Aggregate formation inhibits proteasomal degradation of polyglutamine proteins

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Insoluble protein aggregates are consistently found in neurodegenerative disorders caused by expanded polyglutamine [poly(Q)] repeats. The aggregates contain various components of the ubiquitin/proteasome system, suggesting an attempt of the cell to clear the aberrant substrate. To investigate the effect of expanded poly(Q) repeats on ubiquitin/proteasome-dependent proteolysis, we targeted these proteins for proteasomal degradation by the introduction of an N-end rule degradation signal. While soluble poly(Q) proteins were degraded, they resisted proteasomal degradation once present in the aggregates. Stabilization was also observed for proteins that are co-aggregated via interaction with the expanded poly(Q) domain. Introduction of a degradation signal in ataxin-1/Q92 reduced the incidence of nuclear inclusions and the cellular toxicity, conceivably by accelerating the clearance of the soluble substrate.

INTRODUCTION

Expansion of polyglutamine [poly(Q)] repeats causes a group of inheritable disorders that are characterized by progressive neuronal loss in specific regions of the central nervous system causing cognitive decline and uncontrolled locomotion (1). These presently incurable and often-lethal diseases comprise Huntington’s disease, Kennedy’s disease and spinocerebellar ataxias (SCA) types 1–3, 6 and 7. Animal models revealed that these diseases are mainly caused by a toxic gain of function, attributable to the expanded poly(Q) repeats (2,3). Despite intensive research, the precise molecular mechanism by which the poly(Q) repeats implement their devastating effect on neurons remains elusive (4).

The expanded poly(Q) repeats can form stable β-sheets that mediate intermolecular contacts between repeats (5,6). Consequently, cells expressing such proteins often contain insoluble cytosolic or nuclear aggregates that are predominantly composed of tightly packed poly(Q)-containing proteins. The inclusions also contain various molecular chaperones and components of the ubiquitin/proteasome system (7,8), which are dedicated to refolding or proteolytic destruction of misfolded proteins (9,10).

Although protein aggregates are generally found in the affected neurons of many neurodegenerative disorders, it is still disputed whether they are a major determinant of cellular pathology or whether they counteract toxicity by sequestering the aberrant proteins (11–13). Recent studies providing evidence for a ‘one-hit model’ in neurodegeneration (14) have inspired Perutz and Windle (15) to postulate that the nucleation of misfolded proteins into aggregates is the single biochemical event that initiates neurodegeneration. Indeed, protein aggregates display an inherent cellular toxicity (16), which may be due to the sequestration of essential components of the ubiquitin/proteasome machinery into aggregates (7). Redistribution of the ubiquitin/proteasome system may promote the accumulation of pro-apoptotic substrates of the proteasome (17,18). In support of the alternative scenario, it should be mentioned that the aggregates found in neurodegenerative disorders share many similarities with the aggresome (19–21), a perinuclear proteolytic center that is induced by overexpression of hydrophobic misfolded proteins or by treatment with proteasome inhibitors (22,23). Hence, the formation of aggregates could be an innate protective response of cells in conditions where the amount of misfolded proteins exceeds the proteolytic capacity (4).

Independently of the pathophysiological significance of the aggregates, the question remains why the aggregation-prone poly(Q) proteins are not efficiently eliminated by proteasomal degradation before they form the insoluble protein deposits. A glycine–alanine repeat (GAr), present in the Epstein–Barr virus nuclear antigen 1, inhibits proteasomal degradation by blocking events downstream of ubiquitination (24,25). It has been proposed that, similar to the viral repeat, the poly(Q) repeat may interfere with proteasomal degradation in a length-dependent manner (26). Indeed, proteasomal degradation of mutant ataxin-1, which is responsible for SCA1, was substantially delayed in vitro by the presence of a pathologic poly(Q) repeat although ubiquitination was not affected (26). More recently, a similar inhibitory effect was reported for the expanded poly(Q) repeat in the N-terminal fragment of huntingtin (18).

