Evidence for proteasome involvement in polyglutamine disease: localization to nuclear inclusions in SCA3/MJD and suppression of polyglutamine aggregation in vitro

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Spinocerebellar ataxia type 3, also known as Machado–Joseph disease (SCA3/MJD), is one of at least eight inherited neurodegenerative diseases caused by expansion of a polyglutamine tract in the disease protein. Here we present two lines of evidence implicating the ubiquitin–proteasome pathway in SCA3/MJD pathogenesis. First, studies of both human disease tissue and in vitro models showed redistribution of the 26S proteasome complex into polyglutamine aggregates. In neurons from SCA3/MJD brain, the proteasome localized to intranuclear inclusions containing the mutant protein, ataxin-3. In transfected cells, the proteasome redistributed into inclusions formed by three expanded polyglutamine proteins: a pathologic ataxin-3 fragment, full-length mutant ataxin-3 and an unrelated GFP–polyglutamine fusion protein. Inclusion formation by the full-length mutant ataxin-3 required nuclear localization of the protein and occurred within specific subnuclear structures recently implicated in the regulation of cell death, promyelocytic leukemia antigen oncogenic domains. In a second set of experiments, inhibitors of the proteasome caused a repeat length-dependent increase in aggregate formation, implying that the proteasome plays a direct role in suppressing polyglutamine aggregation in disease. These results support a central role for protein misfolding in the pathogenesis of SCA3/MJD and suggest that modulating proteasome activity is a potential approach to altering the progression of this and other polyglutamine diseases.

INTRODUCTION

Polyglutamine expansion is now recognized to be a major cause of inherited neurodegenerative disease (1). At least eight disorders are due to expansion of a CAG triplet repeat that encodes polyglutamine in the specific disease protein: Huntington’s disease, spinobulbar muscular atrophy, dentato-rubral pallidoluysian atrophy and five dominantly inherited spinocerebellar ataxias (SCA), types 1, 2, 3, 6 and 7 (reviewed in refs 1–3). Although these diseases differ clinically and pathologically, a common pathogenic mechanism likely underlies them all (except possibly SCA6, which is due to a much smaller polyglutamine expansion in a calcium channel). Increasing evidence indicates that expanded polyglutamine confers a novel toxic property upon the otherwise unrelated polyglutamine disease proteins. Polyglutamine expansion leads to an altered, presumably misfolded, domain within the protein (4–6). One clear manifestation of this misfolding is the formation of intracellular aggregates by the disease protein, in particular intranuclear aggregates or nuclear inclusions (NI). NI have now been observed in at least six polyglutamine diseases and numerous transgenic animal models and thus represent a common hallmark of polyglutamine diseases (reviewed in refs 3, 7, 8; see also refs 9–11). NI are most frequently found in neurons from brain regions known to be susceptible to degeneration, suggesting that their formation may be important in disease pathogenesis.

The fact that NI are ubiquitinated supports the view that they contain misfolded and aggregated protein. The presence of ubiquitinated aggregates implies that alterations in the major intracellular system for degrading proteins, the ubiquitin–proteasome pathway, may contribute to the pathogenesis of polyglutamine diseases. The proteasome is a large multicatalytic protease complex that is critical for many cellular processes including cell cycle control, differentiation, antigen presentation and cell survival (12). It is responsible for the ubiquitin-dependent degradation of most cytosolic proteins, including the regulated destruction of short-lived cellular proteins and the elimination of misfolded or damaged proteins. Although proteasomes are abundant in the brain (13), their precise functions in neurons are still not fully understood. Recently, however, it has become clear that the control of protein degradation affects neuronal function.
under normal conditions and in disease states (14–16). The function of the proteasome in neurodegenerative diseases is particularly relevant since many neurodegenerative diseases are associated with protein misfolding and aggregation (17,18).

