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Misfolding Promotes the Ubiquitination of Polyglutamine-Expanded Ataxin-3, the Defective Gene Product in SCA3/MJD

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A major hallmark of the polyglutamine diseases is the formation of neuronal intranuclear inclusions (NIIs) of the disease proteins that are ubiquitinated and often associated with various chaperones and proteasome components. Recently, misfolding has come to be considered one of the primary factors for polyglutamine protein aggregation, although, the nature of misfolding and the relationship between misfolding and ubiquitination of the expanded polyglutamine protein is not yet known. By using ataxin-3, the defective gene product of SCA3/MJD, we demonstrate here that the misfolding propensity and the cellular toxicity of a polyglutamine protein is directly proportional to the length of the glutamine repeats and inversely dependent on the size of the corresponding protein. The size of the polyglutamine bearing protein also inversely influences the binding of 1C2 antibody (an antibody that selectively recognizes polyglutamine expansion) to the polyglutamine protein and determines the minimum length of glutamine expansion to be recognized by 1C2 antibody, which suggests that the critical pathological range of glutamine repeats could also be dependent on the size of the corresponding protein. Ataxin-3 (both full length and truncated) with normal glutamine repeats are not ubiquitinated, however, ataxin-3 with expanded polyglutamine is ubiquitinated and the ubiquitination depends on the misfolding propensity of the polyglutamine expanded ataxin-3.

Keywords: Polyglutamine; Misfolding; Ubiquitination; Ataxin-3

INTRODUCTION

The pathological expansion of unstable trinucleotide repeats has recently been found to cause 14 neurological diseases, eight of which are neurodegenerative diseases (also referred to as polyglutamine diseases) resulting from the expansion of CAG repeats within the coding region of the responsible genes. Those 8 include Huntington's disease (HD), dentatorubral pallidoluysian atrophy (DRPLA), X-linked spinal bulbar muscular atrophy (SBMA), and several spinocerebellar ataxias (SCA1, SCA2, SCA3, SCA6, and SCA7). All eight disorders are progressive, dominantly inherited (except SBMA), typically begin in mid-life, and result in severe neuronal dysfunction and neuronal cell death. Increasing length of glutamine repeats in the affected individual strongly correlates with earlier age of onset and disease severity (Paulson and Fischbeck, 1996; Reddy and Housman, 1997; Cummings and Zoghbi, 2000; Zoghbi and Orr, 2000).

Evidence suggests a toxic gain-of-function effect of the polyglutamine expansion on the protein, and this novel neurotoxic property most likely involves an increased propensity for the disease protein to aggregate (Paulson, 1999; Ferrigno and Silver, 2000; Sherman and Goldberg, 2001). In human disease tissue, transgenic animal models, and transfected cells, expanded polyglutamine proteins have been shown to undergo intracellular aggregation, in most cases forming NIIs (Ross, 1997; Kim and Tanzi, 1998; Lin *et al.*, 1999). The mechanism that causes the polyglutamine proteins to aggregate is still not clear. Though Max

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Perutz's polar zipper hypothesis (Perutz *et al.*, 1994; Stott *et al.*, 1995) or protein misfolding (Paulson, 1999; Ferrigno and Silver, 2000; Sherman and Goldberg, 2001) are considered to be the primary factor in aggregation, there are several other factors that might also enhance the aggregation process, such as transglutaminase-catalyzed cross-linking of polyglutamine protein (Kahlem *et al.*, 1996), aberrant interaction with other proteins dependent on polyglutamine length (Li *et al.*, 1995; Sittler *et al.*, 1998), or proteolytic processing of the full-length disease protein (Ellerby *et al.*, 1999a ; Wellington *et al.*, 2000). However, the discovery of ubiquitinated aggregates and the association of various chaperones and proteasome components with the aggregates suggest that the cells recognize the aggregated disease protein as abnormal, and may represent an appropriate cellular response to refold or degrade aggregated mutant protein (Cummings *et al.*, 1998; Chai *et al.*, 1999a,b; Stenoien *et al.*, 1999; Warrick *et al.*, 1999; Jana *et al.*, 2000). Consistent with this idea, it has been experimentally demonstrated that over expression of selective chaperones in the mammalian cell culture suppresses the aggregate formation and cell death (Cummings *et al.*, 1998; Chai *et al.*, 1999a; Stenoien *et al.*, 1999; Jana *et al.*, 2000), and that the proteasome system is indeed involved in the degradation of polyglutamine proteins although the rate of degradation might depend on the glutamine repeat length (Cummings *et al.*, 1998; Cummings *et al.*, 1999; Jana *et al.*, 2001). However, very little is known about the nature of misfolding of the polyglutamine proteins and the delivery of the misfolded polyglutamine proteins to the ubiquitin proteasome pathway (UPP) for degradation, nor is the failure of the degradation mechanism of UPP to efficiently clear out the misfolded polyglutamine proteins clearly understood.

In our model of glutamine repeat disease, we studied ataxin-3, the defective gene product in spinocerebellar ataxia type 3/Machando-Joseph disease (SCA3/MJD), the most common dominantly inherited ataxia, which is characterized by selective neuronal loss within the brain stem, deep basal ganglia, cerebellum, and spinal cord (Rosenberg, 1992). Ataxin-3 is a novel protein of unknown function with a molecular mass of 42 kDa, and contains glutamine repeats at its C-terminus (Kawaguchi *et al.*, 1994). Studies have shown that ataxin-3 is localized in the cytoplasm or in both the cytoplasm and nucleus (Paulson *et al.*, 1997; Wang *et al.*, 1997; Tait *et al.*, 1998).

