Molecular clearance of ataxin-3 is regulated by a mammalian E4

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Insoluble aggregates of polyglutamine-containing proteins are usually conjugated with ubiquitin in neurons of individuals with polyglutamine diseases. We now show that ataxin-3, in which the abnormal expansion of a polyglutamine tract is responsible for spinocerebellar ataxia type 3 (SCA3), undergoes ubiquitylation and degradation by the proteasome. Mammalian E4B (UFD2a), a ubiquitin chain assembly factor (E4), copurified with the polyubiquitylation activity for ataxin-3. E4B interacted with, and thereby mediated polyubiquitylation of, ataxin-3. Expression of E4B promoted degradation of a pathological form of ataxin-3. In contrast, a dominant-negative mutant of E4B inhibited degradation of this form of ataxin-3, resulting in the formation of intracellular aggregates. In a *Drosophila* model of SCA3, expression of E4B suppressed the neurodegeneration induced by an ataxin-3 mutant. These observations suggest that E4 is a rate-limiting factor in the degradation of pathological forms of ataxin-3, and that targeted expression of E4B is a potential gene therapy for SCA3.

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Introduction

Various inherited neurodegenerative diseases result from an increase in the number of glutamine codon repeats within the open reading frame of the responsible gene (Koshy and Zoghbi, 1997). Such disorders include Huntington's disease,

spinobulbar muscular atrophy, dentatorubral pallidoluysian atrophy (DRPLA), and spinocerebellar ataxia (SCA) types 1, 2, 3, 6, and 7 (Koshy and Zoghbi, 1997; Lunkes and Mandel, 1997). The presence of insoluble aggregates in neurons is a hallmark of these polyglutamine diseases as well as of many other neurodegenerative disorders, including Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, and prion diseases. Furthermore, these intracellular aggregates are conjugated with ubiquitin (Alves-Rodrigues *et al*, 1998; Hayashi *et al*, 1998). Expansion of polyglutamine repeats likely alters the conformation or results in misfolding of the disease-associated protein, thereby conferring a toxic gain of function that is selectively deleterious to neurons. Although many studies have suggested that an alteration in the ubiquitin-dependent pathway of protein degradation might underlie the pathogenesis of polyglutamine diseases, the molecular mechanism of the ubiquitylation of the disease proteins has been unclear.

The ubiquitylation of proteins serves to mark them for degradation by the proteasome, an ATP-dependent multi-protease complex. Protein ubiquitylation is achieved by a multistep mechanism involving several enzymes: a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and a ubiquitin-protein ligase (E3) (Hershko *et al*, 1983; Pickart, 1997; Hershko and Ciechanover, 1998). A new class of ubiquitylation enzyme, a ubiquitin chain assembly factor (E4), the prototype of which is yeast Ufd2, was recently identified (Koegl *et al*, 1999). In conjunction with E1, E2, and E3, E4 is required for the assembly of a polyubiquitin chain on certain types of substrate. In yeast, Ufd2 is implicated in cell survival under stressful conditions and is associated with Cdc48 (Koegl *et al*, 1999), which belongs to the large family of AAA-type ATPases that are thought to possess chaperone activity (Cruciat *et al*, 1999; Golbik *et al*, 1999). Ufd2 and its homologs in other eukaryotes share a conserved domain designated the U box. The U box of Ufd2 mediates the interaction of this protein with ubiquitin-conjugated targets and therefore appears to be an essential functional domain of E4 enzymes. We recently demonstrated that mammalian U-box proteins, including E4B (UFD2a), an ortholog of yeast Ufd2, also possess E3 activity (Hatakeyama *et al*, 2001). E4 activity might therefore reflect a specialized type of E3 activity that targets oligo-ubiquitylated proteins for further ubiquitylation (Aravind and Koonin, 2000; Hatakeyama *et al*, 2001; Cyr *et al*, 2002). Despite the conservation of U-box proteins from yeast to humans, however, the physiological significance of E4 enzymes remains unknown.

We have now shown that ataxin-3, the abnormal expansion of a polyglutamine tract that is responsible for SCA3 (also known as Machado-Joseph disease), is degraded by the ubiquitin-proteasome pathway. To identify the enzymes that mediate the ubiquitylation of ataxin-3, we partially purified a polyubiquitylation activity for this protein. We found that E4B was copurified with this activity and that E4B was required for the polyubiquitylation and degradation of, and

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Figure 1 Degradation of ataxin-3 by the ubiquitin-proteasome pathway. **(A)** HEK293T cells were transiently transfected with an expression vector encoding Myc-ataxin-3(13Q) or Myc-ataxin-3(79Q), or with the empty vector as a control (mock), and then cultured in the absence or presence of 10 μ M LLnL for 6 h. Cell lysates were then subjected to immunoprecipitation (IP) with anti-Myc, and the resulting precipitates were subjected to immunoblot (IB) analysis with either anti-ubiquitin (anti-Ub) or anti-Myc (upper and lower panels, respectively). **(B)** An *in vitro* ubiquitylation assay was performed with rabbit reticulocyte lysate and either GST-ataxin-3(13Q) or GST-ataxin-3(79Q). The reaction was terminated at the indicated times by the addition of SDS sample buffer, and the reaction mixture was subjected to IB analysis with anti-GST. **(C)** Extracts of control (mock) or Myc-ataxin-3(79Q)-expressing HEK293T cells were boiled in the presence of 1% SDS for 5 min. After dilution to 0.2% SDS, the extracts were subjected to IP with anti-Myc and the resulting precipitates were subjected to IB analysis with either anti-ubiquitin or anti-Myc. **(D)** HEK293T cells were transiently transfected with vectors for Myc-ataxin-3(79Q) and His₆-ubiquitin (His₆-Ub), as indicated. Ubiquitylated proteins were purified from cell lysates with a Ni-based resin in the presence of 4 M urea and then subjected to IB analysis with anti-Myc (upper panel). A portion (10%) of the input lysates was also subjected directly to IB analysis (lower panel). **(E)** HEK293T cells were transfected with the Myc-ataxin-3(13Q) vector and then labeled with [³⁵S]methionine for 1 h. The cells were then washed, cultured for the indicated times in the presence of dimethyl sulfoxide (DMSO, vehicle control) or 100 μ M LLnL, and lysed. Cell lysates were subjected to IP with anti-Myc, and the resulting precipitates were subjected to SDS-PAGE and autoradiography. **(F)** HEK293T cells expressing Myc-ataxin-3(13Q) or Myc-ataxin-3(79Q) were subjected to pulse-chase analysis (without LLnL) as described in (E).