Studies of the polyglutamine disease SCA1 recently demonstrated redistribution of the proteasome into NI formed by the disease protein, ataxin-1 (19). To determine whether the proteasome is involved more generally in the polyglutamine diseases and to gain insight into its possible mode of action with respect to pathogenesis, we have examined the role of the ubiquitin–proteasome pathway in another polyglutamine disease, SCA3, also known as Machado–Joseph disease (SCA3/MJD). The most common dominantly inherited ataxia, SCA3/MJD is typically characterized by progressive ataxia with variable degrees of brainstem dysfunction, parkinsonism, dystonia and neuropathy [reviewed in ref. 20]. Pathological findings in SCA3/MJD also vary, but commonly include degeneration within the globus pallidus, regions of the brainstem and spinal cord (21–23). SCA3/MJD is due to a polyglutamine expansion in the gene product of the MJD1 gene, called MJD1p or ataxin-3 (24). At 42 kDa, ataxin-3 is the smallest of the polyglutamine diseases proteins. Its glutamine repeat lies near the C-terminus of the protein, where it is normally 14–40 repeats in length and is expanded in disease to between 55 and 80 repeats [reviewed in ref. 20]. Ubiquitinated NI containing ataxin-3 have been observed in regions of SCA3/MJD brain susceptible to degeneration, including the pons and other brainstem structures (25,26).

Using cell-based models and human disease tissue, we demonstrate here that the ubiquitin–proteasome pathway is involved in SCA3/MJD. We show that the proteasome complex redistributes into polyglutamine aggregates formed by the disease protein ataxin-3, both in human disease tissue and in vitro. Moreover, we present findings that suggest proteasome function is closely linked to aggregate formation, as inhibitors of proteasome activity promote aggregation in transfected cells. Taken together with similar findings in SCA1 (19), our studies implicate the ubiquitin–proteasome pathway as a general rule in polyglutamine diseases and suggest possible avenues for future therapeutic intervention.

RESULTS

The proteasome is recruited into NI of SCA3/MJD brain

NI appear to be a constant pathologic finding in SCA3/MJD, as we have observed NI in all 13 SCA3/MJD cases we have examined (25; H. Paulson and S. Subramony, unpublished data) and others have also reported them using different antisera against the disease protein, ataxin-3 (26). To determine whether the proteasome co-localizes to NI in SCA3/MJD, we performed immunohistochemical staining on brainstem sections of an SCA3/MJD brain known to have abundant NI (25). As shown in Figure 1, NI immunostained positively for the two major macromolecular complexes comprising the 26S proteasome complex: the 20S core that forms the central proteolytic chamber and the 19S cap, also known as PA700, that is necessary for the ATP-dependent delivery of unfolded polypeptide to the 20S core (12). NI also immunostained for a third proteasome macromolecular complex, the 11S regulator, which is believed to play a role in antigen processing in certain cell types (12). For each of these three proteasome components, the frequency of immunopositive NI was lower than that seen with anti-ubiquitin staining of adjacent sections, which labels essentially all NI (25). This variable staining with anti-proteasome antibodies indicates that NI in SCA3/MJD are heterogeneous with respect to molecular composition, with the proteasome being redistributed into a subset of NI. Staining was negative for several other components of the ubiquitin-mediated degradation pathway, including the ubiquitin conjugating enzyme E2 and ubiquitin C-terminal hydrolase (data not shown).

In vitro modeling of proteasome recruitment into polyglutamine aggregates

To better define the molecular determinants of proteasome recruitment, we employed a cellular model of polyglutamine aggregation. Previously we showed that a truncated ataxin-3 fragment with 78 glutamine repeats (Q78) is a potent aggregant, forming intranuclear or perinuclear aggregates in every cell line we have tested (e.g. the cell lines HEK 293, HEK293T, Cos-7, HeLa, MN-1 and PC12, as well as cultured primary neurons; 25,27). From its N-terminus, Q78 consists of an epitope tag [either hemagglutinin (HA) or myc], the 13 amino acids upstream of the glutamine repeat, the expanded glutamine repeat and the subsequent 43 amino acids comprising the C-terminus of the protein as originally published (24). In HeLa cells, myc–Q78 forms intranuclear and perinuclear aggregates. As shown in Figure 2A, the 20S proteasome core was found to redistribute into polyglutamine aggregates formed in both subcellular locations. The 19S cap of the 26S proteasome complex also redistributed into polyglutamine aggregates, as shown by immunofluorescence studies with an antibody against a protein from the 19S cap, ubiquitin isopeptidase Uch37 (28). Similar results were obtained in Cos-7 and 293 cells (data not shown). In all three cell types, nearly all polyglutamine aggregates stained positively for the 20S proteasome core.

