Proteolytic cleavage of polyglutamine-expanded ataxin-3 is critical for aggregation and sequestration of non-expanded ataxin-3

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Spinocerebellar ataxia type 3 (SCA3), like other polyglutamine (polyQ) diseases, is characterized by the formation of intraneuronal inclusions, but the mechanism underlying their formation is poorly understood. Here, we tested the ‘toxic fragment hypothesis’, which predicts that proteolytic production of polyQ-containing fragments from the full-length disease protein initiates the aggregation process associated with inclusion formation and cellular dysfunction. We demonstrate that the removal of the N-terminus of polyQ-expanded ataxin-3 (AT3) is required for aggregation in vitro and in vivo. Consistently, proteolytic cleavage of full-length, pathogenic AT3 initiates the formation of sodium dodecylsulfate-resistant aggregates in neuroblastoma cells. Although full-length AT3 does not readily aggregate on its own, it is susceptible to co-aggregation with polyQ-expanded AT3 fragments. Interestingly, interaction with soluble polyQ-elongated fragments causes a structural distortion of wild-type AT3 prior to the formation of stable co-aggregates. These results establish the critical role of C-terminal, proteolytic fragments of AT3 in the molecular pathomechanism of SCA3, in strong support of the toxic fragment hypothesis.

INTRODUCTION

Among the heterogeneous group of autosomal dominantly inherited ataxias, spinocerebellar ataxia type 3 (SCA3) is the most common. SCA3 is caused by a CAG trinucleotide expansion in the MJD1 gene, which is translated into a polyglutamine (polyQ) stretch in the ataxin-3 (AT3) protein. Compared with other known polyQ disease proteins, AT3 is unusual in that the expanded glutamine segment is located at the C-terminus of the protein, and the repeat length threshold of ~60 glutamine residues for disease development is exceptionally high (1). AT3 consists of a structured N-terminus with deubiquitinating activity, termed the Josephin domain (2–4), two ubiquitin-interacting motifs (UIMs) and a polyQ tract (Fig. 1), which in some AT3 isoforms are followed by a third UIM. In contrast to the globular Josephin domain, the C-terminus of AT3 is thought to be unstructured (5). AT3 does not have significant homology to other ubiquitin proteases; however, the catalytic triad (Cys14, His119, Asn134) and other amino acids within the Josephin domain are conserved. NMR analysis has revealed the close proximity of these three residues, consistent with their proposed catalytic function (6,7). As is the case for most of the roughly 100 identified deubiquitinating enzymes, the physiological function of AT3 is not known (8). It has been suggested, however, that AT3 is a component of the ubiquitin proteasome system (4). Recent studies also suggest that AT3 may be a regulatory component of aggresome formation (9) and may have a neuroprotective function (10). Furthermore, AT3 may be involved in transcriptional regulation (11,12).

A common feature of polyQ diseases is the accumulation of insoluble intracellular deposits containing the aggregated disease protein (13–15). Although the relationship of these inclusions to cellular dysfunction remains controversial, it is clear that these inclusions are associated with pathogenesis. It has been proposed that proteolytic cleavage of polyQ proteins is required for inclusion formation, because polyQ-containing fragments of the disease protein are the predominant species detected in neuronal inclusions in human brain (16–18). Indeed, AT3 fragments

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have been detected in staurosporine-treated mammalian cells (19) and in postmortem brain tissue of SCA3 patients (20). Ikeda et al. (21) additionally showed that expression of polyQ-expanded AT3 fragments in cell culture resulted in inclusion formation and found that mice transgenic for AT3 (79Q) fragments displayed ataxic phenotypes, whereas mice transgenic for full-length AT3 (79Q) were without symptoms. More recently, in a transgenic mouse model expressing full-length AT3 (71Q), intranuclear inclusions and fragments of AT3 in the brain were associated with a neurodegenerative phenotype (20). These combined observations are consistent with the ‘toxic fragment hypothesis,’ which predicts that proteolytically derived fragments of AT3 are required for initiating the aggregation process associated with toxicity (22,23).

Neuronal inclusions in human brain are composed of polyQ-containing fragments as well as other components, such as non-pathogenic polyQ proteins including AT2/3 and the transcription factors, TATA-binding protein (TBP) and CREB-binding protein (CBP) (24,25). To analyze this co-aggregation phenomenon in cell culture, a polyQ-expanded AT3 fragment was co-expressed with full-length AT3Q78 or AT3Q27 or polyQ-deleted AT3 (D228–354) and revealed that only polyQ-expanded AT3 (AT3Q78) was sequestered into sodium dodecylsulfate (SDS)-resistant complexes.