prevented aggregate formation by, ataxin-3. In addition, we showed that expression of E4B prevented neurodegeneration in a fly model of SCA3. Our data thus provide biochemical and genetic evidence that a mammalian E4 enzyme plays an important role in the degradation of ataxin-3.

Results

Degradation of ataxin-3 by the ubiquitin-proteasome pathway

Although a proteasome inhibitor was previously shown to promote the formation of aggregates containing ataxin-3 in cultured cells (Chai *et al*, 1999), it has not been clear whether ataxin-3 itself is ubiquitylated and degraded by the proteasome. To determine whether ataxin-3 is a substrate for the

ubiquitin-proteasome pathway, we transfected HEK293T cells with expression vectors for Myc epitope-tagged forms of ataxin-3 containing a polyglutamine tract of either 13 (normal) or 79 (pathological) residues (13Q or 79Q, respectively), and then cultured the cells in the absence or presence of the proteasome inhibitor LLnL. Both forms of Myc-ataxin-3 were immunoprecipitated from cell lysates with antibodies to (anti-) the Myc epitope and then subjected to immunoblot analysis with anti-ubiquitin. The immunoprecipitates yielded a smeared signal with anti-ubiquitin (Figure 1A), indicating that they contained ubiquitylated proteins. The abundance of unmodified ataxin-3(79Q) was higher than that of unmodified ataxin-3(13Q), even though the cells were transfected with identical amounts of the corresponding expression vectors, suggesting that the stability of ataxin-3(79Q) was

greater than that of ataxin-3(13Q). In the absence of LLnL, the amount of ubiquitylated intermediates of ataxin-3(79Q) was greater than that of such intermediates of ataxin-3(13Q). LLnL treatment increased the level of ubiquitylated intermediates of ataxin-3(13Q) but not that of the intermediates of ataxin-3(79Q) ubiquitylation. The increased abundance of ubiquitylated intermediates of ataxin-3(79Q) as well as the reduced sensitivity of these intermediates to LLnL were suggestive of a defect in the proteasomal degradation, but not in the ubiquitylation, of this form of ataxin-3. Consistent with this notion, the rates of ubiquitylation of ataxin-3(13Q) and ataxin-3(79Q) *in vitro* were similar (Figure 1B). To confirm that the ubiquitin signal obtained with the ataxin-3 immunoprecipitates (Figure 1A) was attributable to ubiquitylation of ataxin-3, we subjected cell lysates to denaturation and subsequent renaturation before immunoprecipitation with anti-Myc and immunoblot analysis with anti-ubiquitin (Figure 1C). The smeared signal was again apparent, confirming that ataxin-3 is itself ubiquitylated. We also cotransfected HEK293T cells with the Myc-ataxin-3(79Q) vector and a vector encoding ubiquitin tagged with six histidine residues (His₆). Proteins modified with His₆-ubiquitin were then purified with a Ni-based resin under denaturing conditions and subjected to immunoblot analysis with anti-Myc. High-molecular-weight (HMW) immunoreactivity was detected by this assay (Figure 1D), confirming that ataxin-3 was conjugated with ubiquitin. The difference in the pattern of signals between Figures 1A and D may be explained if polyubiquitylated Myc-ataxin-3 contains several ubiquitin epitopes recognized by anti-ubiquitin, whereas only a single epitope is recognized by anti-Myc. Protein molecules with longer ubiquitin chains near the top of the gel may thus give rise to stronger signals with anti-ubiquitin, whereas the signal obtained with anti-Myc is likely proportional to the number of ataxin-3 molecules.

To examine whether ataxin-3 is degraded by the proteasome, we performed pulse-chase analysis with Myc-ataxin-3(13Q)-expressing cells incubated in the absence or presence of the proteasome inhibitor LLnL. Myc-ataxin-3(13Q) was rapidly degraded in the absence of LLnL, but the rate of degradation was markedly reduced by LLnL (Figure 1E). Furthermore, comparison of the turnover rates of Myc-ataxin-3(13Q) and Myc-ataxin-3(79Q) revealed that, although the size of the polyglutamine tract did not appear to affect the extent of ubiquitylation, the half-life of ataxin-3(79Q) was substantially greater than that of ataxin-3(13Q) (Figure 1F), being similar to that of ataxin-3(13Q) in cells treated with LLnL. These data suggested that ataxin-3 is ubiquitylated and subsequently degraded by the proteasome, and that pathological expansion of the polyglutamine tract renders this protein resistant to proteasomal degradation after its ubiquitylation. To examine the generality of this stabilizing effect of polyglutamine expansion, we performed similar experiments with atrophin-1, the protein in which expansion of a polyglutamine tract causes DRPLA. Increasing the size of the polyglutamine tract in atrophin-1 also rendered the protein resistant to proteasomal degradation (data not shown). Thus, polyglutamine expansion appears to confer abnormal stability on, and thereby facilitates the accumulation of, disease proteins, an effect that might contribute to the pathogenesis of polyglutamine diseases.