Aggregates formed by Q78 are highly insoluble, migrating as high molecular weight complexes in the stacking portion of SDS–polyacrylamide gels (25). Immunoblot analysis suggested that the proteasome complex is not trapped within these insoluble complexes since 20S core proteins did not co-migrate with polyglutamine aggregates in the stacking gel (data not shown). Thus, proteasomes that redistribute into inclusions are apparently not irreversibly bound to the aggregated polyglutamine protein.

We confirmed recruitment of the proteasome using a second expanded polyglutamine protein in primary neuronal cultures. Cerebellar neurons were transfected with an expression construct that encodes green fluorescent protein (GFP) fused to a glutamine tract of 80 residues derived from the DRPLA protein, atrophin (29). In transfected neurons, GFP–Q80 readily formed cytoplasmic inclusions. As shown in Figure 2B, the 20S proteasome core was found to redistribute into polyglutamine aggregates formed in both subcellular locations. The 19S cap of the 26S proteasome complex also redistributed into polyglutamine aggregates, as shown by immunofluorescence studies with an antibody against a protein from the 19S cap, ubiquitin isopeptidase Uch37 (28). Similar results were obtained in Cos-7 and 293 cells (data not shown). In all three cell types, nearly all polyglutamine aggregates stained positively for the 20S proteasome core.

Full-length mutant ataxin-3 forms NI that recruit the proteasome and correspond to promyelocytic leukemia antigen (PML) oncogenic domains (PODs)

Compared with Q78, full-length ataxin-3 with 78 repeats is relatively poor at forming aggregates in cells. In our hands,
Figure 1. The proteasome localizes to nuclear inclusions in SCA3/MJD. Pontine neurons in SCA3/MJD brain immunostained with antisera to ubiquitin (Ubi) or to components of the proteasome complex: the 20S proteasome core (20S), the 19S proteasome cap (TBP7) and the 11S proteasome regulator (PA28). In each case, spherical NI stain positively for the indicated antigen (TBP7 and PA28 are subunits of the 19S and 11S complexes, respectively). The inset (upper right) shows a higher power view of one of the NI-containing neurons positively stained for the 20S core (NI indicated by arrow, nucleolus indicated by arrowhead). Neurons in control brain did not show discrete immunostaining of subnuclear structures. Tissue sections were not counterstained.

full-length ataxin-3 with a normal or expanded repeat (27 or 78 residues) usually remains diffusely distributed in transfected Cos-7, HeLa or 293 cells (25,27,30). Most of the expressed ataxin-3 is found in the cytoplasm, with only a small amount in the nucleus. Intriguingly, however, in the few cells where full-length mutant ataxin-3 does form aggregates (<1% of transfected cells), the inclusions are found exclusively within the nucleus. Thus, in an effort to promote aggregate formation by the full-length protein, we targeted ataxin-3 to the nucleus by adding a nuclear localization signal (NLS) to the N-terminus of the protein. Addition of an NLS resulted in a redistribution of ataxin-3 into the nucleus (Fig. 3). In HeLa cells, NLS–ataxin-3 with a non-pathogenic repeat of 27 residues localized diffusely to the nucleus with rare exceptions (Fig. 3A). In contrast, NLS–ataxin-3 with 78 repeats formed NI in 20–40% of transfected cells. A subset of these NI sequestered the proteasome (Fig. 3B). The results demonstrate that the full-length disease protein is capable of forming intranuclear aggregates that lead to a redistribution of the proteasome.

We wished to determine whether NI formed by full-length ataxin-3 resided within particular subnuclear structures. This has been shown to be the case for aggregates formed by the SCA1 disease protein, ataxin-1 (31). In transfected cells, mutant ataxin-1 forms inclusions that co-localize to PODs, also known as nuclear domain 10 (ND10) domains (32). In cells transfected with nuclear targeted mutant ataxin-3, staining for PML confirmed that ataxin-3 NI also co-localized to PODs (Fig. 3C and D). Further immunofluorescence studies confirmed that these ataxin-3/POD structures were clearly distinct from other subnuclear domains including nucleoli, coiled bodies, gem structures and DNA replication domains (data not shown).