We have investigated the susceptibility of poly(Q) proteins to proteasomal degradation by introducing poly(Q) repeats of various lengths into designed proteasomal substrates or by introducing a strong degradation signal in mutant ataxin-1.
We show that soluble poly(Q) proteins that contain a strong degradation signal are efficiently degraded independently of the length of the repeat. However, once captured in the aggregates, both poly(Q) proteins and co-aggregating proteins are stable despite the presence of strong degradation signals. Thus, the formation of aggregates renders the toxic proteins resistant to proteasomal degradation and initiates the accumulation of poly(Q) proteins and poly(Q)-interacting proteins.

RESULTS

Efficient proteasomal degradation of poly(Q) proteins provided with a strong degradation signal

To evaluate the effect of poly(Q) repeats on proteasomal degradation, a poly(Q) repeat of 16 amino acids or pathologic expanded repeats of 65 or 112 residues were linked to the C terminus of the rapidly degraded N-end rule proteasomal substrate Ub–R–green fluorescent protein (GFP) or the control Ub–M–GFP, which lacks such degradation signal (27) (Fig. 1A). The ubiquitin moiety of these Ub–X–GFP fusions is cleaved by endogenous ubiquitin C-terminal hydrolases upon which the variable X amino acid is exposed as the new N terminus of the GFP moiety. Processed X–GFP that contains an N-terminal arginine residue is subject to rapid ubiquitination and proteasomal degradation, as predicted according to the N-end rule (28), while X–GFP with an N-terminal methionine is not recognized by the N-end rule pathway. A Ub–R–GFP–GAr chimera containing a 239-amino-acid long–GAr sequence, which was previously shown to protect Ub–R–GFP from proteasomal degradation (29), was used as a control.

In a first set of experiments, HeLa cells were transiently transfected with the Ub–M–GFP and Ub–R–GFP chimeras, with or without the addition of the proteasome inhibitors lactacystin or carboxybenzyl-leucyl-leucyl-leucine vinyl sulfonyl (Z-L3-VS) (30) (Fig. 1B and data not shown). High expression levels were obtained with Ub–M–GFP, which were not enhanced by administration of inhibitors, while the Ub–R–GFP gave very low steady-state expression levels and strong accumulation in the presence of inhibitors, confirming that this reporter is indeed targeted for proteasomal degradation (Fig. 1B). Introduction of the GAr abrogated proteasomal degradation of the Ub–R–GFP reporter resulting in expression levels that were comparable to Ub–M–GFP. In contrast, insertion of the short or expanded poly(Q) repeats gave expression levels very similar to those observed with unmodified Ub–R–GFP, demonstrating that the poly(Q) repeat fusions are still subject to proteasomal degradation. To quantify the effect of the repeats, flow cytometric analysis was performed of cells transfected with the different fusions, and the ratio of the percentages of fluorescent cells in the absence and presence of Z-L3-VS was determined. This demonstrated that the GAr stabilized the reporter while insertion of the poly(Q) repeats resulted in a minor increase in the stability of the Ub–R–GFP (Fig. 1C and D). Notably, the minor but significant effect observed with the poly(Q) repeats was independent of the length of the repeat. Taken together, these data show that proteins containing poly(Q) repeats can be efficiently degraded by the proteasome once provided with a functional degradation signal.

Aggregated poly(Q) proteins resist proteasomal degradation

We then asked whether poly(Q) proteins containing an N-end rule degradation signal differ in their ability to form aggregates as compared with their Ub–M–GFP counterparts. Expression of a GFP reporter with a poly(Q) stretch of 16 residues gave a homogenous fluorescence staining (data not shown). However, a substantial fraction of the cells transfected with Ub–M–GFP–Q112 and Ub–R–GFP–Q112 developed strongly fluorescent aggregates in the cytosol and nucleus (Fig. 2A). The GFP fluorescence appeared in these cells as multiple small dots (Fig. 2A: left panel) or single bright fluorescent aggregates (Fig. 2A: right panel). There were no apparent differences in the morphology or intracellular localization of aggregates induced by Ub–M–GFP–Q112 or Ub–R–GFP–Q112.