Figure 1. Schematic representation of the AT3 constructs used in this study. FLAG- and myc-tags are depicted with open boxes. Josephin, UIM and polyQ domains are highlighted. The PP cleavable GST tag was used for protein purification. His6-tagged and cell culture expression constructs were without the GST tag. Some constructs contain a TEV consensus site at the indicated position. The scaled upper bar contains information about predicted extended strands (c) and random coil (r) regions. Two predicted coiled-coil domains are shown. N-terminal truncation mutants are named by a ‘N’ and the last encoded residue number and by a ‘c’. When the protein contains a polyQ stretch, the protein’s name is followed by a ‘Q’ and the number of glutamines contained. The numbering refers to the wild-type AT3 with 22 glutamines.
(26,27). Interestingly, immunofluorescence microscopy showed that all three full-length AT3 species co-localized with cellular inclusions of polyQ-expanded AT3 fragments, presumably via a polyQ-independent recruitment mechanism. Because neuronal polyQ inclusions in human brain also contain ubiquitin, a recruitment mechanism based on the ability of AT3 to bind polyubiquitin chains via its UIMs was proposed. Indeed, Donaldson et al. (28) observed that co-localization of AT3 species with aggregating AT3 fragments was dependent only on the presence of the UIMs. Taken together, the results of these studies suggest that targeting of full-length AT3 to inclusions is influenced by regions other than the polyQ stretch, including the UIMs, whereas formation of SDS-resistant co-aggregates is dependent on the presence of an elongated polyQ stretch in full-length AT3.

Most mechanistic studies in this active field have been performed with N-terminal fragments of the large protein huntingtin (∼350 kDa). The relatively small size of AT3 (∼42 kDa) allowed us to systematically analyze the aggregation properties of a full-length polyQ disease protein. Considering the aforementioned studies, we hypothesized that SCA3 pathogenesis is caused by initial fragmentation of polyQ-elongated AT3 that results in aggregation and recruitment of polyQ-containing proteins, which may include non-pathogenic AT3. Our analyses of the aggregation properties of a series of truncation mutants show that polyQ-expanded and non-expanded full-length AT3 proteins have very little propensity to aggregate, whereas only the shortest of the polyQ-expanded truncation mutants efficiently aggregate. Likewise, proteolytic cleavage of polyQ-expanded full-length AT3 initiates the formation of SDS-resistant aggregates in vivo. Aggregates of polyQ fragments are able to sequester full-length AT3 early in the aggregation process, in a polyQ-dependent manner. In neuroblastoma cells, recruitment of full-length AT3 into aggregates is efficient and is characterized by a 50% loss of soluble AT3. In vitro, full-length AT3 is structurally rearranged during or immediately after the appearance of polyQ-expanded fragments, but before SDS-resistant aggregates are detected. This observation is consistent with a mechanism recently proposed for polyQ toxicity on the basis of inactivation of transcription factors, like TBP and CBP, by aggregation intermediates (29–31). Here, we demonstrate that the emergence of proteolytic fragments of AT3 containing an extended polyQ segment is essential to initiate the aggregation process and the sequestration of full-length, non-pathogenic AT3 in cell culture. This sequestration of non-pathogenic AT3 and the corresponding reduction of soluble, intracellular AT3 levels could in turn impair AT3 function, thereby contributing to SCA3 pathogenesis. Preventing the fragmentation of AT3 and/or specifically inhibiting the recruitment process could be attractive approaches for preventative or curative treatments of this fatal disease.

RESULTS

N-terminal sequence prevents aggregation of polyQ-expanded AT3 in vitro

To analyze the aggregation properties of AT3 in vitro, glutathione S-transferase (GST) fusion proteins were purified (Fig. 1). The full-length proteins, AT3Q22, AT3Q43 and AT3Q71, were tagged at the N- and C-terminus with a FLAG and myc epitope, respectively. C-terminal fragments were designed on the basis of the results of limited proteolysis experiments with His-AT3Q22 (data not shown) and were myc-tagged at the C-terminus. N-terminal AT3 fragments were FLAG-tagged at the N-terminus. Fusion to GST typically renders polyQ proteins soluble, enabling their purification, as was the case for all analyzed AT3 proteins (Fig. 2A and B, upper panels). To assess the aggregation properties of AT3 proteins, proteolytic removal of the GST by PreScission Protease (PP) was followed by 16 h of incubation. A filter retardation assay was then used to detect SDS-resistant aggregates, as previously described (32). Full-length AT3 proteins with 22, 43 and 71Q did not form SDS-resistant aggregates (Fig. 2A, left panel), as was the case for AT3 fragments without the polyQ region (N256, 318c) (Fig. 2B), C-terminal fragments of AT3 with non-pathogenic polyQ stretches (257cQ22, 257cQ43) (Fig. 2A, left panel) and large fragments of AT3 with 71Q (79cQ71, 162cQ71, 186cQ71, 221cQ71, 3118Q71) (Fig. 2A and B). In contrast, only the smallest C-terminal fragments, 242cQ71 and 257cQ71, readily formed SDS-insoluble material that was retained on the filter (Fig. 2A, right panel). Likewise, without addition of PP, no aggregates were detected (data not shown). Quantification showed that the presence of an additional 15 residues in 242cQ71 reduced the aggregation efficiency by 80%, relative to 257cQ71 (Fig. 2C). We further characterized the aggregation properties of 257cQ71 over time. After a lag-phase of ∼1.5 h, SDS-resistant material was detected, reaching a plateau of aggregation after 8 h (Fig. 2D). This kinetic behavior is compatible with a nucleated polymerization reaction, as described for amyloid and amyloid-like proteins (33–35).