Proteasome inhibition increases polyglutamine aggregation

The redistribution of proteasomes into polyglutamine aggregates suggested to us that proteasome function might play a critical role in the disease process. One possibility is that proteasome recruitment into NI represents an effort by the cell to 'handle' misfolded polyglutamine protein. For example, the proteasome may serve to recognize and eliminate misfolded polyglutamine protein, thereby reducing aggregate formation. To determine if this might be the case, we tested whether inhibitors of the proteasome could promote polyglutamine aggregation. For these experiments, we chose to use a glutamine repeat that is capable of forming aggregates, but less efficiently than Q78. We took advantage of a truncated ataxin-3 construct that contains an intermediate length repeat of 49 glutamine residues (Q49) generated by PCR. A repeat length of 49 falls squarely in the gap between normal and disease repeat length distributions for SCA3/MJD (20). Although shorter than the shortest repeat known to cause SCA3/MJD, a repeat of 49 residues is well within the pathologic range for many polyglutamine diseases, including HD, SCA1, SBMA and SCA2. In transfected HeLa cells, HA–Q49 typically showed a diffuse staining pattern, forming aggregates only infrequently (<5% of cells in each of four experiments). Treatment of transfected HeLa or Cos-7 cells with the specific proteasome inhibitor lactacystin (33) led to a marked
Figure 2. The proteasome redistributes into polyglutamine aggregates in transfected cells. (A) In HeLa cells expressing the mutant ataxin-3 fragment, myc–Q78, polyglutamine aggregates form either in the perinuclear region (top) or in the nucleus (bottom). Co-immunofluorescence staining demonstrates that the 20S proteasome core (red) redistributes into polyglutamine aggregates (green) in either cellular location, confirmed by coincident immunostaining in the merged images. (B) In primary cultured neurons transfected with GFP fused to a Q80 repeat, the proteasome (red) is recruited into many but not all aggregates (green). (Compare upper and lower panels, which show proteasome redistribution only in the upper panel.)

increase in Q49 aggregate formation, both intranuclear and perinuclear (Fig. 4). This increased aggregate formation occurred in a glutamine repeat length-dependent manner, since aggregation was not increased in cells expressing Q27 (Fig. 4A and B) or GFP fused to polyglutamine domains of 19 or 35 glutamine residues (data not shown). As expected, aggregation of Q78 occurred readily even in the absence of lactacystin and was not further increased by inhibiting the proteasome (Fig. 4B). In the presence of lactacystin, the 20S proteasome core still redistributed into aggregates even though the proteasome’s proteolytic activity was inhibited. Quantitation of cell death indicated that lactacystin-induced aggregate formation did not cause increased cell death in HeLa cells (data not shown).

Aggregate formation was promoted by lactacystin in a dose- and time-dependent manner (Fig. 4C and D). Although doses of lactacystin >10 µM were equally or more effective at promoting aggregation, cells showed signs of cytotoxicity to lactacystin at these higher concentrations (20 and 50 µM). Two additional proteasome inhibitors, ALLN and MG132, also increased aggregate formation but caused significant cytotoxicity and thus were not further used (data not shown). Increased aggregate formation appears to be specific to inhibitors of the proteasome since aggregation was not increased by inhibitors of lysosomal proteases (Fig. 4E).

Immunoblot analysis confirmed that, in lactacystin-treated cells, Q49 aggregates resembled aggregates formed by the pathogenic Q78 fragment (25); both were insoluble to boiling, reducing agents and SDS treatment and both migrated in the stack of SDS gels (Fig. 5A and B). Cell fractionation experiments confirmed that Q49 aggregates sedimented in the insoluble fraction (Fig. 5B). Immunoblots also indicated that total Q49 protein levels did not increase dramatically in lactacystin-treated cells. This fact argues against a potentially trivial explanation for increased aggregate formation: a simple rise in the intracellular concentration of Q49 that might be expected to drive the protein toward aggregation.

Importantly, lactacystin also promoted aggregation by full-length mutant ataxin-3. In the presence of lactacystin, NLS–myc–ataxin-3 with a 78 glutamine repeat formed extensive cytoplasmic and intranuclear aggregates in most cells (data not shown), whereas under control conditions it formed exclusively nuclear inclusions in 20–40% of transfected cells (as illustrated in Fig. 3B and D). The formation of cytoplasmic aggregates despite the presence of an NLS in the protein suggests that lactacystin caused NLS–ataxin-3(78) to aggregate before it could be transported to the nucleus, essentially trapping the protein in the cytoplasm.