To investigate the stability of the aggregates, we monitored the number of GFP fluorescent cells, distinguishing between cells with homogenous and punctuate fluorescence. In line with the anticipated rapid turnover of the Ub–R–GFP reporter, transfection with Ub–R–GFP–Q16 yielded a much lower number of cells with detectable GFP fluorescence as compared with Ub–M–GFP–Q16. The expression of these reporters reached a maximum after 2–3 days of transfection, and then gradually declined to background levels. This kinetics of expression was observed for cells that displayed a homogenous staining with the Ub–M–GFP–Q112 and Ub–R–GFP–Q112 constructs. In contrast, after an initial increase during the first 2 days, the number of cells with GFP aggregates remained constant for up to 9 days post transfection when the experiment was terminated (Fig. 2B). Viability assays showed that a large fraction of the aggregate-bearing cells were dead 6 days post transfection, while the fluorescent aggregates remained (see below). Importantly, there was no difference in the stability of aggregates composed of the Ub–M–GFP–Q112 or the N-end rule substrate Ub–R–GFP–Q112.

Stabilization of co-aggregated proteins

Natural proteins bearing poly(Q) repeats of a non-pathologic length co-aggregate with expanded poly(Q) proteins (31), a process that can be mimicked experimentally with GFP variants carrying short poly(Q) repeats (21). We used this characteristic to test whether the inhibitory effect of the poly(Q) repeats depends on their presence in the aggregates.

In order to investigate whether our artificial reporters co-aggregate in the nuclear inclusions (NIs) formed by a natural poly(Q) protein, HeLa cells were co-transfected with the mutant ataxin-1/Q92, together with Ub–M–GFP, Ub–M–GFP–Q16 or Ub–M–GFP–Q112. Reporters containing short or long poly(Q) repeats co-localized in the NIs, while the poly(Q)-less reporter was not found in the inclusions (Fig. 3A). The GFP reporter containing a Q112 repeat also formed large cytosolic aggregates devoid of ataxin-1/Q92 (Fig. 3A).

Next, we analyzed whether the localization of the Q16-containing proteins in the NIs affected their proteasomal degradation. GFP fluorescence was measured in cells transfected with ataxin-1/Q92 together with Ub–R–GFP or Ub–R–GFP–Q16. GFP-containing NIs were observed in cells co-transfected with ataxin-1 and Ub–R–GFP–Q16 after 2 days,
while the NIs of Ub–R–GFP-transfected cells did not emit detectable GFP fluorescence (Fig. 3B). Counting of the cells containing fluorescent NIs 4 days post transfection revealed that the presence of the short poly(Q) repeat stabilized the Ub–R–GFP reporter, resulting in a ∼10-fold increase in the number of GFP-fluorescent cells (Fig. 3C). Similar data were found with Ub^{G76V}-GFP (Fig. 3B and C), which, like Ub–R–GFP, is rapidly degraded by the proteasome but is recognized as a ubiquitin fusion degradation substrate (27). Importantly, the observation that two repeat-less reporters were recognized as a ubiquitin fusion degradation substrate (27).

Accelerated proteasomal degradation reduces aggregate formation

In order to allow accurate estimation of the incidence of aggregate formation, we modified the GFP reporters by tagging the ubiquitin moiety with an eight-amino-acid FLAG epitope, resulting in FLAGUb–X–GFP (Fig. 4A). Upon cleavage of the reporter, the FLAGUb moiety is stable, allowing identification of all transfected cells regardless of their levels of the GFP reporter. We shall refer to this method, which is a modification of the ubiquitin/protein/reference technique developed by Varshavsky and co-workers (32), as the ubiquitin/fluorescence/reference (UFR) technique.

Staining of HeLa cells transfected with FLAGUb–X–GFP constructs demonstrated that all the cells transfected with the stable FLAGUb–M–GFP that expressed the FLAG tag also emitted GFP fluorescence, while many of the cells transfected with destabilized FLAGUb–R–GFP were specifically stained with the anti-FLAG antibody but had undetectable levels of GFP (not shown). Quantitative analysis with the UFR technique confirmed that there were more GFP-positive cells in the FLAGUb–M–GFP–Q112-transfected cells compared with the FLAGUb–R–GFP–Q112-transfected cells (Fig. 4B). More importantly, a 4-fold lower incidence of cells with aggregates was observed in cells transfected with FLAGUb–R–GFP–Q112 compared with its counterpart lacking the N-end rule degradation signal (Fig. 4C and D). We conclude that introduction of a degradation signal into the GFP–Q112 protein reduced the incidence of aggregate formation.