These experiments show that the full-length AT3 proteins are either unable to form SDS-resistant fibrillar aggregates under physiological buffer conditions or do so with extremely low efficiency, whether a polyQ tract above the pathological threshold is present or not. In contrast, certain C-terminal AT3 fragments with an extended polyQ segment readily aggregate, emphasizing the influence of the full-length sequence context on the aggregation properties of AT3.

N-terminal sequence does not protect AT3 from sequestration into aggregates in vitro

The aggregation incompetence of intact AT3 could be due to its general inability to participate in polyQ aggregation or to initiate an aggregation process. To distinguish between these two possibilities, aggregation reactions with the 257cQ71 fragment were carried out in the presence of full-length AT3 proteins and analyzed with the filter assay. Immunodetection with an antibody against the N-terminal FLAG-tag of the full-length AT3 proteins showed that AT3 was recruited into aggregates of 257cQ71, in a process dependent on its polyQ stretch, but independent of its UIMs (Fig. 3A). Quantification of the data shown in Figure 3B revealed that full-length AT3Q22 was robustly recruited and the efficiency of recruitment further increased with expansion of its polyQ stretch.

Next, we addressed the question of whether full-length AT3 stably interacts with 257cQ71 in co-aggregates. Co-aggregates
produced upon incubation of a His$_6$-tagged AT3 protein with 22 glutamines (His-AT3Q22) together with 257cQ71 were bound to Ni$^{2+}$-NTA beads followed by stringent washing and specific elution with imidazole. When His-AT3Q22 was present during the aggregation reaction, a substantial amount of aggregated material specifically bound to the resin (Fig. 3C). In contrast, when PP-cleaved GST–AT3Q22 lacking a His$_6$-tag was used instead, co-aggregates were unspecifically bound and removed during the washing steps. In a separate experiment, the specific co-aggregates in the eluate were pelleted and dissociated with formic acid (36). It was previously determined that the formic acid procedure did not result in hydrolysis of AT3 proteins (data not shown). Western blotting showed the presence of both

Figure 2. Aggregation of AT3 in vitro requires N-terminal truncation and an expanded polyQ segment. (A) Aggregation behavior of purified full-length AT3 proteins and C-terminal AT3 fragments. The recombinant GST-fusion proteins indicated were analyzed by SDS–PAGE and Coomassie blue staining (upper panels). After PP cleavage of the recombinant GST-fusion proteins (3 μM) and aggregation for 16 h, the accumulation of SDS-resistant aggregates was analyzed via the filter assay (lower panels). (B) Aggregation behavior of purified N-terminal AT3 fragments and a C-terminal fragment without a polyQ stretch. The recombinant GST-fusion proteins indicated were analyzed by SDS–PAGE and Coomassie blue staining or western blotting (upper panels). After PP cleavage of the recombinant GST-fusion proteins (3 μM) and aggregation for 16 h, SDS-resistant aggregates were analyzed via filter assay (lower panels). (C) Quantification of results of filter assays, as shown in (A). (D) Time course of the 257cQ71 aggregation reaction. The GST–257cQ71 fusion protein (3 μM) was incubated with PP for up to 10 h. Several dilutions within the linear range of the ECF signal were analyzed by the filter assay (anti-myc). Values shown are the mean values ± the standard deviation of four experiments.
His-AT3Q22 and the polyQ-expanded fragment, 257cQ71 (Fig. 3D). Thus, intact AT3 proteins, including the wild-type protein, can be stably incorporated into SDS-resistant aggregates by a polyQ-dependent mechanism, demonstrating that they can participate in the process of aggregation. This strongly suggests that the full-length AT3 proteins are unable to aggregate on their own due to their inability to initiate this process.

