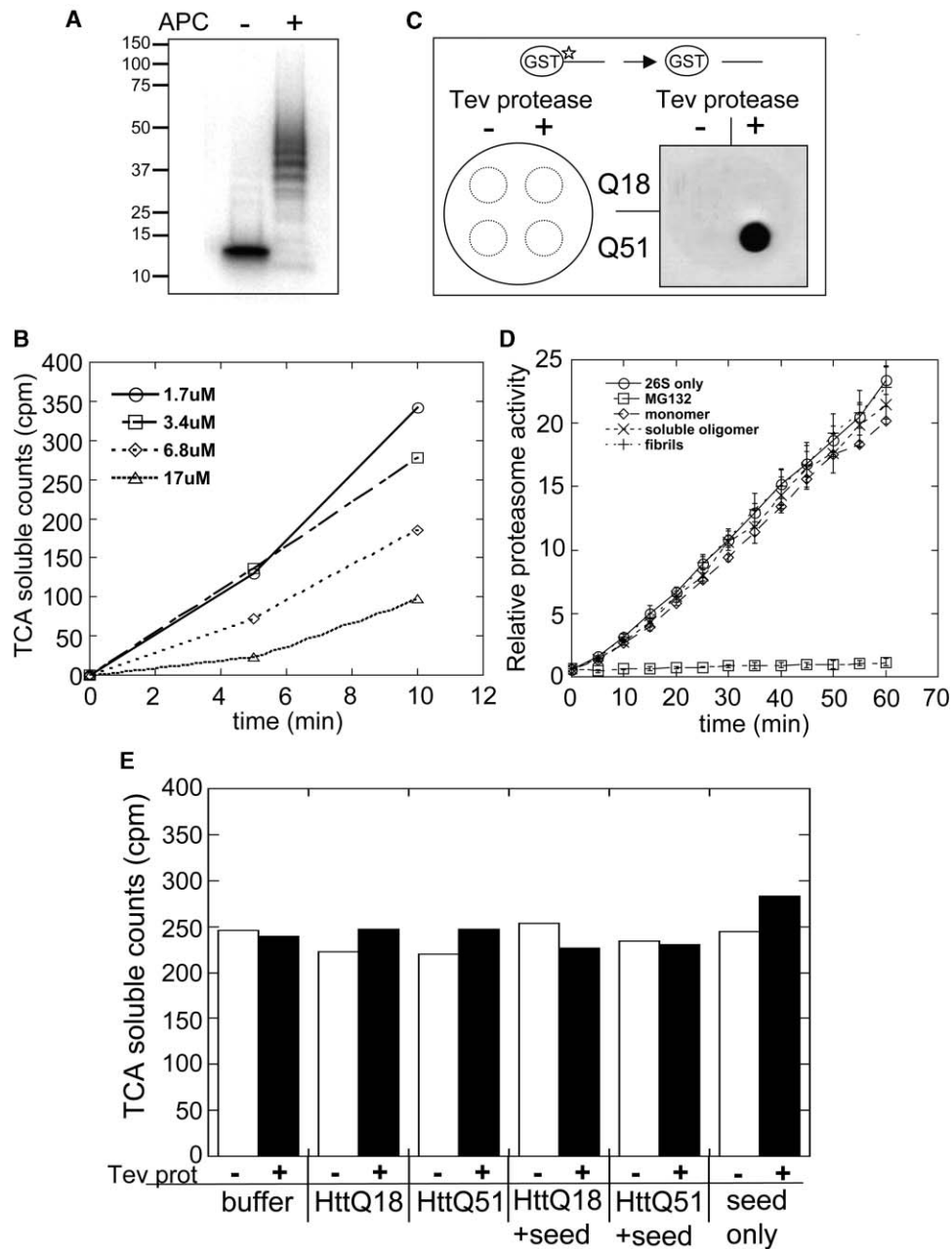


Figure 7. Protein Aggregates Do Not Impair Proteasome Function In Vitro

(A) Purified cyclin B N100 fragment containing the destruction box was iodinated and added to buffer (-) or buffer containing purified E1, UbcH10, ATP, ubiquitin, and anaphase promoting complex (APC) activated with recombinant cdc20 (+).