Figure 1. Efficient proteasomal degradation of poly(Q) proteins provided with a strong degradation signal. (A) Schematic illustration of the Ub–X–GFP reporter constructs carrying repeats of 16, 65 or 112 Q in the C terminus. (B) Western blot analysis probed with an anti-GFP antibody, showing HeLa cells transiently transfected with Ub–M–GFP, Ub–R–GFP, Ub–R–GFP–Q16, Ub–R–GFP–Q65, Ub–R–GFP–Q112 and Ub–R–GFP–GA239. Two days post transfection, the cells were left untreated or treated with 10 μM lactacystin. It should be noted that the poly(Q) fusions migrate more slowly in the denaturating gel than anticipated from their size in amino acids owing to their repeat composition. One representative experiment out of three is shown. (C) Flow cytometric analysis of cells transiently expressing Ub–R–GFP–Q16, Ub–R–GFP–Q112 and Ub–R–GFP–GA239. Cells were treated as in (B) but with 10 μM Z-L3-VS instead of lactacystin. The percentages of GFP-positive cells are indicated in the upper right quadrants. (D) The ratios of the percentages of fluorescent cells in untreated cells versus cells treated with 10 μM Z-L3-VS are plotted. A ratio of 1 represents a stable protein, whereas a lower value indicates proteasomal degradation of the protein. Values shown are mean ± SD from three independent experiments. Significantly different values as compared to Ub–R–GFP are indicated with an asterisk (Student’s t-test, P < 0.05) or two asterisks (Student’s t-test P < 0.01).
Figure 2. Expanded poly(Q)-containing proteins are protected from degradation through the formation of aggregates. (A) Representative micrographs of HeLa cells transiently transfected with Ub–M–GFP–Q112 (upper panels) and Ub–R–GFP–Q112 (lower panels). The micrographs are overlays of the same field of cells where aggregates are green (GFP) and nuclei are blue (Hoechst 33258 staining). The micrographs show the cells with several small aggregates (left panel) and a single large aggregate (right panel) that were observed with the polyQ constructs. (B) HeLa cells were transiently transfected with Ub–M–GFP–Q16, Ub–R–GFP–Q16, Ub–M–GFP–Q112 or Ub–R–GFP–Q112, and the numbers of GFP-positive cells with punctuate and homogenous GFP staining were scored at the indicated time points.
Proteins with short poly(Q) repeats are co-stabilized. (A) HeLa cells were co-transfected with equimolar ratio of an ataxin-1/Q92-encoding plasmid together with plasmids encoding Ub–M–GFP, Ub–M–GFP–Q16 or Ub–M–GFP–Q112. Cells were stained at 2 days post transfection with an anti-ataxin-1 antibody, and ataxin-1 NIs (upper panel) and GFP fluorescence (lower panel) were detected in the same cells. Ub–M–GFP–Q16 and Ub–M–GFP–Q112 co-localized in the ataxin-1 NIs (arrows), while Ub–M–GFP was not recruited in the NIs. Ub–M–GFP–Q112 also formed large cytosolic aggregates that were devoid of ataxin-1 (open arrow). (B) Experimental conditions were as in (A). Cells were transfected with an ataxin-1/Q92-encoding plasmid together with Ub–R–GFP, Ub–R–GFP–Q16, Ub^{G76V}–GFP and Ub^{G76V}–GFP–Q16. Four days post transfection, the NIs in ataxin-1/Q92+Ub–R–GFP–Q16 or Ub^{G76V}–GFP–Q16 are still GFP-fluorescent, while the NIs in cells co-transfected with their repeat-less counterparts are devoid of GFP fluorescence. (C) Quantification of the experiment shown in (B), but at 4 days post transfection. Cells containing ataxin-1/Q92 NIs were scored for the presence or absence of GFP fluorescence. Mean ± SD from three independent experiments. Significantly different values are indicated with asterisks (Student’s t-test, P < 0.01).
Figure 4. Accelerated proteasomal degradation reduces aggregate formation. (A) Schematic presentation of the FLAGUb–X–GFP–Q112 construct used for the ubiquitin/fluorescence/reference (UFR) technique. Upon expression of the construct, the ubiquitin is cleaved from the fusion by endogenous ubiquitin C-terminal hydrolases (indicated by scissors), and the FLAGUb can serve as an internal stable control protein to be compared with the stability of X–GFP–Q112. (B) Quantification of FLAG-positive cells expressing detectable levels of M–GFP–Q112 and R–GFP–Q112. (C) As in (B), but FLAG-positive cells were scored for the presence of GFP aggregates. (D) Representative low-magnification micrographs show expression of FLAGUb–M–GFP–Q112 and FLAGUb–R–GFP–Q112. FLAG staining (left panel), GFP fluorescence (middle panel) and Hoechst 33258 staining (right panel) are shown. These micrographs were taken with a short exposure, and show only the aggregates. Values shown in (B) and (C) are mean ± SD from three independent experiments. Significant differences are indicated with an asterisk (Student’s t-test, \( P < 0.05 \)).
Effect of degradation signal on the toxicity of poly(Q) proteins