Wild-type AT3 is structurally altered and rapidly incorporated into early aggregation intermediates formed by 257cQ71 in vitro

To analyze early stages of the aggregation and recruitment reaction, we determined the time dependence of AT3Q22 distribution between supernatant and pellet fractions by centrifugation and subsequent SDS treatment (Fig. 4A). Aggregation reactions were initiated by PP cleavage of the GST moiety from either the 257cQ22 or the 257cQ71 fragment. Upon incubation with 257cQ22, or uncleaved GST–AT3 fragments, wild-type AT3Q22 remained in the supernatant and SDS-soluble over a time period of 8 h at 30°C (Fig. 4B) (data not shown). In contrast, upon incubation with the polyQ-expanded fragment, 257cQ71, <10% of AT3Q22 was detected in the soluble supernatant 2 h after PP addition and migrated as the monomeric species on SDS–polyacrylamide gel electrophoresis (PAGE) (Fig. 4C). Of the remaining 90% of AT3Q22 in the pellet, the SDS-soluble portion detected by western blotting accounted for 50%, whereas the SDS-resistant portion caught in the stacking gel likely accounted for the leftover 40%. At later time points, the amount of AT3Q22 found in the SDS-soluble pellet fraction gradually decreased, reflecting a corresponding increase in the amount of AT3Q22 found in the SDS-resistant fraction. These results suggest a two-step mechanism consisting of early recruitment of full-length AT3 into SDS-soluble aggregates, followed by conversion into SDS-resistant fibrillar co-aggregates. To better define the mechanism of recruitment, earlier time points were analyzed. After addition of PP, samples were incubated for the indicated time and subsequently centrifuged for 10 min (Fig. 4D). At the earliest time point (T = 0), PP was added and samples were immediately centrifuged. PP cleavage

Figure 3. In vitro, full-length AT3 stably associates in a polyQ-dependent manner with SDS-insoluble aggregates of 257cQ71. (A) Co-aggregation of full-length AT3 proteins with 257cQ71. The indicated GST–AT3 proteins (0.3 μM) and GST–257cQ71 (3 μM) were incubated with PP for 16 h and subjected to the filter assay. Membranes were probed with anti-myc and anti-FLAG antibodies in parallel. (B) Quantification of (A). Several dilutions were analyzed. The ratio of the anti-FLAG signal (recruitment) to the anti-myc signal (total aggregation) was calculated. This ratio was defined as 100% for AT3Q71. Calculated values are mean ± standard deviation of five independent experiments. (C) Affinity purification of co-aggregates under stringent conditions. GST–257cQ71 (3 μM) was incubated with His-AT3Q22 or GST–AT3Q22 (0.5 μM) and PP in the aggregation reaction. Incubation with Ni-NTA was followed by denaturing washes and specific elution with imidazole. The flow-through (FT) and the wash fractions (W 1–5) were analyzed together with the eluate (E) by the filter assay (anti-myc). (D) Dissociation of eluted co-aggregates with formic acid. Co-aggregates from the eluate of (C) were dissociated with formic acid and further analyzed by western blotting (anti-AT3). The arrow indicates the 257cQ71 fragment, which was also detectable by anti-myc (data not shown).
Figure 4. Wild-type AT3 is structurally distorted and rapidly incorporated into aggregation intermediates formed by 257cQ71. (A) Schematic outline of the experimental procedure used in (B–D). (B) Analysis of the solubility of AT3Q22 upon incubation with 257cQ22. Aliquots of aggregation reactions containing 2 μM 257cQ22 and 0.1 μM wild-type protein (AT3Q22) were analyzed over time by centrifugation at 20 000g for 10 min followed by SDS treatment and western blotting (anti-FLAG). The distribution of the wild-type AT3 into SDS-soluble pellet and supernatant fractions was quantified. (C) Analysis of the solubility of AT3Q22 upon incubation with aggregating 257cQ71. Aliquots of co-aggregation reactions containing 2 μM 257cQ71 and 0.1 μM AT3Q22 were analyzed as in (B). (D) Analysis of the solubility of AT3Q22 in the first 2 h of the reaction described in (C). GST–257cQ71 was mixed with GST–AT3Q22, and the reaction was started by adding PP. Aliquots were taken at the indicated time points and immediately centrifuged at 20 000g for 10 min. The separated supernatant and pellet fractions were boiled in SDS-sample buffer to stop the reactions. Amounts of SDS-soluble AT3Q22 were quantified by western blotting. (E) Conformational change of His-AT3Q22 upon 257cQ71 aggregation. IAEDANS and IANBD doubly labeled His-AT3Q22 (0.1 or 0.2 μM) was incubated with 257cQ71 (1 μM), and changes in IAEDANS fluorescence upon 257cQ71 aggregation at 30°C were followed. IAEDANS and IANBD are donor–acceptor dyes participating in intramolecular FRET. The increase of IAEDANS fluorescence reflects the increase in dye distance due to conformational changes in the AT3Q22 Josephin domain. As a control, 257cQ22 was used.
had a half-time of 10 min and proceeded during this centrifugation period (data not shown). Approximately 36% of AT3Q22 was found in the soluble supernatant, and ~42% of AT3Q22 was detected in the SDS-soluble pellet fraction (Fig. 4D, T = 0), suggesting an immediate interaction of AT3Q22 with the 257cQ71 fragment generated upon cleavage. During the first 1.5 h of the reaction, the amount of AT3Q22 detected in the SDS-soluble pellet fraction remained constant, but the amount of AT3Q22 in the supernatant decreased with time. Considering that this time period corresponds to the lag phase of the fibrillization reaction (compare with Fig. 2D), our results suggest an immediate recruitment of AT3Q22 into SDS-soluble aggregation intermediates that apparently must reach either a critical size or concentration to be converted into SDS-resistant aggregates with time (Fig. 4D).