Purification of the polyubiquitylation complex for ataxin-3

To characterize the molecular mechanism of the ubiquitylation of ataxin-3, we established an *in vitro* ubiquitylation assay. Glutathione S transferase (GST) alone or a GST-ataxin-3(79Q) fusion protein was incubated with a rabbit reticulocyte lysate or mouse brain extract in the presence of an ATP-regenerating system. The reaction mixture was then subjected to immunoprecipitation with anti-GST, and ubiquitylated proteins were detected by immunoblot analysis with anti-ubiquitin. GST-ataxin-3(79Q), but not GST, underwent marked polyubiquitylation on incubation with either rabbit reticulocyte lysate or mouse brain extract (Figure 2A).

To purify the polyubiquitylation activity, we subjected rabbit reticulocyte lysate to fractionation by several steps of column chromatography (Figure 2B). The resulting fractions were assayed for ubiquitylation activity in the presence of ubiquitin, E1, and E2. Size-exclusion chromatography yielded two peaks of ubiquitin-conjugation activity for ataxin-3, in HMW fractions (fractions 11–14; ~400–1000 kDa) and a low-molecular-weight (LMW) fraction (fraction 17; ~100 kDa) (Figure 2C). The HMW fractions contained polyubiquitylation activity and the LMW fraction contained mostly oligo-ubiquitylation activity (also see Figure 2G). These results suggested that the LMW fraction contained a partial complex that lacks a component (or components) required for extension of the ubiquitin chain of oligo-ubiquitylated ataxin-3, whereas the HMW fractions contained the complete complex responsible for polyubiquitylation. By analogy to the yeast Ufd pathway, in which E1, E2, and E3 mediate oligo-ubiquitylation but E4 (Ufd2) is also required for polyubiquitylation, the factor missing from the LMW fraction was likely a putative mammalian E4.

Further purification of the polyubiquitylation activity present in the HMW fractions was unsuccessful. With the use of a surface plasmon resonance sensor, however, we observed that the HMW fractions contained an ataxin-3-binding factor (data not shown). This factor was purified by ataxin-3-based affinity chromatography, which yielded a predominant protein of 97 kDa (Figure 2D). This ataxin-3-binding protein was subjected to in-gel digestion to identify its amino-acid sequence. The amino-acid sequences of three peptides generated from this protein were almost identical to that of mouse VCP, an AAA-type ATPase (Figure 2E); the observed sequence differences likely reflect the difference in species (mouse and rabbit).

E4B as a mammalian E4 for ataxin-3 polyubiquitylation

Cdc48, a *Saccharomyces cerevisiae* homolog of VCP, is implicated in the Ufd (ubiquitin-fusion degradation) pathway, by which artificial ubiquitin-fusion proteins are degraded (Johnson *et al*, 1995; Ghislain *et al*, 1996). Cdc48 binds to both Ufd2 (E4) and the ubiquitin-fusion protein (substrate) (Koegl *et al*, 1999). The analogy with Cdc48 and Ufd2 in yeast and our observation that VCP copurified with the polyubiquitylation activity in the HMW fractions suggested that E4B, a mammalian homolog of Ufd2, might contribute to the polyubiquitylation of ataxin-3. Immunoblot analysis revealed the presence of E4B in the HMW fractions but not in the LMW fraction (Figure 2F). Furthermore, the addition of recombinant E4B to the LMW fraction restored polyubiquitylation activity for ataxin-3 (Figure 2G), indicating that the missing

Figure 2 Copurification of VCP and E4B with polyubiquitylation activity for ataxin-3. **(A)** Recombinant GST or GST-Myc-ataxin-3(79Q) was incubated for 0 or 1 h (reaction: (-) and (+), respectively) with rabbit reticulocyte lysate (RRL) or mouse brain extract (MBE) in the presence of an ATP-regenerating system. The reaction mixture was then subjected to immunoprecipitation with anti-GST, and the resulting precipitates were subjected to immunoblot analysis either with anti-ubiquitin (upper panel) or with anti-Myc (lower panel). The ubiquitylated material smaller than the full-length substrate corresponds to ubiquitylated degradation products of Myc-ataxin-3(79Q). **(B)** Protocol for purification of polyubiquitylation activity from rabbit reticulocyte lysate. **(C)** Rabbit reticulocyte lysate fractions from the Superose 6 gel filtration column were assayed for polyubiquitylation activity with GST-ataxin-3 as in **(A)**. The positions of polyubiquitylated (poly-Ub) and oligo-ubiquitylated (oligo-Ub) GST-ataxin-3 are indicated on the right. The elution of molecular size standards from the column is indicated by arrowheads. L, sample loaded onto the Superose 6 column. **(D)** The HMW fractions from the Superose 6 column were subjected to chromatography on a GST or GST-ataxin-3(79Q) affinity column. Portions of the applied sample and the eluates were analyzed by SDS-PAGE and silver staining. The position of a 97-kDa ataxin-3-binding protein (MBP97) is indicated. **(E)** Comparison of the amino-acid sequences of three peptides derived from MBP97 with internal sequences of mouse VCP. **(F)** Superose 6 column fractions were subjected to immunoblot analysis with anti-E4B. **(G)** The *in vitro* ubiquitylation assay was performed with GST-ataxin-3(79Q) as substrate and the indicated combinations of purified enzymes (E1, E2, and E4B) and the LMW fraction from the Superose 6 column as a crude source of E3. **(H)** The *in vitro* ubiquitylation assay was performed with GST-ataxin-3(79Q) and the HMW fractions from the Superose 6 column in the absence or presence of recombinant E4BAU (1 μ g).

factor was indeed E4B. In contrast, the addition of the recombinant mutant E4BAU, which exerts a dominant-negative effect on endogenous E4B (see Figure 6), inhibited the polyubiquitylation activity in the HMW fractions (Figure 2H). Given that E4B did not support polyubiquitylation in the presence of only E1 and E2 (in the absence of the LMW fraction), E4B alone did not show E3 activity for ataxin-3.

These data suggest that E4B functions as an E4 for polyubiquitylation of ataxin-3.