In the present investigation, we demonstrate that the misfolding propensity of mutant ataxin-3 is directly proportional to the glutamine repeat length and inversely proportional to the corresponding protein size. The critical pathological range of glutamine repeat could also be dependent on the corresponding protein size, as evidenced by the nature of 1C2 antibody binding. Though the chaperone binding (degree of misfolding) is followed by 1C2 binding (most likely induced β-sheet conformation), the length of glutamine repeats required for chaperone binding is comparatively much higher than that for 1C2 antibody binding. Finally, we show that the misfolded ataxin-3 is ubiquitinated.

MATERIALS AND METHODS

Expression Plasmids

The isolation of normal and mutant full-length cDNA of ataxin-3 (the *MJD*1 gene product) containing 20 (normal), 68 (mutant), and 80 (mutant) CAG repeats and the construction of plasmids pAS2-1-MJD-20CAG, pAS2-1-MJD-68CAG, and pAS2-1-MJD-80CAG has been described earlier (Wang *et al.*, 2000). The full-length ataxin-3 cDNA with normal and expanded CAG repeats were excised from the pAS2-1 vector with *Bam*HI and *Stu*I, and subcloned into the pEGFP-N1 vector (Clontech) at *Bgl*II and *Sma*I sites. The plasmids were named pEGFP-N1-MJD(f)-20CAG, pEGFP-N1-MJD(f)-68CAG, and pEGFP-N1-MJD(f)-80CAG. Using the instability of CAG repeats, we obtained a further expanded construct containing 130CAG repeats in pEGFP-N1 vector, and named as pEGFP-N1-MJD(f)-130CAG. The N-terminal deletion mutant (deletion of 1-246 amino acids) of ataxin-3 were constructed by digesting the full length MJD cDNA with *Bgl*II and *Stu*I, and subcloning the resulting fragment into the *Bgl*II and *Sma*I site of pEGFP-N1 vector. The plasmids were named pEGFP-N1-MJD(t)-20CAG, pEGFP-N1-MJD(t)-80CAG, and pEGFP-N1-MJD(t)-130CAG.

Cell Culture, Transfection, Cell Viability Assay and Counting of Aggregates

Mouse neuro2a cells were cultured in Dulbecco's modified Eagle's medium (Life Technologies Inc., Gaithesburg, MD) supplemented with 10% heat-inactivated fetal bovine serum and antibiotics penicillin/streptomycin. One day prior to transfection, cells were plated into 6-well tissue cultured plates at a subconfluent density. Cells were transiently transfected with expression vectors using LipofectAMINE 2000 reagent (Life Technologies Inc.) according to the manufacturer's instruction. Transfection efficiency was about 80-90%. After 24 h or 48 h of transfection, cells were used for immunofluorescence staining, co-

immunoprecipitation, and immunoblotting. For cell viability assay, cells (5 x 10^3 cells/well) were seeded into 96-well plates and 24 h after seeding, cells were transfected with the expression plasmids. On the following day, medium was changed and the cells were differentiated with 5 mM of dbcAMP (N6,2'-O-dibutyriladenosine-3',5'-cyclic monophosphate sodium salt; Nacalai Tesque, Kyoto, Japan) for 3 days. Cell viability was measured by MTT assay as described previously (Wang *et al.*, 1999). Statistical analysis was performed using Student *t*-test, and $P < 0.05$ was considered to indicate statistical significance. Aggregate formation was manually counted under a fluorescence microscope (approximately 500 transfected cells in each case) and the cells containing more than one aggregate were considered to have a single aggregate.

Co-Immunoprecipitation and Immunoblotting Experiment

After 24 or 48 h of transfection, cells were washed with cold PBS, scraped, pelleted by centrifugation, and lysed on ice for 30 min with RIPA buffer (10 mM Hepes [pH 7.4], 150 mM NaCl, 10 mM EDTA, 2.5 mM EGTA, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate, 10 mM NaF, 5 mM $Na_4P_2O_7$, 0.1 mM Na_2VO_5, 1 mM PMSF, 0.1 mg/ml Aprotinin). Cell lysates were briefly sonicated, centrifuged for 10 min at 15000*g* at 4°C and the supernatants (total soluble extract) were used for immunoprecipitation. Protein concentration was measured according to the method of Bradford, using Bio-Rad Protein Assay reagent (Bio-Rad Laboratories, Hercules, CA, USA) and BSA as a standard. For each immunoprecipitation experiment, 200 μg protein in 0.2 ml RIPA buffer was incubated either with 5 μl (2 μg) of GFP antibody (mouse monoclonal from Boehringer Mannheim, Indianapolis, IN, USA) or 4 μl (2 μg) of normal mouse IgG. After 5-6 h of incubation at 4°C with rotation, 10 μl of magnetic protein G beads (Perspective Biosystem, Framingham, MA, USA) were added, and incubation was continued at 4°C overnight. The beads were pulled down with a magnet (Dynal, Oslo, Norway) and washed 6 times with RIPA buffer. Bound proteins were eluted from the beads with SDS (1X) sample buffer, vortexed, boiled for 5 min, and analyzed by immunoblotting. The total cell lysate or the immunoprecipitated proteins were separated through SDS-polyacrylamide gel electrophoresis and transferred onto PVDF membranes (Immobilon-P; Millipore, Bedford, MA, USA). The membranes were successively incubated in blocking buffer (5% skim milk in TBST [50 mM Tris; pH 7.5, 0.15 M NaCl, 0.05% Tween]), with primary antibody in TBST, and then with secondary antibody conjugated with HRP (Amersham Life Science, Buckinghamshire, UK) in TBST. Detection was carried out with enhanced chemiluminescence reagent (ECL; Amersham Life Science). The primary antibodies used in the immunoblotting experiments were obtained from the following sources. The rabbit polyclonal anti-Hdj-1 (SPA-400) and mouse monoclonal Hsp70 (SPA-800 and SPA-810) were purchased from Stressgen Biotechnologies (Victoria, British Columbia, Canada). Mouse monoclonal anti-Hdj-2 (MS-225) was from Neomarkers (Union City, CA) and mouse monoclonal antibody 1C2 was from Chemicon International (Tenecula, CA). Rabbit polyclonal anti-ubiquitin was from Dako (Dako Japan Co., Kyoto). All primary antibodies were used in 1:1000 dilutions for immunoblotting.