To analyze the influence of 257cQ71 on the AT3Q22 structure during this recruitment process, we performed intra-molecular fluorescence resonance energy transfer (FRET) measurements. His-AT3Q22 was labeled with IAEDANS and IANBD as a fluorescence donor–acceptor pair for energy transfer interactions at distances up to 50 Å. IAEDANS was covalently attached to the active site Cys14 and IANBD to Cys172 within the Josephin domain. From the decrease of IAEDANS fluorescence change had been reached (Fig. 4E). We calculated a distance of 40.5 Å between donor and acceptor chromophores after a plateau in fluorescence change remains to be explored in more detail, but it is of note that the presence of 257cQ71 results in a conformational change of AT3Q22, which may deplete soluble AT3 levels through a co-aggregation mechanism. Alternatively, transient interaction of AT3Q22 with aggregating AT3 fragments may induce a misfolding event that leads to its efficient degradation.

Coexpression of full-length AT3 with 257cQ71 results in co-aggregation and depletion of soluble AT3 levels in N2a neuroblastoma cells

To confirm our findings from in vitro experiments in a cellular environment, the aggregation and recruitment properties of AT3 were analyzed in mouse neuroblastoma cells (N2a). Immunofluorescence microscopy 24 h post-transfection showed that polyQ-expanded 257cQ71 formed inclusions in almost all transfected cells (Fig. 5A; Supplementary Material, Table S1). Non-truncated AT3 proteins were largely diffusely distributed within the cells, irrespective of the polyQ length (Fig. 5A). However, aggregate structures were observed in ~5–10% of cells transfected with the polyQ-expanded protein, AT3Q71 (Supplementary Material, Table S1). When FLAG-AT3 proteins were co-expressed with the 257cQ71-myc fragment, they distributed into cellular inclusions (Fig. 5B). This co-localization was dependent on the presence of a polyQ segment in AT3, and therefore was not observed with the polyQ deletion mutant, AT3QΔ. Deletion of both UIMs, however, had no influence on the co-localization of AT3 with 257cQ71. To determine whether full-length AT3 was stably sequestered into SDS-resistant aggregates, as observed in vitro, the filter assay was used to analyze lysates derived from cells co-expressing FLAG-AT3 proteins and 257cQ71-myc. Quantitative analysis revealed that AT3 proteins accumulated in SDS-resistant aggregates of 257cQ71 in a polyQ-dependent manner (Fig. 6A). Furthermore, the efficiency of recruitment of AT3 proteins into 257cQ71 aggregates correlated with polyQ length, suggesting that the polyQ stretch is the sole determinant mediating co-aggregation. These findings supported our in vitro findings, which together suggest a polyQ-dependent mechanism of recruitment of full-length AT3.

To corroborate that AT3Q22 co-aggregation was dependent on the presence of aggregation competent fragments, we employed doxycycline-responsive N2a cells. Co-aggregation of AT3Q22 required synthesis of the 257cQ71 fragment (~DOX), whereas in the absence of 257cQ71 synthesis (+DOX), no co-aggregation was detected (Fig. 6B, upper panel). After 24 h of expression of 257cQ71 (~DOX), western blot analysis showed that the levels of SDS-soluble AT3Q22 diminished to ~50% compared with repressed (+DOX) cells (Fig. 6B, lower panel) and coincided with the appearance of SDS-insoluble material containing AT3Q22. Notably, AT3Q22 had identical expression levels and remained SDS-soluble after co-expression of 257cQ22 (Supplementary Material, Fig. S5). Thus, the strong reduction of SDS-soluble AT3Q22 can be attributed to the presence of a polyQ-expanded AT3 fragment, which may deplete soluble AT3 levels through a co-aggregation mechanism. Alternatively, transient interaction of AT3Q22 with aggregating AT3 fragments may induce a misfolding event that leads to its efficient degradation.