Next, we asked whether, under our experimental conditions, cellular toxicity correlated with the presence of aggregates. Nuclear condensation and fragmentation, which are indicative of apoptosis, were evaluated in cells expressing FLAGUb–M–GFP–Q112 or FLAGUb–R–GFP–Q112. Transfected cells without GFP aggregates displayed normal nuclear morphology, while a large fraction of the cells with aggregates contained condensed or fragmented nuclei (Fig. 5A and B). This was observed for both FLAGUb–M–GFP–Q112 and FLAGUb–R–GFP–Q112.

We also studied the kinetics of cell death of FLAGUb–M–GFP–Q112– and FLAGUb–R–GFP–Q112-transfected cells as compared with FLAGUb–M–GFP–Q112 (Fig. 5D), which reflected the 4-fold lower incidence of aggregate formation with FLAGUb–R–GFP–Q112 (Fig. 4C). We conclude that the reduced formation of aggregates accomplished by the insertion of a degradation signal is accompanied by a decrease in the cellular toxicity of the poly(Q) proteins. Yet, the correlation between aggregate formation and cell death does not imply that the aggregates cause the toxic effect, since they may equally well be a consequence of a protective reaction in cells that express high concentrations of the toxic poly(Q) proteins.

Destabilizing ataxin-1

The GFP reporters described in this work are in many respects different from the natural proteins associated with poly(Q) disorders. For example, they harbour C-terminal repeats, while in natural poly(Q) proteins the repeats are often positioned close to the N terminus. Moreover, the GFP reporters cause aggregates in the cytosol and nucleus, while natural poly(Q) proteins mainly cause NIs. We therefore chose to test some of the key findings obtained with the GFP constructs with human mutant ataxin-1/Q92. To this end, we constructed FLAGUb–M–ataxin-1/Q92 and FLAGUb–R–ataxin-1/Q92 chimeras. Expression of these modified ataxin-1/Q92 constructs in HeLa cells resulted in the formation of the characteristic NIs (Fig. 6A). Immunostaining of NIs for ataxin-1 and a ubiquitin reporter did not induce proteasomal degradation. The functional assay revealed that this repeat forms a flexible domain (40). An intriguing hypothesis is that mobilization of the proteasomes against the poly(Q) proteins may be initiated in the process of aggregate formation. Indeed, it was recently proposed that aggregation and targeting for degradation of the androgen receptor with expanded poly(Q) repeat are linked events (35). Moreover, earlier work indicates that the misfolding of aggregation-prone proteins is a consequence of their aggregation (36). In the present study, we have approached this question using designed and natural poly(Q) proteins.