(B) 125 I-Ub_n-cyclin N100 was added at the indicated concentrations to purified rat liver 26S proteasomes, and degradation was monitored over time as trichloroacetic acid (TCA) soluble radioactivity.

(C) GST-fused huntingtinQ18 or Q51 was purified with glutathione agarose and treated with buffer (-) or with buffer containing TEV protease (+). The cleavage reaction mixture was vacuum filtered through a 0.2 μ m cellulose acetate membrane. Trapped material was visualized by incubation with S protein-coupled horseradish peroxidase.

(D) 10 nM purified rat liver 26S proteasomes were either untreated or treated with 2 μ M MG132, 1 μ M polyglutamine spherical, or fibrillar peptide aggregates, and the "chymotrypsin-like" peptidase activity was determined by using the fluorogenic substrate Suc-LLVY-AMC.

(E) Purified GST-fused huntingtinQ18 or Q51 was either untreated or treated with TEV protease overnight to generate aggregates. The cleavage reaction was also done in the presence of a seed of preformed polyglutamine peptide aggregates. These reaction mixtures were added to 25 nM proteasomes and 125 I-Ub_n-cyclin N100. Degradation of cyclinN100 was measured as TCA soluble 125 I radioactivity and compared to a degradation mixture with buffer alone.

within IBs whereas only a minor fraction of proteasomes are recruited to the IBs, thus it is highly unlikely that the majority of proteasomes are tightly bound to aggregated

protein. Moreover, the observed severe impairment of UPS function in *trans* compartments from which both the aggregates and the aggregation-prone monomers

are excluded (by the presence or absence of an NLS) argues strongly against a requirement for direct physical interaction between proteasomes and aggregates for UPS impairment. Our data also show that protein aggregates *in vitro* do not inhibit 26S proteasome-mediated degradation of ubiquitin-dependent and ubiquitin-independent substrates *in vitro*, even when present in vast molar excess.

These data do not exclude the possibility that ubiquitylated aggregates might interact more tightly with proteasomes. Recent studies suggest that even short, non-pathogenic (Q19) polyglutamine tracts are inefficiently degraded (Holmberg et al., 2004) or completely indigestible (Venkatraman et al., 2004) by proteasomes *in vitro* and *in vivo*, despite the presence of endogenous ubiquitylation. Indeed, FRAP and FRET studies indicate that proteasomes interact tightly with polyQ proteins in IB, where they are effectively immobile (Holmberg et al., 2004). Thus, although IB-associated proteasomes may be inactivated by tightly bound indigestible ubiquitylated aggregates, because the majority of proteasomes in cells containing highly aggregated proteins are excluded from IB, it is unlikely that such direct interactions can account for the global impairment of proteasomes observed in the present work. Taken together with our finding that IB formation is not required for UPS impairment, it is unlikely that a direct proteasome choking or proteasome sequestration model can account for our observation of global UPS impairment.

If protein aggregates do not interfere with UPS function by sequestering or directly clogging proteasomes, it is possible that they could impair UPS function by influencing the activity or distribution of UPS modulators. For example, formation of highly ubiquitylated aggregate complexes could, in principle, deplete intracellular levels of free ubiquitin. However, measurement of free ubiquitin levels in cells with large aggregate burdens does not support this hypothesis (Bence et al., 2001). Likewise, overexpression of ubiquitin together with aggregation prone proteins does not enhance clearance (data not shown), suggesting that ubiquitin levels are not limiting.

Proteins containing expanded polyglutamine have been shown to interact with and inactivate polyglutamine-containing transcription factors (Kazantsev et al., 1999; Nucifora et al., 2001); recent studies have suggested that this interaction can occur with an early, oligomeric form of Htt and may depend more on the polyglutamine conformation than on its aggregation state (Schaffar et al., 2004). Also, studies from other labs have demonstrated that smaller intermediate forms of protein aggregates are more toxic to cells than fibrillar forms (Bucciantini et al., 2002; Kaye et al., 2003). Consistent with this, it has been argued that the formation of higher order aggregates and their subsequent coalescence into IBs may be cytoprotective (Taylor et al., 2003; Arrasate et al., 2004). Our finding of UPS impairment in the absence of IBs suggests that intermediate forms of protein aggregates may be able to inhibit UPS function. Finally, it should be noted that nonnative pathogenic conformers of polyglutamine proteins could interact with and activate UPS inhibitors. For example, the presence of nonnative-undegraded protein aggregates could initiate caspase activation; one recent study reported that caspase acti-