DISCUSSION

Redistribution of the 26S proteasome complex into NI in SCA3/MJD brain and cellular models implicates the ubiquitin–proteasome degradation pathway in SCA3/MJD pathogenesis. Together with similar findings in SCA1 (19), the results presented here suggest that proteasome involvement may be generalized to most if not all polyglutamine diseases. They further support the view that expanded polyglutamine domains promote misfolding of the disease protein, resulting in aggregation. Moreover, our finding that proteasome inhibition accelerates aggregate formation implies that the proteasome may play a direct role in modulating aggregation in the disease state.

A fundamental question prompted by our results is: does proteasome redistribution into polyglutamine aggregates represent an appropriate and beneficial response by the cell or is it rather a pathologic event that has deleterious consequences for the cell? The fact that proteasome inhibitors increase polyglutamine aggregation favors the view that the proteasome represents a first-line cellular defense to recognize and eliminate misfolded...
polyglutamine protein before it aggregates. The simplest model to explain our results with proteasome inhibitors is that, under normal conditions, the proteasome selectively degrades misfolded, expanded polyglutamine protein, thereby reducing the concentration of aggregation-prone polypeptide. Since polyglutamine aggregation is likely to proceed through a nucleation step that is dependent upon the concentration of misfolded protein (6,34; E. Wanker, personal communication), this function of the proteasome would reduce the rate of aggregation. When proteasome activity is blocked with lactacystin or other inhibitors, the concentration of misfolded polyglutamine protein would increase, favoring nucleation and aggregation. Alternatively, proteasome inhibitors could increase polyglutamine aggregation indirectly through effects on other cellular proteins or metabolic pathways.

Our results are further consistent with a model in which the proteasome acts upon polyglutamine protein after it has formed aggregates, perhaps further processing the aggregated polypeptide. An intriguing hypothesis, one that is still consistent with a separate beneficial role for the proteasome, is that expanded polyglutamine protein directly inhibits the proteasome to which it is targeted. Inhibition of the proteasome by expanded polyglutamine would decrease cellular levels of functioning proteasomes. Our lactacystin results suggest that this, in turn, would further promote aggregation in a deleterious feed-forward manner. How might expanded polyglutamine inhibit the proteasome? To be degraded by the proteasome a protein must first be unfolded in order to enter the central proteolytic chamber. Expanded polyglutamine may form a highly stable β-sheet hairpin structure (4) that resists unfolding and thus blocks entrance to the chamber. A similar model has been proposed for β-amyloid effects on the proteasome (35). In vitro reconstitution experiments clearly will be required to determine whether expanded polyglutamine does in fact inhibit the proteasome.

A much debated issue in polyglutamine diseases (36,37) is whether NI are pathogenic structures, bystanders in disease or perhaps even beneficial to the cell (38). Recent studies in SCA1 transgenic mice have shown that macroscopic aggregates are not necessary for initiating disease pathogenesis (39). Whether, however, aggregates contribute to later stages of disease remains unknown. It is important to note that neurological symptoms in polyglutamine diseases progress over decades, suggesting that at the cellular level there may be an initial and prolonged period of neuronal dysfunction before a later period of neuronal demise. Our current view of polyglutamine pathogenesis is that it is a multistep process in which misfolding of the disease protein is central to all steps of disease, including the early period of neuronal dysfunction, but in which macroscopic intranuclear aggregates are late participants, contributing principally to the final period of neuronal demise. The tools are now in hand to test directly the role of aggregation in neurons. The ability to increase aggregation by inhibiting the proteasome (this study) or to decrease aggregation by overexpressing certain chaperonins (19; Y. Chai and H. Paulson, unpublished data) should now allow...
Inhibiting the proteasome promotes polyglutamine aggregation. Treating transfected HeLa or Cos-7 cells with the specific proteasome inhibitor lactacystin causes a marked increase in polyglutamine aggregation that is dependent upon repeat length. (A) Immunofluorescence of Cos-7 cells transfected with HA–Q27 or HA–Q49 and incubated with or without lactacystin (controls were treated with the carrier, DMSO). In cells expressing the intermediate repeat length ataxin-3 fragment HA–Q49, but not in cells expressing HA–Q27, lactacystin causes a marked increase in intranuclear and cytoplasmic aggregation. (The arrow identifies a Q49-expressing cell that has both intranuclear aggregates and diffuse cytoplasmic staining.) (B) Quantitation of lactacystin-induced aggregation by HA–Q49-expressing cells. (C) Dose dependence of lactacystin-induced aggregation. HeLa cells expressing HA–Q49 were incubated for 24 h with the indicated concentrations of lactacystin and scored for aggregate formation. (D) Time dependence of aggregate formation. Aggregation in Cos-7 cells expressing HA–Q49 was quantitated at the indicated times after lactacystin was added. (E) Aggregate formation is not increased by inhibitors of lysosomal proteases. Aggregation was quantitated in HeLa cells for 24 h after addition of the indicated protease inhibitors: lac, lactacystin; leup, leupeptin; chlor, chloroquine. In (B) and (C), results are the means of two separately performed experiments; in (D) and (E), results are from single representative experiments.