Aggregation of full-length pathogenic AT3 is initiated by proteolytic cleavage in neuroblastoma cells

As previously mentioned, AT3Q71 could form aggregate structures with low efficiency in the absence of 257cQ71,
possibly through proteolytic removal of its N-terminus. To further investigate this phenomenon, AT3Q71 aggregates were quantified using the filter assay. Consistent with the immunofluorescence microscopy data (Supplementary Material, Table S1), the quantity of SDS-resistant AT3Q71 aggregates was 5–10% the quantity of 257cQ71 aggregates (Fig. 6C). Dissociation of these SDS-resistant AT3Q71 aggregates with formic acid, followed by western blotting, showed the presence of polyQ-containing AT3 fragments that ranged in size from 32 to 62 kDa, the former fragment being

![Figure 5. PolyQ-dependent co-localization of full-length AT3 with inclusions of polyQ-expanded AT3 fragments in N2a neuroblastoma cells. (A) Localization of wild-type AT3, polyQ-expanded AT3 and 257cQ71 fragment in N2a cells via immunofluorescence microscopy. Transiently transfected N2a cells were co-stained with an affinity-purified anti-AT3 serum followed by a CY3-coupled secondary antibody and DAPI. (B) Localization of wild-type AT3, polyQ-expanded, polyQ-, and UIM-deleted AT3 upon co-expression of aggregating fragment. N2a cells were transiently cotransfected with 257cQ71 and indicated full-length AT3. Co-staining with rabbit anti-FLAG and mouse anti-myc antibodies was followed by anti-rabbit-CY3, anti-mouse-FITC and DAPI.](https://academic.oup.com/hmg/article-abstract/15/4/555/641824)
Figure 6. PolyQ-containing fragments produced in N2a cells result in aggregation and sequestration of full-length AT3, thereby diminishing soluble AT3 levels. (A) Co-aggregation of full-length AT3 proteins with 257cQ71. N2a cells were transiently transfected with the indicated constructs and 257cQ71. After 48 h, SDS-resistant aggregates in cell lysates were detected by the filter assay. The ratio of the anti-FLAG signal (recruitment) to the anti-myc signal (total aggregation) was calculated (ratio for AT3Q71 = 100%). Values shown are mean ± standard deviation of four independent experiments. (B) Decreased SDS-soluble AT3Q22 levels in N2a cells expressing 257cQ71. Doxycycline-responsive N2a cells were transiently transfected with AT3Q22 under control of the constitutive CMV promoter of the pcDNA3.1 plasmid. Expression of the aggregation-competent 257cQ71 and the aggregation-incompetent 257cQ22 fragment (Supplementary Material, Fig. S3) was under the control of the Tet response element encoded on the pTRE plasmid. Lysates derived from cells expressing (+DOX) or not expressing (+DOX) 257cQ71 were analyzed at the indicated time points for the sequestration of wild-type AT3Q22 into SDS-resistant aggregates using the filter assay (upper panel). Identical cell lysates were analyzed for SDS-soluble AT3Q22 by western blotting with anti-AT3 antibody. The lower panel shows the reduction in the amount of SDS-soluble AT3Q22 over time in response to 257cQ71 expression. Values shown are mean ± standard deviation of four independent transfections. In response to 257cQ22, AT3Q22 remained SDS-soluble (Supplementary Material, Fig. S3). (C) Inefficient AT3Q71 aggregation. Cell lysates of N2a cells transiently transfected with the AT3Q71 construct were analyzed for the accumulation of SDS-resistant aggregates by the filter assay (anti-myc) at the indicated time points after transfection. For comparison, lysates from 257cQ71 expressing cells were analyzed in parallel. (D) Composition of dissociated AT3Q71 and 257cQ71 aggregates. N2a cell lysates were prepared 60 h after transient transfection. SDS-resistant material was pelleted and dissociated with formic acid followed by western blotting (anti-AT3/anti-polyQ). (E) TEV cleavage of mutant full-length AT3. N2a cells were transfected with the indicated AT3 constructs together with the TEV protease construct. Cell lysates after 72 h were analyzed for the accumulation of SDS-resistant aggregates by the filter assay (anti-myc). Aggregation was defined as 100% for 257cQ71. Calculated values are mean ± standard deviation of three independent transfections.
similar in size to 257cQ71 and the latter having the expected size of the full-length protein (Fig. 6D). Thus, the aggregation observed upon expression of AT3Q71 was likely related to the proteolytic generation of polyQ-expanded fragments. To clearly demonstrate that the aggregation of AT3 required the proteolytic production of polyQ-expanded fragments, we introduced a highly specific tobacco etch virus (TEV) protease cleavage site into AT3, close to amino acid 257. Upon co-expression of the TEV protease, cleavage of AT3Q71 occurred (Fig. 6E), and an increase in the amount of SDS-resistant aggregates was detected (Fig. 6F). Thus, proteolytic release of polyQ-containing AT3 fragments in a cellular environment can initiate aggregation. On the basis of these combined data, we propose that expanded polyQ segments must be liberated from a protective sequence context of the full-length AT3 protein in order to initiate the aggregation process, whereas the intact protein preserves its ability to co-aggregate.