vation results in irreversible inhibition of proteasomes via cleavage of 19S regulatory particle subunits S5a, S6', and S1 (Sun et al., 2004). However, immunoblot analysis of extracts from cells expressing high levels of GFP-HttQ150 revealed no evidence of cleavage of S5a, S6', S2, or S1 proteasome subunits (data not shown). Moreover, treatment of NES and NLSGFP^u lines transfected with Atx1Q82 or HttQ103 with the broad caspase inhibitor Z-VAD-FMK had no effect on the aggregate-associated increase in NES or NLSGFP^u fluorescence (data not shown).

Are Inclusion Bodies Pathogenic?

The observation that neurofibrillary tangles in Alzheimer's disease are enriched in ubiquitin conjugates suggested a linkage between protein aggregation and UPS dysfunction more than 20 years ago (Koo et al., 1999). Nonetheless, the mechanism by which IB formation is linked to cellular dysfunction has remained an unresolved mystery. IBs containing ubiquitylated proteins are a nearly invariant feature of neurodegenerative disease in autopsy material. However, close examination of the time course of aggregate formation and cell death in an *in vitro* model of Htt-induced cytotoxicity (Arrasate et al., 2004) and of pathogenesis in animal models (Johnston et al., 2000) suggest that functional deficits associated with expression of pathogenic, aggregation-prone proteins can be observed significantly prior to the point where IBs can be observed. These observations suggest that IB formation is not requisite for aggregate-associated toxicity and may in fact contribute to cytoprotection (Arrasate et al., 2004; Kopito, 2000). Our data suggest that, although IBs are a measure of total aggregate burden in cells expressing aggregation-prone proteins, detectable UPS impairment can be observed in the absence of any detectable IBs. Thus, we conclude that global UPS impairment is an early response to the presence of protein aggregates and not a consequence of IB formation. These findings suggest that the search for potential therapeutics that target cellular aggregation pathways will need to focus on blocking early events in aggregate formation while not affecting—or even possibly enhancing—IB formation.

Experimental Procedures

Antibodies

Monoclonal c-Myc antibody was obtained from Oncogene, M2 FLAG monoclonal antibody from Sigma, polyclonal ubiquitin antibody UG-9510 from Affiniti, polyclonal Hsp70 (Spa812) from Stressgen, monoclonal GFP antibody from Roche, and monoclonal 20S α -subunit antibody 21D11 from Mount Sinai Medical Center Hybridoma Core Facility. Polyclonal antibodies against S7, S14, S4, and REG α were kind gifts from Martin Rechsteiner and Carlos Gorbea (University of Utah).

Plasmids

See Supplemental Data for construction of NESGFP^u and NLSGFP^u. Huntingtin exon 1 Myc-his plasmid was described previously (Bence et al., 2001). The NLS huntingtin exon 1 plasmid was created by inserting the SV40 nuclear localization sequence into the KpnI site in the N terminus of Htt exon 1. Full-length ataxin-1Q30, ataxin-1Q82, and ataxin-1Q82K772T plasmids were generous gifts from Harry Orr (University of Minnesota). Wt rhodopsin and the P23H mutant were described previously (Illing et al., 2002). Ornithine decarboxylase-expressing plasmid was received from Phil Coffino

(University of California, San Francisco), and FLAG-tagged L158P VHL plasmid was a gift from Judith Frydman (Stanford University). CyclinB N100 fragment (Chen and Fang, 2001) was obtained from Guowei Fang (Stanford University). The original GST-huntingtin fusion constructs were obtained from Erich Wanker (Max-Delbrück-Centrum, Germany). These constructs were modified by the addition of a TEV protease cleavage site between the GST and huntingtin exon 1 coding regions and the addition of an S tag C-terminal to the huntingtin exon 1 coding region.