Figure 5. Lactacystin treatment causes an increase in SDS-insoluble polyglutamine aggregation. Shown are immunoblots of lysates from transfected HeLa cells incubated in the absence or presence of lactacystin. (A) Equal lysates from control cells and cells expressing HA–Q49 or HA–Q78 were examined by immunoblot with anti-HA antisera. In the presence of lactacystin, HA–Q49 forms SDS-insoluble complexes that migrate in the stacking gel (brackets). Note that HA–Q78 forms insoluble complexes even under control conditions and these are not measurably increased by lactacystin treatment. (B) Lactacystin causes a marked increase in high molecular weight aggregates of HA–Q49 (arrowhead) which sediment in the insoluble fraction. Fractionation of lysates into soluble and insoluble fractions was carried out before immunoblot analysis with anti-HA antisera. In (A) and (B), arrows indicate monomeric Q49 protein.

Moreover, as modifier genes are identified through genetic screens in invertebrate models of disease (9,40), the precise role of cellular chaperone systems, including chaperonins and the 26S proteasome complex, in polyglutamine diseases may become clear.

The results presented here indicate previously unrecognized similarities between SCA3/MJD and SCA1 that may have broader implications for the entire class of polyglutamine diseases. First, studies of both diseases now show that the full-length disease proteins are capable of forming inclusions, provided the protein is resident within the nucleus. The finding of aggregation by the full protein suggests that proteolysis may not be necessary to drive the misfolding and aggregation that occurs in disease. Secondly, nuclear inclusions formed by full-length ataxin-1 and ataxin-3 both reside in a specific subnuclear domain known as PODs or ND10 domains. The function of PODs is unclear, but recent studies suggest they may mediate certain forms of cell death, in part through the recruitment of other proteins (41,42). The relationship of NI to PODs in other polyglutamine diseases clearly will need to be pursued. Thirdly, the nuclear environment appears to promote aggregation of both disease proteins; when present in the cytoplasm, mutant forms of ataxin-1 and ataxin-3 do not form detectable aggregates (25,27,39). Fourthly, the proteasome and certain cellular chaperone proteins redistribute into NI formed by the two disease proteins, both in vivo and in vitro (19; this study and unpublished data). Despite these unifying features there are still important differences, the most obvious being a difference in the normal subcellular distribution of the two proteins. Ataxin-1 is almost exclusively nuclear in neurons (43), whereas ataxin-3 shows a variable cytoplasmic and nuclear pattern of expression (25–27,30,44–46). Our results suggest the possibility that even though most ataxin-3 protein in neurons is cytoplasmic (25,26), some is nuclear, and it is this intranuclear pool of ataxin-3 that precipitates disease. It will thus be critical to identify the factors regulating nuclear transport of ataxin-3 and to address, in vivo, the
importance of nuclear localization that the present cell-based studies of ataxin-3 suggest.