Immunofluorescence Techniques

Cells grown in chamber slides or in 6-well tissue cultured plates were transiently transfected with different MJD constructs. Forty-eight hours after transfection, cells were washed twice with PBS, fixed with 4% paraformaldehyde in PBS for 20 min, permeabilized with 0.5% Triton X-100 in PBS for 5 min, washed extensively, then blocked with 5% nonfat dried milk in TBST for 1 h. Primary antibody incubation was carried out overnight at 4°C. After several washings with TBST, cells were incubated with the appropriate secondary antibody for 1 h, washed several times, and mounted in antifade solution (Vecashield Mounting Media, Vector Laboratories, Berlingame, CA). In some cases, immunofluorescence staining was carried out directly into the 6-well tissue cultured plates, and in those cases, after final washings, cells were visualized using a water lance in the confocal microscope. The primary antibodies against lamin B (goat polyclonal; from Santa Cruz, CA) and 1C2 were used in 1:500 dilution, and the secondary antibodies conjugated with CY3 (purchased from Molecular Probes, Eugene, OR, USA) were used in 1:500 dilution. Samples were observed using a confocal microscope (Fluoview, Olympus, Japan), and digital images were assembled using Adobe Photoshop.

RESULTS

Truncation of Ataxin-3 Increases Aggregate Formation and Cell Death

We created several full-length and truncated (C-terminal) ataxin-3 expression constructs (FIG. 1A) with normal and expanded glutamine repeats through fusion

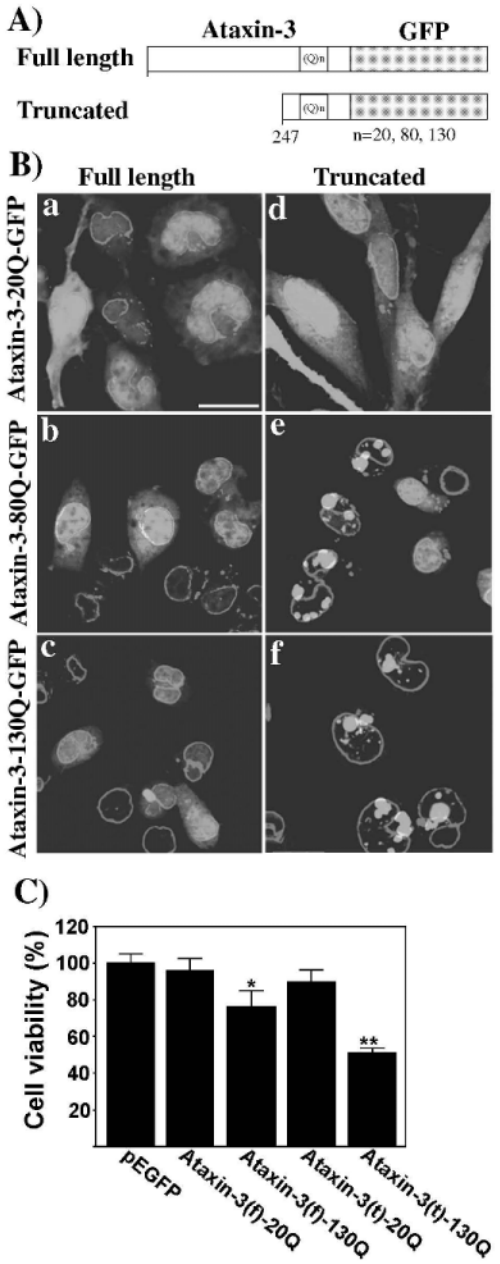

FIGURE 1 Truncation of ataxin-3-EGFP fusion protein with expanded polyglutamine enhances aggregate formation and cell death. (**A**) The full-length and truncated ataxin-3-EGFP expression constructs with different glutamine repeats. (**B**) Subcellular localization and aggregate formation of the different ataxin-3-EGFP fusion constructs. Neuro2a cells were transiently transfected with different ataxin-3-EGFP fusion constructs. Two days after transfection, cells were processed for immunofluorescence staining for lamin B. A CY3 labelled secondary antibody was used to label the lamin B. Aggregate formation was manually counted under the fluorescence microscope, and the cells containing more than one aggregate were considered to have a single aggregate. Scale bar; 20 μm. (**C**) Effect of the different full-length and truncated ataxin-3-EGFP fusion proteins on cell viability. Neuro2a cells were transiently transfected with different ataxin-3-EGFP fusion constructs in a 96-well tissue cultured plate. On the following day, medium was replaced and the cells were differentiated for another three days. Cell viability was measured by MTT assay. Values are the mean ± SD; n=6. *P <0.01 and *P <0.001 as compared to pcDNA-transfected control.

with enhanced green fluorescence protein (EGFP). Using these expression constructs, we first checked the subcellular distribution of both normal and polyglutamine-expanded ataxin-3, then investigated the influence of the glutamine repeats and the corresponding protein size on aggregation and cell death (FIG. 1B, 1C). Mouse neuro2a cells were transfected with the ataxin-3 expression constructs, and after two days of transfection, cells were either directly visualized under the confocal microscope or processed for immunofluorescence staining for lamin B to clearly visualize the nuclear boundary. The normal full-length and truncated ataxin-3 were predominantly localized in the nucleus with diffuse cytoplasmic distribution. Though the