Cell Lines

HEK293 cells were selected for stable low-level expression of NESGFP^u and NLSGFP^u by G418 resistance. Clonal selection was performed by limiting dilution as described (Bence et al., 2001). Cells were grown in DMEM (Gibco) with 10% FBS and antibiotics. The HT1080 fibrosarcoma cells stably expressing LMP2-GFP were a gift from John Trowsdale (University of Cambridge).

Microscopy

Cells were removed from the plate with trypsin and fixed in solution with 4% paraformaldehyde. Cells were permeabilized in 0.2% Triton X-100 and then incubated with primary antibody against the aggregation substrate in 0.1% bovine serum albumin. DNA was stained by 10 μ g/mL bisbenzimidazole. Anti-mouse Alexa 594 (1:5000) was used as the secondary antibody to visualize the transfected plasmid expression. GFP^u fluorescence quantification in transfected cells was performed as described previously (Bence et al., 2001). The nuclear and cytoplasmic distribution of the various UPS components was measured by delineating the cellular and nuclear boundaries and measuring the total fluorescent intensities with Metamorph software (Universal Imaging). The values were background corrected and plotted as a ratio of cytoplasmic fluorescence to nuclear fluorescence.

Proteasome Activity Assay

Cells were untreated or treated with lactacystin (Calbiochem) for 5 hr. Cells were harvested and lysed under hypotonic conditions with a dounce homogenizer by using pestle A in buffer containing 10 mM Tris (pH8.0), 1 mM EDTA, 20% glycerol, and 2 mM ATP. Cell lysate was mixed 1:1 with 200 μ M Suc-LLVY-AMC (Calbiochem) in a 96-well clear bottom/black side plate in triplicate. Fluorescence intensity was measured by excitation at 360 nm and emission at 440 nm in a SpectraMax GeminiEM fluorescence plate reader (Molecular Devices). The rate of Suc-LLVY-AMC hydrolysis in cells treated with 5 μ M lactacystin for 5 hr was set as 100% proteasome inhibition.

Cyclin Ubiquitylation

Xenopus cyclin B (N-terminal 100 amino acids) was radioiodinated by using the chloramine T method. Ubiquitylation reactions were performed with anti-cdc27 immunopurified anaphase promoting complex (APC) from *Xenopus* interphase egg extracts. Immunopurified APC was activated by the addition of Sf9/baculovirus purified his-tagged Cdh1. Activated APC/Cdh1 was washed to remove unbound Cdh1, and the activated APC/Cdh1 was combined with ATP, Ub (Sigma), Ubc10 (6xhis recombinant), E1 (recombinant), and Ubiquitin aldehyde (Calbiochem) to generate ubiquitylated cyclin N100.

Cyclin Degradation Assay

15 μ g of GST Q18/51S incubated in 50 μ l final volume TEV protease buffer (1 mM DTT, 10mM Tris [pH 8.0], 150 mM NaCl, and 0.5 mM EDTA) with 50 μ g TEV protease. Cleavage reactions were incubated overnight at 37°C. Cleavage reactions were diluted 1:4 into degradation buffer (25 mM Tris [pH 7.5] and 2 mM MgATP 2 mM MgCl₂) with 25 nM 26S proteasomes (Calbiochem). Cleavage reactions and proteasomes were incubated together for 30 min at 37°C. 1000 cpm of Ub_(N)-Cyclin N100 was added and incubated at 37°C for 30 min. Proteins were precipitated with 5% trichloroacetic acid (TCA) and TCA soluble radioactivity was measured in a gamma counter over 1 min integration time.

Polyglutamine Peptide Aggregation

Small polyglutamine peptides were synthesized (KK-biotinQ₄₄-KK) and solubilized as described previously (Chen and Wetzel, 2001).

Spherical aggregates were prepared essentially as described by Poirier et al. (2002). Briefly, hexafluoroisopropanol processed KK-biotinQ₄₄-KK was dissolved in 8 mM phosphate buffer, 2.2 mM KCl, 110 mM NaCl, and (pH 7.4) and incubated for 4 hr at 20°C in the presence of 20% glycerol.

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