Finally, the role of proteasomes in neurodegenerative diseases extends beyond the polyglutamine diseases. Many inherited and acquired neurodegenerative diseases are proteinopathies, diseases in which a particular protein or set of proteins misfold and aggregate (17,18). Ubiquitin stains proteinaceous deposits in many classes of neurodegenerative disease, including Alzheimer’s disease and other dementias, Parkinson’s disease, prion diseases, motor neuron disease and, of course, polyglutamine diseases (47,48). Moreover, the proteasome localizes to patho- logical structures in most of these diseases (19,49,50). Despite the clear differences between these diseases, perturbations of the proteasome degradation pathway may represent a common link. To this end, manipulations of proteasome function may prove to be of therapeutic use not only in the polyglutamine diseases, but in other neurodegenerative diseases as well.

MATERIALS AND METHODS

Expression plasmids

The ataxin-3 expression constructs pJ3M-ataxin-3(Q27), pcDNA3-myc-ataxin-3(Q78), pcDNA3-HA-Q27, pcDNA3-HA-Q78 and pJ3M-Q78 were described previously (25,30). PCDNA3-HA-Q49 was derived by PCR amplification of the C-terminus of ataxin-3 (Q78) during which a spontaneous reduction in CAG repeat size occurred. Automated DNA sequencing (University of Iowa DNA Facility) confirmed that pCDNA3-HA-Q49 was identical to pCDNA3-HA-Q78 except for its shorter repeat of 49 residues. Expression vectors pAG-NLS-myc-ataxin-3(Q27) and pAG-NLS-myc-ataxin-3(Q78) contain full-length ataxin-3 cDNA modified to contain an NLS at the N-terminus (the NLS sequence is MPKKRKRK). These modified cDNAs were generated by PCR and cloned into pAG-3, an expression vector in which the expressed cDNA is tagged at the C-terminus with a myc–hexahistidine epitope tag (51). To generate NLS constructs, we used the forward primer (5′-GGATCCACCATGCCCCAAGAG-AAGCGGAAAGTGCGT TCCATCTTCC AGCAGAAACAAGAAGGC-3′) and the reverse primer (5′-GGCGGCCGGCTCT-GTCAGATTTAGTGAGGATAGG-3′). The GFP–polyglutamine fusion constructs with Q19, Q35 or Q80 repeats (29) were generously provided by W. Strittmatter (Durham, NC).

Cell culture and transfection

HeLa cells were grown in minimum essential medium (MEM) and Cos-7 cells and 293 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco BRL, Grand Island, NY). All cell lines were supplemented with 10% fetal calf serum, 100 U/ml penicillin–50 µg/ml streptomycin (Gibco BRL) and 1% goat serum in 0.05% Triton X-100. HeLa cells were also supplemented with 1% MEM non-essential amino acid solution (Sigma, St Louis, MO). For transient transfections, 50% confluent 35 mm dishes of HeLa, Cos-7 or 293 cells were transfected with 1 µg of expression plasmid DNA using Lipofectamine PLUS reagent (Gibco BRL, Grand Island, MD). Twenty-four hours after transfection, cells were transferred to chamber slides coated with 0.1% gelatin/phosphate-buffered saline (PBS) for an additional 24 h before immunofluorescence.

For transfection of cultured neurons, cerebella were removed from 3-day-old Sprague–Dawley rat pups, incubated in dissociation medium twice for 30 min each (52), triturated with a 5 ml pipette and centrifuged at 300 g for 5 min. The pellet was resuspended in Earle’s salts with 3 mg bovine serum albumin (BSA), 3 mg ovomucoid and 300 U DNase, then carefully added to 5 ml BSA/ovomucoid (10 mg/ml) and centrifuged at 70 g for 6 min. The pelleted, dissociated neurons were resuspended in DMEM, 10% calf serum, 10% Ham’s F-12, Pen/Strep/Fungizone and plated onto polyornithine-coated glass coverslips in 6-well chamber dishes at a density of 5 × 10^6 cells/dish. The cultures were treated with cytosine-β-d-arabinofuranoside on the second day after seeding and then transfected on day 3 using an established calcium phosphate precipitation protocol for cortical neurons (53) with the following modifications: 4 µg DNA/well was used and kynurenic acid or APV were not included in the transfection.