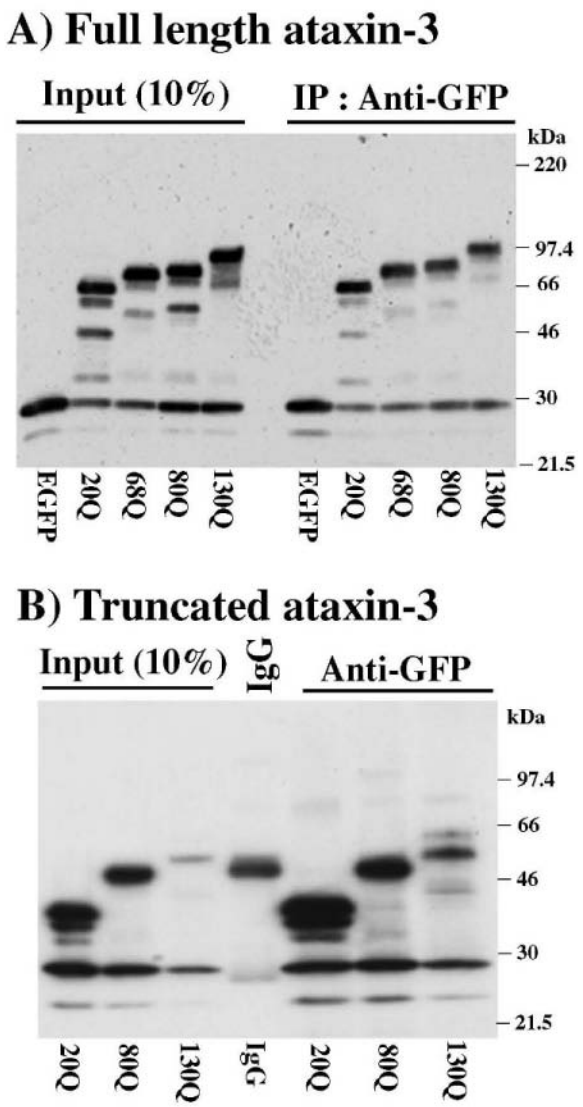

FIGURE 2 Immunoprecipitation of ataxin-3-EGFP fusion proteins by anti-GFP. The EGFP and the ataxin-3-EGFP expression constructs with different glutamine length were transfected to the neuro2a cells. Two days after transfection, cells were collected and processed for immunoprecipitation by GFP antibody. The blots were probed with GFP antibody. (**A**) Immunoprecipitation of full-length ataxin-3-EGFP fusion proteins. (**B**) Immunoprecipitation of truncated ataxin-3-EGFP fusion proteins.

nuclear localization signal (NLS) for ataxin-3 has not yet been identified and characterized, it has been predicted (Tait *et al.*, 1998) that an NLS is located near the glutamine repeats (amino acids 281-285). Both the full-length and truncated ataxin-3 constructs used in this study contained this NLS site. The full-length ataxin-3 with 80Q did not form any aggregates, whereas the truncated ataxin-3 with 80Q formed massive aggregates after two days of transfection. Similarly, the transfection of full-length ataxin-3 with 130Q for two days caused aggregate formation in about 2-4% cells, which dramatically increased to 80-90% when the ataxin-3 with 130Q was truncated (deletion of 246 amino acids). Aggregates were mostly localized in the nuclear compartment. To evaluate the effect of glutamine repeats and the corresponding protein size on the cellular toxicity, we transfected the ataxin-3 constructs to the mouse neuro2a cells, then the cells were differentiated and the cell viability was measured by MTT assay after three days. Differentiation protocol was used to enhance the cell death mediated by polyglutamine-expanded ataxin-3. We had observed earlier that the differentiation enhances the cell death mediated by mutant huntingtin (Wang *et al.*, 1999; Jana *et al.*, 2000) As shown in figure 1C, truncated ataxin-3 with 130Q caused a dramatic increase in cell death compared to full-length ataxin-3 with 130Q. The full-length and the truncated ataxin-3 with normal glutamine repeats had no influence on cell death. Results strongly suggest that the polyglutamine aggregates are linked to the cell death, and that not only the length of the glutamine repeats but also the corresponding protein size influences the aggregate formation and cell death.

Influence of Length of Glutamine Repeats and Size of Corresponding Protein on the Misfolding Propensity of Ataxin-3

Given that both the length of glutamine repeats and the size of the corresponding protein affect the ataxin-3 aggregation, we assumed that an increase in glutamine repeat length or decrease in protein size might disturb the proper folding of ataxin-3. Recently, several reports have demonstrated the association of the Hsp70 and Hsp40 family of chaperones with polyglutamine aggre-