DISCUSSION
Proteolytic cleavage of pathogenic AT3 as a prerequisite for aggregation

We have systematically analyzed the influence of structural elements N-terminal to the polyQ segment on the aggregation properties of AT3. An expanded polyQ segment in the context of full-length AT3 remains soluble, whereas removal of the protective N-terminal sequence allows for efficient aggregation under physiological conditions (Figs 2 and 5). Upon expression of AT3Q71 in neuroblastoma cells, a low level of SDS-resistant aggregates is detectable, but these aggregates are likely to be a result of AT3 fragmentation (Fig. 6C and D). We furthermore demonstrate that liberation of fragments from their natural context by specific cleavage of full-length AT3Q71 containing a TEV site causes enhanced aggregation (Fig. 6E and F). These combined results indicate a requirement of proteolytic cleavage for initiating aggregation of polyQ-expanded AT3.

The detailed in vitro analysis of AT3 truncation mutants suggests a proteolytic cleavage site near residue 250 of AT3 (Fig. 2A). Consistently, aggregates resulting from expression of AT3Q71 in cells are composed of a subset of fragments that are similar in size to 257cQ71 (Fig. 6D). AT3 fragments of approximately this size have indeed been detected in human SCA3 brain lysates and not in control samples (20). It has long been thought that proteolytic processing of neurodegenerative disease proteins is required for disease progression, as suggested by the toxic fragment hypothesis. Clear support of this principle stems mostly from studies of Alzheimer’s disease, where specific cleavage of amyloid precursor protein by β- and γ-secretases is essential for pathogenesis (reviewed in 38). Our studies in neuroblastoma cells suggest that an unknown endogenous protease(s) is responsible for generating the detected fragments of AT3 (Fig. 6D). Candidate proteases involved in this processing would include members of the caspase and calpain families, which have been previously implicated in the processing of polyQ proteins (19,39–41).

Recruitment of full-length AT3 into aggregates composed of polyQ-expanded AT3 fragments

Although strongly restraining aggregation of the protein itself, the N-terminal sequence does not inhibit polyQ-mediated sequestration of intact AT3 proteins into aggregates formed by polyQ-expanded fragments. Recruitment of full-length AT3 into SDS-insoluble aggregates is strictly dependent on the presence of a polyQ stretch in AT3, both in vitro and in vivo (Figs 3A and 6A). These results are compatible with previous studies describing the polyQ-dependent recruitment of factors, such as TBP and CBP, into aggregates of mutant huntingtin fragments (29,30). We additionally show using immunofluorescence microscopy that co-localization of full-length AT3 with aggregates is similarly dependent on its polyQ stretch and not on its UIMs (Fig. 5B). These results are inconsistent with a previous report that describes UIM-dependent co-localization of full-length AT3 proteins with aggregates of polyQ-expanded AT3 fragments (28). This discrepancy could be due to the nature of the AT3 constructs used in the previous study, which were fusions of green fluorescent protein (GFP) to full-length AT3 proteins. Similar polyQ-containing fusions were reported to have altered aggregation properties (29,32), and it is possible that the GFP–AT3 fusions used in the previous study do not exhibit the actual co-aggregation behavior of AT3 alone.

In vitro analyses of the recruitment of AT3 into polyQ aggregates have revealed several new aspects of this process. We show that full-length AT3Q22 is directly recruited into pelletable SDS-soluble aggregation intermediates, and the resulting co-aggregates are later converted into SDS-resistant fibrils (Fig. 4C and D). During the early steps of the recruitment process that are characterized by the accumulation of SDS-soluble aggregates, AT3 becomes structurally distorted upon interaction with emerging, polyQ-expanded fragments in a monomeric or oligomeric state (Fig. 4E). This interaction likely results in the observed stable association of AT3 with fibrillar aggregates late in the co-aggregation process (Fig. 3C).