Binding of VCP to ataxin-3 in a polyglutamine-dependent manner

The possible interaction between ataxin-3 and VCP *in vivo* was examined by a co-immunoprecipitation assay with

Figure 3 Interaction between VCP and ataxin-3 *in vivo* and *in vitro*. **(A)** HEK293T cells were transiently transfected with an expression vector for Myc-ataxin-3(79Q) or with the empty vector as a control. Cell lysates were subjected to immunoprecipitation with anti-Myc, and the resulting precipitates as well as the original lysates (load) were subjected to immunoblot analysis with anti-VCP or anti-Myc. **(B)** GST, GST-ataxin-3(0Q, 13Q, or 79Q), or GST-fusion proteins of the indicated ataxin-3 deletion mutants were incubated for 30 min at room temperature with recombinant HA-VCP. GST or the GST-fusion proteins were then precipitated with glutathione-sepharose beads and subjected to immunoblot analysis with anti-HA (top panel) or anti-GST (middle panel). A portion (10%) of the input binding mixture was also subjected directly to immunoblot analysis with anti-HA (bottom panel). **(C)** Schematic representation of the ataxin-3 deletion mutants and summary of the data obtained from the *in vitro* assay of the binding of ataxin-3 derivatives to VCP.

HEK293T cells transiently expressing Myc-ataxin-3(79Q). Immunoblot analysis revealed that endogenous VCP was specifically precipitated with anti-Myc (Figure 3A). The reciprocal co-immunoprecipitation experiment confirmed the interaction between ataxin-3(79Q) and VCP (data not shown). The interaction of ataxin-3(79Q) with VCP was also apparent with purified recombinant proteins *in vitro* (Figure 3B), suggesting that VCP directly associates with ataxin-3(79Q). Although ataxin-3(13Q) and an ataxin-3 mutant lacking a polyglutamine tract (0Q) also interacted with VCP *in vitro*, the amount of VCP bound to ataxin-3 decreased as the size of the polyglutamine tract decreased. Furthermore, we tested a series of ataxin-3 deletion mutants for the ability to bind VCP in order to determine the region of ataxin-3 required for binding (Figures 3B and C). Two NH₂-terminal deletion mutants (ΔN79QC and 79QC) of ataxin-3(79Q) interacted with VCP, whereas a deletion mutant lacking the COOH-terminal region and the polyglutamine tract (ΔQC) as well as an NH₂-terminal deletion mutant (13QC) of ataxin-3(13Q) failed to bind to VCP. Thus, VCP appears to bind to a region of ataxin-3 that includes the polyglutamine tract in a manner dependent on the size of the latter.

Formation of a trimolecular complex by ataxin-3, VCP, and E4B

To determine whether VCP interacts with E4B *in vivo*, we performed co-immunoprecipitation analysis with transfected HEK293T cells. FLAG epitope-tagged E4B was detected in hemagglutinin epitope (HA)-tagged VCP immunoprecipitates (Figure 4A). This interaction was further confirmed by an *in vitro* binding assay with purified recombinant FLAG-E4B and GST-VCP proteins (Figure 4B). Wild-type E4B thus bound to

GST-VCP but not to GST. A deletion mutant of E4B that lacks the COOH-terminal U-box domain (E4BΔU) also bound to VCP, whereas a mutant that lacks the NH₂-terminal domain (E4BΔN) did not. The association of E4B with VCP thus does not require the U-box domain and appears to be mediated through the NH₂-terminal region of E4B. Interaction between endogenous VCP and E4B was confirmed in an HMW fraction of rabbit reticulocyte lysate (Figure 4C).

We also examined the interactions among ataxin-3(79Q), VCP, and E4B *in vitro* (Figure 4D). Purified recombinant GST-ataxin-3(79Q) was mixed with recombinant FLAG-E4B, and GST-ataxin-3(79Q) was then precipitated with glutathione-sepharose beads. The low level of association apparent between E4B and ataxin-3(79Q) was markedly increased by the inclusion of HA-VCP in the original incubation mixture. In contrast, VCP interacted with ataxin-3(79Q) to similar extents in the absence or presence of E4B. Together, these results suggest that VCP binds directly to ataxin-3 and to E4B, and thereby mediates the interaction between ataxin-3 and E4B. They also suggest that this interaction does not require the ubiquitylation of ataxin-3.

Promotion by E4B of ataxin-3 degradation

The association of E4B with ataxin-3 prompted us to determine whether E4B affects ataxin-3 degradation. Coexpression of recombinant E4B reduced the abundance of both ataxin-3(13Q) and ataxin-3(79Q) in HEK293T cells, although the effect on the amount of ataxin-3(13Q) was more marked (Figure 5A). Given that the amount of the corresponding ataxin-3 mRNAs was not affected by overexpression of E4B, the effect of this protein on ataxin-3 abundance appears to be mediated at the post-transcriptional level. Pulse-chase

Figure 4 Interaction of E4B with VCP and ataxin-3. (A) HEK293T cells transiently expressing HA-VCP and FLAG-E4B were subjected to immunoprecipitation with anti-FLAG, and the resulting precipitates were subjected to immunoblot analysis with anti-HA or anti-FLAG. A portion (10%) of the input lysates was also subjected directly to immunoblot analysis with anti-HA and anti-FLAG. (B) Recombinant GST or GST-VCP was mixed with recombinant FLAG-tagged wild-type (Wt) E4B, a deletion mutant lacking the U-box domain (ΔU), or an NH₂-terminal deletion mutant (ΔN). Proteins precipitated with glutathione-sepharose beads were then subjected to immunoblot analysis with anti-FLAG (top panel) or anti-GST (middle panels). A portion (10%) of the input binding mixture was also subjected directly to immunoblot analysis with anti-FLAG (bottom panel). (C) An HMW fraction (Superose 6, fraction 13) of rabbit reticulocyte lysate was subjected to immunoprecipitation with anti-E4B or normal immunoglobulin, and the resulting precipitates were subjected to immunoblot analysis with anti-VCP and anti-E4B. (D) GST or GST-ataxin-3(79Q) was mixed with FLAG-E4B and HA-VCP, as indicated, and then precipitated with glutathione-sepharose beads. The precipitated proteins were subjected to immunoblot analysis with anti-FLAG, anti-HA, or anti-GST.

analysis revealed that coexpression of E4B markedly increased the rates of degradation of both ataxin-3(13Q) and ataxin-3(79Q) by similar extents (Figure 5B). Overexpression of E4B had no effect on the turnover rate of cyclin E, which is also degraded by the ubiquitin-proteasome pathway, excluding the possibility that the promotion of ataxin-3 degradation by E4B reflects a nonspecific activation of protein ubiquitylation.