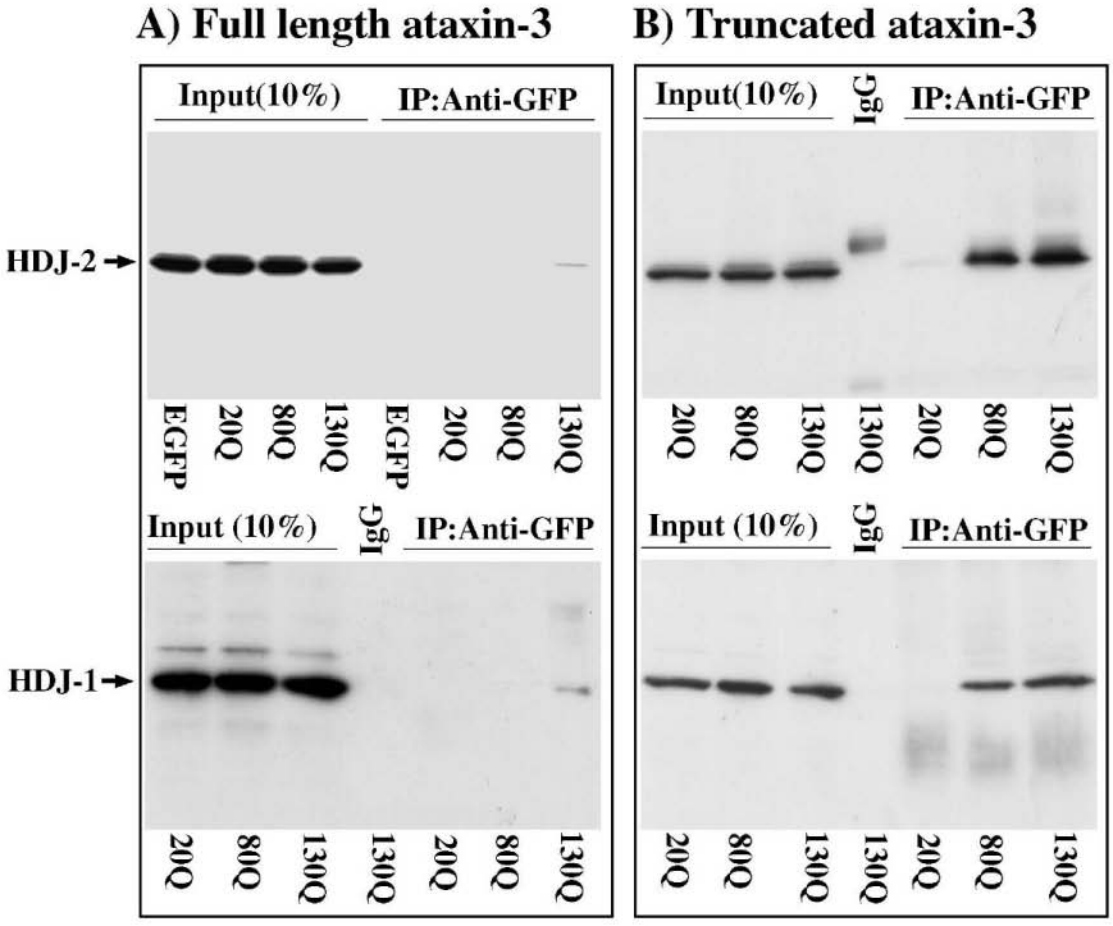

FIGURE 3 Truncation enhances the interaction of Hdj-1 and Hdj-2 chaperones with the polyglutamine-expanded ataxin-3. Neuro2a cells were transiently transfected with the different full-length and truncated ataxin-3-EGFP fusion constructs. After two days of post-transfection, cells were harvested and processed for immunoprecipitation by anti-GFP. Blots were probed with either Hdj-2 (upper panel) or Hdj-1 (lower panel) antibody. (**A**) Full-length ataxin-3-EGFP fusion proteins. (**B**) Truncated ataxin-3-EGFP fusion proteins.

gates. We used the interaction of those chaperones with the ataxin-3 as a tool with which to investigate ataxin-3 misfolding. Because all of the ataxin-3 proteins used in this study were expressed by fusion with GFP, we took advantage of the GFP antibody for co-immnoprecipitation experiments. As shown in figure 2A and 2B, the full-length and the truncated ataxin-3 with different glutamine repeats were immunoprecipitated well with GFP antibody. In the case of truncated ataxin-3 with 130Q, the soluble pool was very low as a result of massive aggregate formation. Next we performed similar co-immunoprecipitation experiments, in which the blots were probed with two different Hsp40 family chaperones, Hdj-1 and Hdj-2 (FIG. 3). Both Hdj-1 and Hdj-2 were weakly interacted with only full-length ataxin-3 with 130Q. However, the interactions were dramatically increased upon truncation of ataxin-3. A strong interaction of those chaperones with the truncated ataxin-3 with 80Q was also observed which was not detected with the full-length ataxin-3 with 80Q. Similar results were also observed when we studied the interaction of Hsc70/Hsp70 with the ataxin-3 (FIG. 4).

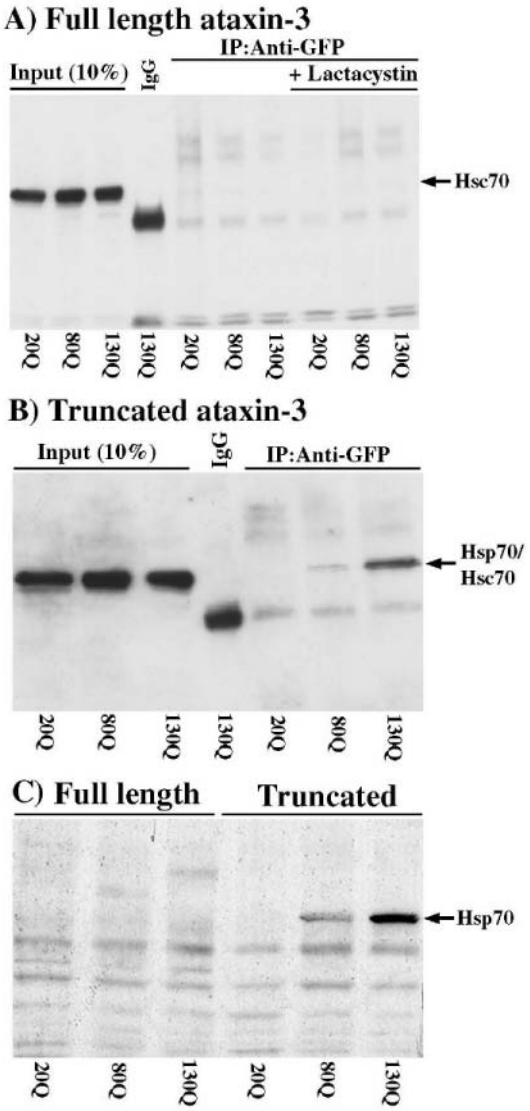

FIGURE 4 Truncated ataxin-3 with expanded polyglutamine interacts with Hsp70/Hsc70 and induces the Hsp70 expression. The transfections of the different ataxin-3-EGFP fusion constructs to the neuro2a cells and the immunoprecipitations of the fusion protein by GFP antibody were performed in a similar way as described in figure 1. (**A**) Full-length ataxin-3-EGFP fusion proteins with expanded polyglutamine do not interact with Hsc70/Hsp70. In some experiments, the transfected cells were treated with lactacystin, a proteasome inhibitor, for 24 h to induce Hsp70. The blot was detected with an antibody that recognizes both Hsc70 (constitutive form) and Hsp70 (inducible form). (**B**) Interaction of Hsc70/Hsp70 with the truncated ataxin-3 with expanded polyglutamine. (**C**) Induction of the Hsp70 upon expression of truncated ataxin-3 with expanded polyglutamine. Cells were harvested after three days of transfection.