A major role of the ubiquitin/proteasome system is the destruction of misfolded and potentially hazardous proteins. Yet, insertion of an expanded poly(Q) repeat domain in the control Ub–M–GFP reporter did not induce proteasomal degradation, suggesting that just the presence of the toxic domain is not sufficient for recognition by the ubiquitin/proteasome system. This is in line with the observation that huntingtin proteins with normal and expanded poly(Q) repeats have very similar half-lives (34). The presence of proteasomes in NIs composed of poly(Q) proteins suggests, however, that the aberrant proteins are at some point targeted for degradation (7). An attractive hypothesis is that mobilization of the proteasomes against the poly(Q) proteins may be initiated in the process of aggregate formation. Indeed, it was recently proposed that aggregation and targeting for degradation of the androgen receptor with expanded poly(Q) repeat are linked events (35). Moreover, earlier work indicates that the misfolding of aggregation-prone proteins is a consequence of their aggregation (36). In the present study, we have shown that, once captured in the aggregates, the poly(Q) proteins become extremely stable, regardless of the presence of a strong N-end rule degradation signal. Hence, it seems that the poly(Q) repeat proteins are only recognized as misfolded substrates that are deemed for proteasomal degradation once they reach the aggregates, but paradoxically it is the aggregation event that renders the proteins resistant to proteasomal degradation.

Recent in vitro studies demonstrate that stable domains in designed substrates resist unfolding by the 19S subunit of the proteasome, which abrogates their degradation (37,38). Conceivably, while the isolated expanded poly(Q)-containing proteins may not form stable structures, aggregated poly(Q) proteins may challenge the unfolding attempts of the proteasome. It is noteworthy that the expanded poly(Q) repeat and the viral GAr appear to exploit different mechanisms for their inhibitory activity on proteasomal degradation. The functional and microscopic analysis of chimeras carrying the GAr suggests that they do not form large aggregates (25,29,39). Moreover, structural analysis of an IxB–GAr chimera revealed that this repeat forms a flexible domain (40). An intriguing possibility is that the viral repeat may adapt a tight conformation upon binding to a yet-unidentified partner; the
Figure 5. Effect of introduction of a degradation signal on the toxicity of poly(Q) proteins. (A) Representative micrographs of cells transfected with FLAGUb–R–GFP–Q112. Staining with anti-FLAG antibody was used to identify transfected cells (left panel) and analyzed for the presence of GFP aggregates (middle panel) and nuclei with a Hoechst staining (right panel). Note that the cells that contain aggregates have fragmented and condensed nuclei, but not the cell that does not contain an aggregate. (B) Quantification of the percentage of FLAGUb–M–GFP–Q112- and FLAGUb–R–GFP–Q112-transfected cells at 3 days post transfection without aggregates (FLAG-positive, no aggregate; gray bar) or with aggregates (FLAG-positive, aggregate; black bar) that displayed apoptotic nuclei. Values shown are mean±SD from three independent experiments. Significant differences from cells without aggregates are indicated with two asterisks (Student’s t-test, P<0.01). (C) Ub–M–GFP–Q112- and Ub–R–GFP–Q112-transfected cells were stained with LIVE/DEAD staining at the indicated time points. The percentage of cells with aggregates that were dead was determined by fluorescence microscopy. (D) Dot plots of flow cytometry analysis of cells transfected with Ub–M–GFP–Q112 and Ub–R–GFP–Q112 and stained with the LIVE/DEAD dye at 3 days post transfection. The percentages of GFP-positive dead cells are indicated in the upper right quadrants.
stable complex may be capable of frustrating the unfolding attempt of the proteasome.

We have observed that the presence of poly(Q) aggregates correlates with enhanced apoptosis. This distinct correlation support the view that the aggregates are the major cause of cellular toxicity. However, this is not conclusive evidence, since the presence of aggregates in the dying cells may also reflect an attempt to restrict toxicity in cells that are exposed to high levels of the soluble toxic protein. Notably, Watase and co-workers (41) presented data suggesting that the soluble mutant ataxin-1 is the main toxic determinant in an animal model of SCA1.