To confirm further the effect of E4B on ataxin-3 abundance, we subjected HeLa cells stably expressing Myc-ataxin-3(79Q) to transient transfection with a vector for HA-E4B. Immunofluorescence analysis revealed that coexpression of E4B resulted in a loss of Myc immunoreactivity (Figure 5C). Given that such an effect was not induced by coexpression of CHIP, another U-box protein, it appeared to be E4B specific.

Quantitative analysis indicated that the abundance of ataxin-3(79Q) was reduced to below the limit of detection in >90% of cells coexpressing E4B (Figure 5D); for comparison, the corresponding values for cells coexpressing CHIP or β-catenin were <13%. Collectively, these data suggest that E4B promotes the degradation of ataxin-3, and that this effect surmounts the stabilization of ataxin-3 conferred by expansion of the polyglutamine tract.

Stabilization and aggregation of ataxin-3 induced by inhibition of E4B function

We recently showed that the U-box domain is functionally indispensable for polyubiquitylation mediated by U-box proteins (Hatakeyama *et al*, 2001). Thus, the E4B mutant E4BΔU,

Figure 5 Promotion by E4B of ataxin-3 degradation. (A) HEK293T cells were transiently transfected with vectors for Myc-ataxin-3(13Q) (30 µg) or Myc-ataxin-3(79Q) (10 µg), in the absence or presence of a vector encoding FLAG-E4B (10 µg). The cells were subsequently lysed and subjected either to immunoblot analysis with anti-Myc (upper panel) or to Northern blot analysis with an ataxin-3 cDNA probe (lower panel). (B) HEK293T cells were transiently transfected with expression plasmids encoding Myc-cyclin E, Myc-ataxin-3(13Q), or Myc-ataxin-3(79Q), in the absence (mock) or presence of a vector for FLAG-E4B. The cells were labeled with [³⁵S]methionine for 1 h, washed, and cultured for the indicated times before lysis. Cell lysates were subjected to immunoprecipitation with anti-Myc, and the resulting precipitates were subjected to SDS-PAGE and autoradiography (left panel). The Myc-ataxin-3 signals from two independent experiments were quantitated (right panel; ataxin-3(13Q) (squares) and ataxin-3(79Q) (circles) in the absence or presence of E4B are represented by open and closed symbols, respectively). (C) HeLa cells stably expressing Myc-ataxin-3(79Q) were transiently transfected with vectors encoding either HA-E4B (left panels) or HA-CHIP (right panels). The cells were then subjected to immunofluorescence analysis with anti-Myc (top panels) or anti-HA (middle panels) and were stained with Hoechst 33258 dye (bottom panels). Myc-ataxin-3(79Q) was not apparent in cells expressing HA-E4B (arrowheads), but was observed in cells expressing HA-CHIP. Scale bars, 50 µm. (D) The number of HA-positive cells in which Myc immunoreactivity was not detected was expressed as a percentage of all HA-positive cells in experiments similar to that shown in (C); in some instances, HA-β-catenin was used as the control protein instead of HA-CHIP. Data are means ± SD of values from three independent experiments (200 HA-positive cells counted in each).

which lacks the U-box domain, might be expected to function in a dominant-negative manner. We therefore examined the effect of expression of this mutant on the degradation of ataxin-3(79Q). Pulse-chase analysis revealed that coexpression of E4BAU resulted in a marked delay in the degradation of ataxin-3(79Q) in HEK293T cells (Figure 6A). We also tested whether endogenous ataxin-3 was affected by expression of E4BAU in the neuronal cell line Neuro2A. Expression of E4BAU almost completely abrogated the degradation of endogenous ataxin-3 in these neuronal cells (Figure 6B), suggesting that E4B contributes to the degradation of ataxin-3 under physiologically relevant conditions.

Full-length ataxin-3 containing an expanded polyglutamine tract has previously been shown not to form aggregates in cultured cells, whereas expression of an NH₂-terminal deletion mutant of pathological ataxin-3 results in the formation of pronounced intracellular inclusions (Ikeda *et al*, 1996). Culture of cells in the presence of a proteasome inhibitor, however, also results in the formation of aggregates by full-length ataxin-3 containing an expanded polyglutamine tract (data not shown). Aggregate formation might thus reflect insufficient clearance of the pathological protein. Given the dominant-negative effect of E4BAU on ataxin-3 degradation, we examined the effect of this mutant

Figure 6 Stabilization and aggregation of ataxin-3 induced by inhibition of E4B function. (A) HEK293T cells were transiently transfected with an expression vector for Myc-ataxin-3(79Q) together with either expression plasmids for wild-type E4B or the mutant E4BΔU or the empty vector alone (mock). Cells were labeled with [³⁵S]methionine for 1 h, washed, and cultured for the indicated times before lysis. Lysates were subjected to immunoprecipitation with anti-Myc, and the resulting precipitates were analyzed by SDS-PAGE and autoradiography. (B) Neuro2A cells were infected either with a control retrovirus (mock) or with a retrovirus encoding FLAG-E4BΔU, and were then incubated with 50 μg/ml cycloheximide for 0–6 h. Cell lysates were then subjected to immunoblot analysis with anti-ataxin-3 and anti-Hsp90 (control). (C) HeLa cells were first infected with a control retrovirus (mock, left panels) or a retrovirus encoding FLAG-E4BΔU (right panels), and were then transiently transfected with a vector for Myc-ataxin-3(79Q). The cells were then stained with Hoechst 33258 (bottom panels) and subjected to immunofluorescence analysis with anti-ataxin-3 (top panels) and anti-FLAG (middle panels). Arrowheads indicate ataxin-3 aggregates in or adjacent to the nucleus. Scale bars, 25 μm. The percentage of inclusion-positive cells was estimated by scoring 200 cells in each of two independent experiments.