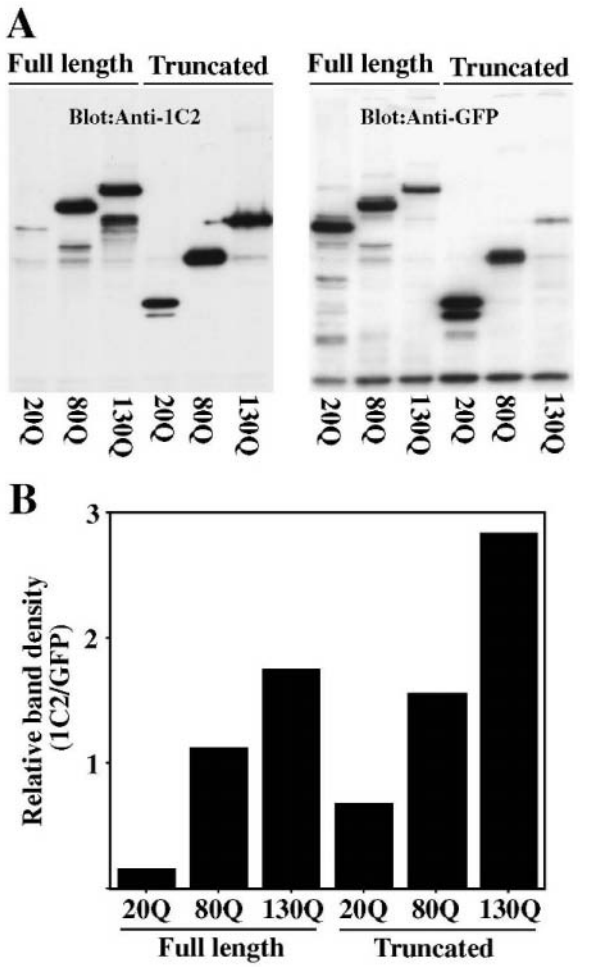

FIGURE 5 Truncation enhances the binding of 1C2 antibody with the ataxin-3-EGFP fusion proteins. Neuro2a cells were transfected with the different ataxin-3-EGFP fusion constructs in a similar manner as described in figure 1. The harvested cells were lysed in RIPA buffer with mild sonication, then the total cell lysate was processed for immunoblotting experiments. The blots were sequentially probed with 1C2 (**A**) and GFP (**B**) antibody. (**C**) The relative 1C2 antibody binding (1C2/GFP band densities) with the different full length and truncated ataxin-3-EGFP fusion proteins. The band intensities were measured using NIH image analyzer.

We used a particular antibody that recognized both Hsc70 and Hsp70. We were unable to detect any interaction of Hsc70/Hsp70 with the polyglutamine expanded full-length ataxin-3, though we were able to detect the interaction of Hsc70/Hsp70 with the truncated ataxin-3 in a polyglutamine length-dependent manner. To confirm the interaction of Hsp70 with the polyglutamine expanded full-length ataxin-3, we treated the transfected cells with lactacystin (a proteasome inhibitor which causes increased expression of Hsp70) 24 h before harvesting the cells. The interaction of both Hsc70 and Hsp70 with the truncated ataxin-3 was independently confirmed by using antibody specific for Hsc70 and Hsp70 (data not shown). We have also observed the association of HDJ-1, HDJ-2 and Hsc70 chaperones with the ataxin-3 aggregates (data not shown). The expression of Hsp70 was dramatically induced upon expression of truncated ataxin-3 with expanded glutamine repeats for three days, but not the full-length ataxin-3 with the same glutamine repeats over the same time period (FIG. 4C).

Increase in Glutamine Repeat Length and Decrease in Corresponding Protein Size Enhances the Binding of 1C2 Antibody with Ataxin-3

The 1C2 antibody has been demonstrated to selectively recognize and bind with the pathological range of polyglutamine expansion, and the binding was increased with the increase in glutamine repeat length without saturation (Trottier *et al.*, 1995). However, the minimum number of glutamine repeats required for 1C2 antibody recognition and also the recognition properties are not yet clear. Here we demonstrated that the full-length ataxin-3 with normal glutamine repeats (20Q) was weakly recognized by 1C2 antibody, and the signal intensity for 1C2 binding was strongly increased with the increase in disease range glutamine repeat length as expected (FIG. 5). Truncation of full-length ataxin-3 dramatically increased the signal intensity of 1C2 antibody binding. Normalization of the band intensity (1C2/GFP) revealed that the truncation of full-length ataxin-3 with 20Q and 130Q increased the 1C2 antibody binding by about 4, 2.5-fold, respectively (FIG. 5B). Results suggested that not only the length of the glutamine repeats but also the size of the corresponding protein modulates the 1C2 antibody binding. The 1C2 antibody also detected another band in full-length ataxin-3 with 80Q and 130Q, but not in their truncated forms, and the same bands could be detected by anti-GFP. This strongly suggests that the full-length ataxin-3 proteins are undergoing proteolytic cleavage, and the cleavage site is located within the truncated region. Figure 6 demonstrated the immunohistochemical staining of both full-length and truncated ataxin-3 containing 20Q and 130Q with 1C2 antibody. The 1C2 antibody was able to weakly detect the truncated ataxin-3 with 20Q but not the full-length ataxin-3 containing 20Q. The full-length and truncated ataxin-3 with 130Q was strongly detected by 1C2 antibody, and there was no apparent difference of the staining pattern between the cytoplasmic and nuclear ataxin-3. However, an earlier report demonstrates that the 1C2 antibody preferentially binds with the nuclear ataxin-3 (Perez *et al.*, 1999). Aggregates were mostly negative for 1C2 antibody staining, which strongly suggests that the polyglutamine domains were buried inside the aggregates. 1C2 antibody also did not detect the polyglutamine aggregates in the HD exon1 transgenic mice brain (data not shown).