Several proteins harbouring short poly(Q) repeats or glutamine-rich domains are captured in the NIs formed by the expanded repeat. Among these are important regulators such as the CREB-binding protein (31) and the TATA-binding protein (42), which have been proposed as mediators of the neuronal toxicity observed in aggregate-containing cells (43). The present study predicts that the co-aggregated proteins are also stabilized as a consequence of their redistribution. The extent to which the anticipated rise in steady-state levels may contribute to the cellular pathology remains to be resolved.

The process of poly(Q) aggregate formation shares many similarities to the orchestrated sequence of events that lead to the formation of aggresomes in cells that overexpress hydrophobic proteasome substrates (20,21). However, it is unclear whether the formation of aggregates contributes to the clearance or inactivation of the toxic proteins. While our data

Figure 6. Targeting ataxin-1/Q92 for proteasomal degradation. (A) Representative micrographs of aggregate formation in cells expressing FLAGUb–M–ataxin-1/Q92 and FLAGUb–R–ataxin-1/Q92 2 days post transfection. Ataxin-1 (red) and HC3 proteasome subunit immunostaining (green), Hoechst 33258 staining (blue) and overlay are shown. (B) Three days post transfection, cells were immunostained for FLAG, to identify transfected cells by FLAGUb and ataxin-1/Q92. FLAG-positive cells were scored for detectable ataxin-1 staining. (C) As in (B), but FLAG-positive cells were scored for the presence of ataxin-1-positive NIs. Values shown in (C) and (D) are mean±SD from three independent experiments. Significant differences are indicated with an asterisk (Student’s t-test P < 0.05).
do not exclude the possibility that aggregate formation may be initiated or accelerated by the same mechanism that directs the generation of aggresomes, they strongly suggest that the formation of these aggresome-like structures do not accomplish destruction of the poly(Q) protein but rather induces further accumulation of the toxic poly(Q) and co-aggregated proteins.

The co-localization of proteasomes within the aggregates in the absence of degradation supports the idea that the redistribution of the ubiquitin/proteasome system may indirectly hinder the proteolysis of other substrates (7,17,18). However, the proteolytic capacity of the ubiquitin/proteasome system largely exceeds the level required for household activities, since as much as 80% of the enzymatic activity of the proteasome can be blocked without affecting cell viability (17,27). Interestingly, we observed that aggregate formation did not cause accumulation of the proteasome substrates Ub–R–GFP or UbG76V–GFP. Different results were reported in a recent study using a similar GFP-based proteasome substrate carrying the CL1 degradation signal, from which it was concluded that aggregates cause a general impairment of the ubiquitin/proteasome system (17). The discrepancy may be explained by experimental procedure, since we transiently expressed the GFP substrates, while Bence and co-workers (17) used a stably expressing cell line. However, studies performed with a stable transfecant carrying the UbG76V–GFP reporter did not reveal accumulation of the GFP substrate in aggregate-containing cells (L.G.G.C. Verhoef, K. Lindsten and N.P. Dantuma, unpublished observations). While a cell-type-specific effect cannot be excluded, a provocative explanation may be that the aggregates block the degradation of some but not all substrates. Further studies are required to resolve this important issue.

In contrast to the aggregates, soluble poly(Q) proteins are efficiently destroyed by the proteasome once provided with a strong degradation signal. Importantly, introduction of a degradation signal dramatically reduced the incidence of cells with aggregates or NIs, as well as the toxicity of poly(Q) protein. Thus, the induction of accelerated degradation could be an interesting therapeutic approach for neurodegenerative disorders associated with poly(Q) proteins. Recently, strategies have been developed to redirect the cellular ubiquitination machinery to target proteins of interest. One method is based on the expression of a chimeric ubiquitin ligase adaptor harboring binding motifs for the target proteins (44), while the second approach utilizes small peptides that form a trimeric complex with the target protein and the ubiquitin ligase (45). We believe that, even though the nature of these compounds will demand state-of-the-art gene therapeutic approaches, the lack of treatments and the fact that genetic tests can identify individuals at risk long before the onset of the disease justify further attempts to develop similarly designed ubiquitin ligase adaptors for poly(Q) proteins.