on ataxin-3 aggregation. HeLa cells stably expressing E4BΔU were transiently transfected with a vector for Myc-ataxin-3(79Q). In control cells not expressing E4BΔU, ataxin-3(79Q) appeared to be distributed evenly throughout the cytoplasm, with <1% of cells exhibiting ataxin-3 aggregates (Figure 6C). In contrast, a markedly larger number of cells expressing E4BΔU (~8%) manifested intra- or perinuclear aggregates containing ataxin-3. These results suggest that perturbation of the E4B-dependent degradation pathway results in ataxin-3 aggregation.

Suppression of ataxin-3 neurotoxicity by E4B in a *Drosophila* model

To examine the effect of E4B on ataxin-3-induced neurodegeneration *in vivo*, we established transgenic flies expressing

Figure 7 Suppression of ataxin-3-induced neurodegeneration by E4B in *Drosophila*. The compound eyes of transgenic flies expressing either GAL4 alone (*sev-Gal4/+*) (A, D), Myc-ataxin-3ΔN79QC (*UAS-ataxin-3ΔN79QC/sev-Gal4*) (B, E), or both Myc-ataxin-3ΔN79QC and FLAG-E4B (*UAS-ataxin-3ΔN79QC/sev-Gal4; UAS-E4B/+*) (C, F) were examined by SEM at low and high (insets) magnification (A–C) and by the optical neutralization method (D–F). Scale bars: 100 μm (entire eye) or 10 μm (insets). (G) Immunoblot analysis for the expression of transgenes. Lane 1: GAL4 (*GMR-Gal4/+*); lane 2: Myc-ataxin-3ΔN79QC (*UAS-ataxin-3ΔN79QC/GMR-Gal4*); lane 3: Myc-ataxin-3ΔN79QC; FLAG-E4B (*UAS-ataxin-3ΔN79QC/GMR-Gal4; UAS-E4B/+*).

E4B or an NH₂-terminal deletion mutant of ataxin-3 with an expanded polyglutamine tract (ataxin-3ΔN79QC), the latter of which efficiently induces aggregate formation in mammalian cells (data not shown). Targeted expression of transgenes was achieved by the GAL4/UAS system (Brand and Perrimon, 1993). Crossing of flies harboring a target gene downstream of the UAS element with a proper *Gal4* line allows expression of the gene in a tissue-specific manner. We first crossed a *UAS-ataxin-3ΔN79QC* line with the *GMR-Gal4* line that induces a strong expression of the transgene in compound eyes and brain during their development (Hay *et al*, 1997). However, the expression of ataxin-3ΔN79QC by this promoter resulted in pupal death. The expression of ataxin-3ΔN79QC was then induced by crossing the transgenic flies with the *sev-Gal4* line, which expresses GAL4 in particular photoreceptor precursor cells in the eye disc during the larval and pupal period (Banerjee *et al*, 1987). Scanning electron microscopy (SEM) revealed a disorganized external morphology of the compound eyes of adult flies that was possibly caused by neurodegeneration induced by ataxin-3ΔN79QC expression (Figure 7). We confirmed that the arrangement of photoreceptor cells was disrupted by using the optical neutralization technique. Images revealed that the trapezoidal organization of photoreceptors was almost completely absent

from the compound eyes of flies expressing the ataxin-3 mutant. The pupal death in *GMR-Gal4* crossed line and the eye phenotype in *sev-Gal4* crossed line were clearly suppressed by E4B in the double-transgenic flies. The expression of transgenes was confirmed by immunoblotting experiments at the pupal stage (Figure 7G). Ataxin-3 Δ N^{QC} was reduced by the expression of E4B in double-transgenic flies. The biochemical data are consistent with the notion that E4B suppresses the neurotoxicity of ataxin-3 proteins with an expanded polyglutamine tract by reducing its abundance through ubiquitin-mediated degradation.

Discussion

We have demonstrated that an ataxin-3 protein with an expanded polyglutamine tract is more stable than the normal ataxin-3 protein. The stability of ataxin-1 is also increased as a result of expansion of its polyglutamine tract (Cummings *et al*, 1999). High concentrations of wild-type ataxin-1 were recently shown to induce a degenerative phenotype similar to that elicited by the pathological protein with an expanded polyglutamine tract (Fernandez-Funez *et al*, 2000). Expansion of the polyglutamine tract thus likely damages neurons not only as a result of its inherent toxic effect but also through the stabilization and consequent accumulation of the responsible protein.

Our data indicate that normal ataxin-3 and pathological ataxin-3 with an expanded polyglutamine tract are ubiquitinated to similar extents, suggesting that stabilization of the abnormal protein is not due to inefficient formation of a polyubiquitin chain. The stabilization of pathological forms of ataxin-3 therefore likely results from an impairment in the recognition of the ubiquitinated protein or in its degradation by the proteasome. E4B might contribute not only to polyubiquitination but also, in conjunction with VCP, to transfer of the substrate to the proteasome.

Our data suggest that E4 is a rate-limiting factor in the degradation of ataxin-3. Furthermore, a qualitative difference in the ubiquitin chain formed by E4B might also be critical for the promotion of ataxin-3 degradation; whereas regular E3 enzymes conjugate the COOH-terminal glycine of one ubiquitin molecule to the internal lysine-48 of the adjacent ubiquitin molecule, E4B does not exhibit a preference for lysine-48 but rather forms polyubiquitin chains by targeting any of the lysine residues of the adjacent ubiquitin molecule (Hatakeyama *et al*, 2001). Such a pattern of ubiquitin linkage formed by E4B might be responsible for the enhanced degradation of pathological ataxin-3 observed in cells overexpressing this E4.