Misfolded Ataxin-3 is Ubiquitinated

Ubiquitin is a well known marker of polyglutamine aggregates, but how and when polyglutamine aggre-

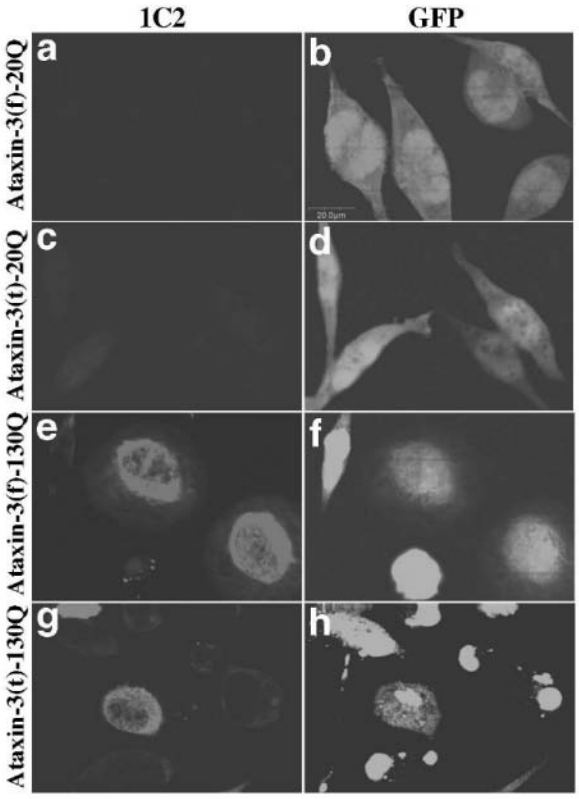

FIGURE 6 Immunofluorescence staining of the different full-length and truncated ataxin-3-EGFP fusion proteins with 1C2 antibody. Neuro2a cells were transfected with different ataxin-3-EGFP fusion constructs and two days after transfection, cells were processed for immunofluorescence staining for 1C2 antibody (**a**, **c**, **e**, and **g**) and compared with the respective fields (**b**, **d**, **f**, and **h**) for the expression of ataxin-3-EGFP using the fluorescence of GFP. **a** and **b**, full-length ataxin-3-EGFP with 20Q; **c** and **d**, truncated ataxin3-EGFP with 20Q; **e** and **f**, full-length ataxin-3-EGFP with 130Q; g and h, truncated ataxin-3-EGFP with 130Q.

gates are ubiquitinated is not yet known. The most likely hypothesis is that the expanded polyglutamine proteins are misfolded, and failure to refold might cause their ubiquitination before they are degraded by proteasome. There is also the possibility that the ubiquitination might occur after the formation of polyglutamine aggregates. Based on our observation that the increase in glutamine repeat length or the decrease in corresponding protein size causes misfolding of the ataxin-3, we decided to investigate whether similar conditions are responsible for the ubiquitination of ataxin-3. Therefore, we transfected the ataxin-3 constructs to the neuro2a cell, and after two days, cells were collected and processed for immunoprecipitation by GFP antibody. Blots were probed with anti-ubiquitin. As shown in figure 7A, the full-length ataxin-3 with 20Q and 80Q were not ubiquitinated, though, the full-length ataxin-3 with 130Q was. Furthermore, truncation of the full-length ataxin-3 containing 130Q massively increased its ubiquitination (Fig. 7B). The full-length ataxin-3 containing 80Q was not ubiquitinated, though truncation markedly increased the ubiquitination of this protein. These results were correlated with the misfolding and the aggregate formation of the full-length and the truncated ataxin-3.

DISCUSSION

Apart from Max Perutz's polar zipper hypothesis, it has recently become evident that the expanded polyglutamine proteins are misfolded, and that misfolding could be another major cause of polyglutamine protein aggregation. Most of the earlier reports regarding the misfolding of polyglutamine protein rely on the recruitment of various chaperones to the polyglutamine aggregates and the suppression of polyglutamine protein aggregation by those chaperones (Cummings *et al.*, 1998; Chai *et al.*, 1999a,b; Stenoien *et al.*, 1999; Warrick *et al.*, 1999; Jana *et al.*, 2000). We previously reported that the chaperones of the Hsp70 and Hsp40 family members interact with the polyglutamine-expanded huntingtin exon1 and suppress their aggregation (Jana *et al.*, 2000). By using those chaperones, we first demonstrate here that the misfolding propensity of the polyglutamine protein is not only directly dependent on glutamine repeat length but also inversely related to the corresponding protein size. Secondly, we show that the size of the polyglutamine proteins also inversely influences the binding of 1C2 antibody, an antibody that selectively recognizes polyglutamine expansion. Thirdly, both aggregation and cell death are dramatically increased as a consequence of the reduction of the polyglutamine protein size. Results strongly suggest that the pathogenesis of polyglutamine diseases is not only dependent on the length of the glutamine repeats but also on the corresponding protein size.