MATERIALS AND METHODS

Construction of plasmids

All open reading frames were expressed from a cytomegalovirus (CMV) promoter in the mammalian expression vectors pBK–CMV (Stratagene, La Jolla, CA) or EGFP–N1 (Clontech, Palo Alto, CA). The poly(Q)-encoding repeats were PCR-amplified from the androgen receptor containing repeats of 16, 65 and 112 CAG (a gift from Dr Christine Bailey, University of Pennsylvania School of Medicine, Philadelphia, PA) and cloned in frame with Ub–X–GFP (27), generating Ub–X–GFP–Q16, Ub–X–GFP–Q65 and Ub–X–GFP–Q112. The tagged GFP-reporter constructs were generated by cloning of a double-stranded DNA oligonucleotide encoding a FLAG epitope in the 5′ NheI and EcoRI restriction sites in the Ub–X–GFP constructs. The Ub–X–ataxin-1/92Q constructs were generated by cloning in the pBK–CMV backbone FLAGUb–X in frame with the ataxin-1/92Q, excised with HindIII from pXHC-92Q (a gift from Dr Huda Zoghbi, Baylor College of Medicine, Houston, TX).

Transfections and tissue culture

The human cervical epithelial carcinoma line HeLa was cultured in Iscove’s modified Eagle’s medium supplemented with 10% fetal calf serum (Life Technologies, Grand Island, NY), 10 U/ml penicillin and 10 μg/ml streptomycin. Cells were transiently transfected with Lipofectamine (Life Technologies) and analyzed at the indicated time points. For treatment with proteasome inhibitor, cells were incubated for 16 h with medium containing 10 μM lactacystin (Affiniti Research Products Ltd, Devon, UK) or Z-L1-VS (a gift from Dr Hidde Ploegh, Harvard Medical School, Boston, MA).

Western blot analysis

Total cell lysates were fractionated by 10% SDS–PAGE and transferred to Protran BA 85 nitrocellulose filters (Schleicher and Schuell). The filters were blocked in phosphate-buffered saline (PBS) supplemented with 5% skim milk and 0.1% Tween-20 and incubated with a rabbit polyclonal anti-GFP antibody (Molecular Probes Europe, Leiden, The Netherlands). After subsequent washings and incubation with peroxidase-conjugated goat anti-rabbit serum, the blots were developed by enhanced chemiluminiscence (Amersham-Pharmacia Biotech, Uppsala, Sweden).

Fluorescence microscopy and flow cytometry

For fluorescence microscopy, the cells were grown and transfected on coverslips. After rinsing in PBS and fixation in 4% paraformaldehyde, immunostaining was performed using a rabbit polyclonal anti-ataxin-1 antibody (a gift from Dr Huda Zoghbi), a mouse anti-FLAG monoclonal antibody (M5, Sigma, St Louis, MO) or a mouse monoclonal antibody directed against the HC3 α-subunits of the proteasome (Affiniti Research Products Ltd, Devon, UK). After subsequent washing steps, cells were incubated with the Alexa Fluor 594 or FITC-labelled secondary antibodies (Molecular Probes and DAKO). All antibodies were diluted in 50 mM Tris pH 7.4, 0.9% NaCl, 0.25% gelatine and 0.5% Triton-X100. Cells were routinely counterstained with Hoechst 33258 (Molecular Probes). Fluorescence was analyzed using a LEITZ-BMRB fluorescence microscope (Leica, Heidelberg, Germany) and images were captured with a cooled CCD camera (Hamamatsu, Osaka, Japan). Analysis of aggregate stability was performed by
counting per field the number of cells with homogenous and punctuate GFP staining at the indicated time points, using an inverted fluorescence microscope (DMIL, Leica). For identification of viable cells, cells were stained with an amine-reactive membrane-impermeable red fluorescent dye at the indicated time points (LIVE/DEAD Cell Viability Kit No. 3, Molecular Probes) and analyzed with flow cytometry and fluorescence microscopy. For quantitative analysis with the fluorescence microscope, 100–400 GFP-positive cells were scored for the presence of punctuate or homogenous staining and cell viability. Flow cytometry was performed with a FACSort flow cytometer (Beckton Dickinson, Mountain View, CA), and data were analyzed with CellQuest software.

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