Protease inhibitor treatment

Three or twenty-four hours after transfection, cells were incubated with the indicated proteasome or lysosomal inhibitors for 24 h or, in the experiment in Figure 4D, for the indicated lengths of time. Control cells were incubated with equivalent amounts of the carrier agent, dimethyl sulfoxide (DMSO). The results shown in the experiments in Figure 4 are from representative experiments duplicated at least twice in all cases. The proteasome inhibitors lactacystin and MG-132 were from Calbiochem (La Jolla, CA). The non-specific proteasome/lysosomal protease inhibitor N-acetyl-leucinyl-leucyl-norleucinal (ALLN) and lysosomal inhibitors leupeptin, E64 and chloroquine were from Sigma. Unless otherwise stated the concentrations used were: 10 µM lactacystin, 10 µM E64, 10 µM MG-132, 15 µM ALLN and 25 µM chloroquine. To confirm that 10 µM lactacystin inhibited proteasome function, we performed anti-ubiquitin immunoblots on lysates, which documented increases in total ubiquitinated protein consistent with the expected failure in degradation.

Immunocytochemistry

Forty-eight hours after transfection, cells were washed with PBS, fixed for 15 min with 4% formaldehyde/PBS, permeabilized with 0.05% Triton X-100, blocked for 15 min with 5% normal goat serum in 0.05% Triton X-100/PBS and incubated for 60 min at 37°C with primary antibody. Subsequently, cells were incubated for 60 min with either anti-mouse–FITC/anti-rabbit–TRITC or anti-rabbit–FITC/anti-mouse–TRITC (1:1000) (Jackson ImmunoResearch Laboratories, West Grove, PA). After extensive washing, chamber slides were counterstained for 10 min in 0.05% Triton X-100/PBS containing 4,6-diamidino-2-phenylindole (DAPI) (2 µg/ml) (Sigma) and mounted in SlowFade (Molecular Probes, Eugene, OR). Samples were observed with a Zeiss Axiosplan fluorescence microscope under 630x magnification and digitized images were collected on separate fluorescence channels using a Diagnostics SPOT digital camera. Digitized images were assembled using Adobe Photoshop.

The 20S proteasome was detected by immunostaining with rabbit polyclonal anti-20S proteasome antibody (1:1,000) (a gift of W. Ward, University of Texas, San Antonio, TX). Immunostaining for other proteins of the ubiquitin–proteasome pathway were assessed using antibodies from Affiniti (Exeter, UK) with dilutions in parentheses: rabbit polyclonal antibodies against the ATPase subunit TB17 of the human 19S proteasome regulator (1:200), ubiquitin C-terminal hydrolase (1:200), ubiquitin conju-
I n Q27-expressing cells or control cells in the presence or absence of lactacystin treatment.

Immunoblots and cell fractionation studies
Transfected HeLa cells were harvested directly into 1× SDS loading buffer (50 mM Tris pH 6.8, 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, 10% glycerol), sonicated briefly and heated to 95°C for 5 min. Equal amounts of protein were electrophoresed on discontinuous 12% polyacrylamide gels (SDS–PAGE) and the gels were transblotted to PVDF membranes. Membranes were blocked for 2 h in blocking solution (10% non-fat powdered milk, 10% glycerol, 0.2% Tween-20 in PBS), incubated for 2 h with primary polyclonal anti-HA antibody Y-11 (Santa Cruz Biotechnology) at a dilution of 1:1000 or anti-20S proteasome antisera at 1:2000, washed five times in blocking solution and incubated for 1 h with peroxidase-conjugated goat anti-rabbit antiserum (1:15 000). Bands were visualized using enhanced chemiluminescence (Amersham, Piscataway, NJ).

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ABBREVIATIONS
ALLN, N-acetyl-leucinyl-leucinyl-norleucinal; BSA, bovine serum albumin; DAB, diaminobenzidine; DAPI, 4,6-diamidino-2-phenylindole; DMEM, Dulbecco’s modified Eagle’s medium; DMSO, dimethyl sulfoxide; GFP, green fluorescence protein; HA, hemagglutinin; MEM, minimum essential medium; MJD, Machado–Joseph disease; ND10, nuclear domain 10; NI, intranuclear inclusions; NLS, nuclear localization signal; PBS, phosphate-buffered saline; PML, promyelocytic leukemia antigen; PODs, PML oncogenic domains; SCA, spinocerebellar ataxia.

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