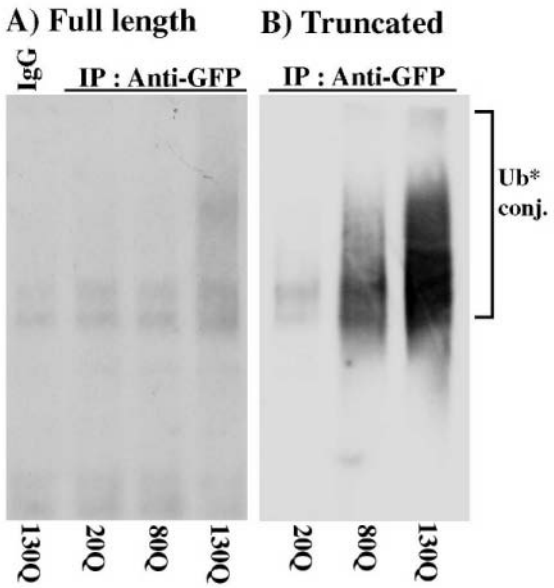

FIGURE 7 Misfolding dependent ubiquitination of ataxin-3. The cells were transfected with the different ataxin-3-EGFP fusion constructs and the total cell lysate was immunoprecipitated as described in figure 2. The blots were probed with ubiquitin antibody. (**A**) Full-length ataxin-3-EGFP fusion proteins. (**B**) truncated ataxin-3-EGFP fusion proteins.

It has been demonstrated that many of the polyglutamine disease proteins like huntingtin, atropin-1 are substrates for protease (Goldberg *et al.*, 1996; Wellington *et al.*, 1998), and therefore it is not surprising that the proteolytic processing of these disease proteins might generate aggregate-prone toxic fragments. In fact some reports have suggested that the inhibition of caspase cleavage of huntingtin, atropin-1, and androgen receptor reduce aggregate formation and cytoxicity (Ellerby *et al.*, 1999a,b ; Wellington *et al.*, 2000). Further, identification of several N-terminal fragments of huntingtin in the HD brain (DiFiglia *et al.*, 1997; Mende-Muller *et al.*, 2001) and of a 42 kDa fragment of ataxin-2 with an expanded glutamine repeat in the SCA2 brain (Huynh *et al.*, 2000) strongly suggest that the proteolytic processing could be a critical event at least in the pathogenesis of some polyglutamine diseases.

We observed that the truncation of the polyglutamine protein dramatically increases its misfolding and ability to bind with 1C2 antibody. However, a polyglutamine aggregate was not recognized by the 1C2 antibody, suggesting that the 1C2 antibody binding site might be buried inside the aggregates. Our results are very similar to the observation reported by others (Perez *et al.*,

1999) and we have also observed that the polyglutamine-expanded huntingtin aggregates are not recognized by 1C2 antibody. 1C2 antibody was demonstrated earlier to recognize the ataxin-3 aggregates (Hayashi *et al.*, 2003). We do not know the exact reason for this discrepancy. It is possible that some of the polyglutamine domains in the aggregates are exposed outside that might be weakly recognized by the 1C2 antibody. A previous report found that polyglutamine protein aggregation was prevented by 1C2 antibody (Heiser *et al.*, 2000). These findings, further suggest that the 1C2 antibody binding site might be involved at least in part in polyglutamine protein aggregation. 1C2 antibody has been demonstrated to detect a unique conformation that requires a minimum length of glutamine repeats and is stabilized by further increase in length (Trottier *et al.*, 1995). The typical conformational epitope recognized by 1C2 antibody is not yet known. We speculate that the 1C2 antibody recognizes a β-sheet conformation that is stabilized and exposed outside because of the truncation of the polyglutamine protein. The stabilized β-sheet conformation could be able to form an intramolecular polar zipper, or could conceivably cause misfolding. Therefore, the truncation-induced polyglutamine protein aggregation is most likely due to both polar zipper formation and misfolding.

Given that 1C2 antibody recognizes the pathological range (a range that could be able to form polar zipper) of glutamine repeats, the truncation induced 1C2 binding most likely indicates that the critical pathological range of glutamine repeats might depend on the corresponding protein context. Similarly, disease onset and severity could also be dependent not only on the glutamine repeats but also on the corresponding protein size.

Another interesting observation made in our investigation was the misfolding-dependent ubiquitination of ataxin-3. The full length ataxin-3 with 20 and 80 glutamine repeats were not ubiquitinated, whereas the full-length ataxin-3 with 130Q was. Truncation causes a massive ubiquitination of the ataxin-3 containing 80Q and 130Q but not of 20Q. The results are correlated with the misfolding propensity of the ataxin-3, and therefore led us to conclude that the misfolding drives the ataxin-3 towards ubiquitination and further degradation by proteasome. It is now well known that the polyglutamine aggregates are ubiquitinated, though how and when the aggregates are ubiquitinated is not yet known. Our results suggest that the misfolded ataxin-3 is ubiquitinated and aggregated before being degraded by proteasome. Similar findings were also reported in the case of polyglutamine-expanded truncated huntingtin (Jana *et al.*, 2001). It was previously demonstrated that the degradation of the polyglutamine protein (SCA1 and huntingtin) depends on the glutamine repeat length (Cummings *et al.*, 1999; Jana *et al.*, 2001) and the inhibition of ubiquitination partially blocks polyglutamine protein aggregation (Saudou *et al.*, 1998). Therefore, the slower degradation of the ataxin-3 containing longer glutamine repeats could be another reason for the massive accumulation of the ubiquitinated derivatives and the formation of ubiquitinated aggregates.

The question now is how the misfolded polyglutamine protein is recognized by the ubiquitination machine and whether chaperones play any role. This aspect of ubiquitination requires further investigation.

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