We identified VCP as an ataxin-3-associated protein that copurified with polyubiquitylation activity for ataxin-3. VCP is thought to function as a multiubiquitin chain-binding protein that is required for the degradation of many substrates by the ubiquitin-proteasome pathway (Dai *et al*, 1998; Dai and Li, 2001). VCP neither exhibits E3 activity nor promotes the activity of E3 enzymes by itself (data not shown). VCP does possess protein 'unfoldase' activity, suggesting that it might mediate the dissociation of ubiquitinated substrates from E3 or E4 in order to facilitate their transfer to the proteasome. On the basis of these observations and on our present demonstration that ataxin-3 forms a ternary complex with VCP and E4B, we propose that VCP mediates

the dissociation of ubiquitinated ataxin-3 from E4B, but that expansion of the polyglutamine tract of ataxin-3 increases its affinity for VCP and thereby inhibits its release from VCP-E4B, delaying its degradation by the proteasome. This hypothesis could be tested by comparing the rates of dissociation of ubiquitinated normal and pathological ataxin-3 proteins from the complex of VCP and E4B.

Cdc48 forms a complex with Ufd1-Npl4 and participates in the endoplasmic reticulum-associated degradation (ERAD) system, which mediates the ubiquitination of misfolded proteins in the endoplasmic reticulum followed by their export to the cytosol and degradation by the proteasome (Ye *et al*, 2001; Braun *et al*, 2002; Jarosch *et al*, 2002; Rabinovich *et al*, 2002). Unlike Ufd1, Ufd2, Ufd4, or Ufd5 did not appear to contribute to this system (Ye *et al*, 2001). However, yeast *ufd2-rpn10* double mutants exhibit increased sensitivity to various stressors (Koege *et al*, 1999), suggesting that ubiquitylation mediated by Ufd2 may play an important role in the clearance of damaged proteins (probably in the cytosol). Neurons and other cells not able to dilute the intracellular concentration of incorrectly folded proteins by cell division require a system for the removal of such molecular waste in order to maintain the integrity of cellular functions for extended periods. E4B is abundant in neurons of both mice and *Caenorhabditis elegans* (data not shown), suggesting that it might contribute to the clearance of damaged proteins in the nervous system. The possible role of E4B in the clearance of abnormal proteins responsible for other neurodegenerative diseases remains to be determined.

The observation that neurodegeneration is reversible in Htt transgenic mice suggests that polyglutamine disease is a result of reversible neuronal dysfunction rather than a consequence of neuronal death (Yamamoto *et al*, 2000). Given that the accumulation of polyglutamine-containing disease proteins is likely the cause of such neuronal dysfunction, one approach to treatment would be to regulate the expression of these proteins. One possible means of achieving this goal might be to increase the activity of the ubiquitin-proteasome system. We have shown that E4B is a rate-limiting factor in the degradation of ataxin-3, and that expression of E4B both promotes the degradation of pathological ataxin-3 in cultured cells as well as prevents neurodegeneration in a fly model of SCA3. Targeted expression of E4B is therefore a potential gene therapy for SCA3.

Materials and methods

Antibodies

Polyclonal antibodies to human ataxin-3, mouse VCP, and GST were generated in rabbits with bacterially produced recombinant proteins as antigens. Rabbit polyclonal antibodies to mouse E4B were produced as described (Hatakeyama *et al*, 2001).

Expression plasmids

Cloning of human ataxin-3(13Q) and ataxin-3(79Q) was described (Ikeda *et al*, 1996). Complementary DNAs for NH₂-terminally Myc-tagged ataxin-3(13Q), ataxin-3(79Q) and their deletion mutants, for NH₂-terminally HA-tagged mouse VCP, and for NH₂-terminally FLAG-tagged mouse E4B and its deletion mutants were subcloned into pcDNA3 (Invitrogen) or pGEX-6P1 (Amersham Pharmacia Biotech). A cDNA for His₆-tagged ubiquitin was subcloned into pRSET (Invitrogen) and into pCI-neo (Invitrogen).

Production of recombinant proteins

GST-fusion proteins were purified from *Escherichia coli* with glutathione-sepharose 4B (Amersham Pharmacia Biotech). In some instances, the GST moiety was removed from the fusion proteins by treatment with Precision protease (Amersham Pharmacia Biotech).

Baculovirus expression system

A cDNA for HA-VCP was subcloned into pBacPAK9-GST, and cDNA for FLAG-E4B was into pFASTBAC HT (Life Technologies). The recombinant baculoviruses were generated with the BacPAK baculovirus expression system (Clontech) or the Bac-to-Bac Baculovirus Expression System (Life Technologies). The recombinant proteins were purified from baculovirus-infected Sf9 cells with glutathione-sepharose or ProBond resin (Invitrogen).

Transfection, immunoprecipitation, and immunoblot analysis

HEK293T cells were transfected by the calcium phosphate method (Wigler *et al*, 1977) and were subjected to immunoprecipitation assays as described (Hatakeyama *et al*, 2001). For the denaturing condition, cell extracts were boiled in the presence of 1% SDS for 5 min and then diluted to 0.2% SDS with 50 mM Tris-HCl (pH 7.4), 150 mM NaCl and 0.5% Triton X-100. Immunoblot analysis was performed with the following primary antibodies: anti-Myc (1 µg/ml; 9E10, Covance), anti-FLAG (1 µg/ml; M5, Sigma), anti-HA (1 µg/ml; HA11, Babco), anti-GST (1:5000 dilution of antiserum) (1 µg/ml; B2, MBL), anti-ubiquitin (1 µg/ml; 1B3, MBL), and anti-E4B (1 µg/ml).

In vitro binding assay

GST or GST-fusion proteins (1 µg) were incubated for 30 min at room temperature with recombinant test proteins (0.5 µg) in 200 µl of a solution containing 50 mM Tris-HCl (pH 7.4), 150 mM KCl, and 0.1% Triton X-100. They were then precipitated with glutathione-sepharose 4B and subjected to immunoblot analysis.

Fractionation of rabbit reticulocyte lysate and purification of ataxin-3-binding protein

Rabbit reticulocyte lysate was prepared as described (Hershko, 1983). To purify the polyubiquitylation activity for ataxin-3, we applied the lysate to a column of Q-sepharose (Amersham Pharmacia Biotech) that had been equilibrated with buffer B (50 mM Tris-HCl (pH 7.4), 0.1 mM dithiothreitol). After washing the column with buffer B, bound proteins were eluted with a linear gradient of 0–1 M KCl in buffer B. Fractions containing the polyubiquitylation activity (which eluted at ~0.4 M KCl) were pooled and subjected to precipitation with 35% ammonium sulfate. The resulting precipitate was dissolved in 5 mM potassium phosphate (pH 7.0) and applied to a hydroxyapatite column. The flow-through fraction was collected and subjected to size-exclusion chromatography on Superose 6 (Amersham Pharmacia Biotech) in buffer B containing 150 mM KCl. For affinity purification of the ataxin-3-binding protein, the HMW fractions (fractions 11–14) from the Superose 6 column were applied to a GST-sepharose column, and the resulting flow-through fraction was applied to a GST-ataxin-3(79Q)-sepharose affinity column. Bound proteins were eluted with 100 mM glycine (pH 2.9) and separated by SDS-polyacrylamide gel electrophoresis (PAGE). The band corresponding to the ataxin-3-binding protein was excised and subjected to in-gel digestion with Lys-C endopeptidase (Wako). The peptides generated from the band were separated by reversed-phase high-performance liquid chromatography and sequenced with an automated gas-phase Edman sequencer. The LMW fraction (fraction 17) from the Superose 6 column was subjected to chromatography on a Mono Q column (Amersham Pharmacia Biotech) with a gradient of 50–500 mM KCl, and the resulting active fraction was used as a source of oligo-ubiquitylation activity.

In vitro ubiquitylation assay

Recombinant GST (control) or GST-ataxin-3(79Q) (0.5 µg each) was incubated for 1 h at 37°C with rabbit reticulocyte lysate (20 µg)

or fractions in a final volume of 20 µl containing an ATP-regenerating system (50 mM Tris-HCl (pH 7.4), 5 mM MgCl₂, 2 mM ATP, 10 mM creatine phosphate, creatine kinase (3.5 U/ml), inorganic pyrophosphatase (0.6 U/ml)), ubiquitin (1 mg/ml), 3 µM ubiquitin aldehyde, rabbit E1 (10 µg/ml; MBL), and UbcH5c (100 µg/ml). GST or GST-ataxin-3(79Q) was immunoprecipitated with anti-GST and subjected to immunoblot analysis with anti-ubiquitin. Alternatively, the reaction was terminated by the addition of SDS sample buffer, and the products were subjected to SDS-PAGE and immunoblot analysis with anti-GST (MBL).

Pulse-chase analysis

HEK293T cells were metabolically labeled with [³⁵S]methionine for 1 h at 37°C, washed with PBS, and cultured in Dulbecco's modified Eagle's medium for 0–9 h. Cell lysates were subjected to immunoprecipitation with anti-Myc, and the resulting precipitates were subjected to SDS-PAGE and analysis with a BAS2000 instrument (Fuji Film). For determination of the turnover rate of endogenous ataxin-3, Neuro2A cells were cultured for 0–6 h in the presence of cycloheximide (50 µg/ml) and cell lysates were subjected to immunoblot analysis by anti-ataxin-3.

Retrovirus-mediated gene transfer

A cDNA for FLAG-E4BAU was subcloned into the pMX-puro vector (kindly provided by T Kitamura, University of Tokyo). The pMX-puro or pMX-puro-E4BAU vectors were transfected into a clone of retrovirus-packaging cells (Plat-A for HeLa cells; Plat-E for Neuro2A cells) (Morita *et al*, 2000). Recombinant retroviruses were used to infect HeLa or Neuro2A cells, and infected cells were selected by culture in the presence of puromycin (2.5 µg/ml).

Immunofluorescence staining

HeLa cells grown on glass cover slips were transfected by calcium phosphate precipitation and prepared for immunostaining as described (Hatakeyama *et al*, 2001).

Northern blot analysis

Total RNA was extracted from HEK293T cells expressing ataxin-3(13Q) or ataxin-3(79Q) with the use of ISOGEN (Nippon Gene) and was then subjected to Northern blot analysis with an ataxin-3 cDNA probe that had been labeled with [α -³²P]dCTP.

Generation of transgenic flies

To construct vectors for the generation of transgenic flies, we subcloned Myc-ataxin-3ΔN^{79QC}, which lacks residues 1–128 of ataxin-3(79Q), or FLAG-E4B cDNA fragments into pUAST (Brand and Perrimon, 1993). Transformed lines were generated by standard procedures with *w¹¹¹⁸*, *Dr¹/TMS*, *Sb¹*, *P{ry⁺17.2=D2-3}99B* as the parental line. *GMR-Gal4* and *sev-Gal4* were obtained from the Bloomington Stock Center. For SEM, flies were fixed and dehydrated in acetone and viewed with a JOEL JSM-5600 LV instrument. Ommatidial structures were observed by the technique of neutralization of the cornea (Franceschini and Kirschfeld, 1971) with an oil-immersion ×20 objective. To confirm the expression of transgenes, we subjected 400 µg protein extracts obtained from pupae collected 24 h after puparium formation to immunoprecipitation and immunoblot analysis with the use of antibodies to Myc or FLAG.

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