Relevance to SCA3 pathogenesis

On the basis of these and other studies, we propose the following model for the aggregation and functional impairment of AT3 (Fig. 7). Short polyQ-expanded fragments of AT3 are released by proteolytic cleavage and undergo the conformational change required for seed formation (nucleation) and aggregation initiation (14,29,34,35,42). Early in the aggregation process, misfolded polyQ-expanded fragments interact with full-length AT3, thereby influencing the structure of its Josephin domain. This interaction may also induce a misfolding event in the polyQ tract of AT3, which facilitates its stable incorporation into the final fibrillar aggregates. Soluble, misfolded AT3 that escapes incorporation into SDS-resistant aggregates of polyQ fragments may be degraded by the ubiquitin–proteasome system. Depletion of active AT3, as a result of either incorporation into fibrils or degradation, could in turn contribute to SCA3 pathogenesis. Such a mechanism could also exist during the pathogenesis of other polyQ diseases, as is suggested by the recruitment of wild-type
huntingtin fragments into aggregates of polyQ-expanded huntingtin fragments (43).

It is widely thought that many polyQ diseases occur, at least in part, because of a dominant gain-of-function associated with polyQ-expanded gene products. In SCA3, however, clinical data suggest that a partial loss of AT3 function may also play a role in pathogenesis. In heterozygous SCA3 patients, the polyQ length of the wild-type gene product is negatively correlated with the age of disease onset (1). In these individuals, disease onset may arise due to a combination of both a toxic gain-of-function of polyQ-expanded AT3 and a dominant negative inactivation of the wild-type AT3 caused by polyQ-mediated sequestration. The age of onset would decrease as the polyQ stretch of the normal gene product lengthened, because of an increased propensity of wild-type AT3 for sequestration into disease aggregates, which is predicted by our results (Fig. 6A). Clinical studies also show that SCA3 patients homozygous for the polyQ-expanded AT3 allele have more severe symptoms at an earlier age compared with heterozygotes (44). According to our hypothesis, disease phenotypes would be enhanced in homozygous individuals because of a relative increase in the abundance of toxic polyQ-expanded fragments and a corresponding increase in the recruitment and inactivation of full-length AT3 and other polyQ-containing proteins (e.g. transcription factors). Such a sequestration mechanism would support the observation that SCA3 brain regions that degenerate also contain inclusions (26). Additionally, other mechanisms may contribute to the increased severity of pathology in homozygous SCA3 patients. The mouse neuroblastoma cells we analyzed here are unlikely to be impaired by a polyQ-mediated sequestration mechanism, as the endogenous AT3 contains only six consecutive glutamines and was not stably seques-tered into aggregates. Consistently, we did not observe overt toxicity and did not analyze this aspect in further detail.

As neurodegenerative diseases, such as SCA3, have an average age of onset of 30–50 years, it is widely thought that age-related changes in cellular homeostasis contribute to pathogenesis. Neurons expressing polyQ-expanded AT3 might be fully functional while not accumulating any toxic agent over decades until an imbalance in the production of toxic species and their clearance sets in. Age-related phenomena, such as activation of calpain and caspase proteases, as well as decreased chaperone activity (45), have been proposed to be involved in initiating the disease. In light of our studies, activation of such proteases could enhance the proteolytic release of toxic AT3 fragments, eventually overwhelming cellular quality-control mechanisms and causing SCA3 symptoms.

**MATERIALS AND METHODS**

**Expression constructs**

FLAG (MDYKDDDKAGEF)- and myc (CEQKLISEEDL)-tagged AT3, truncated N-terminal FLAG-tagged and truncated C-terminal myc-tagged expression constructs were generated by standard PCR techniques using PSG5 vectors containing human AT3 cDNAs (GenBank accession no. AAB33571) as templates (a kind gift from E. Wanker, Max-Delbrück-Center, Berlin, Germany). Constructs were cloned into pGEX-6P-1 (Amersham) for bacterial expression and into
pTRE-2hyg (Clontech) and pcDNA3.1/Hygro vectors (Invitrogen) for mammalian expression via BamHI and XhoI/Sall restriction sites. The construct N318Q71 was generated by cleaving the plasmid pGEX-6P-1-AT3Q71 with PpuMI and NotI and by ligating the PpuMI/NotI linker (composed of the DNA oligonucleotides GACTGACTGACTGA and GCCTCAGTCAGTCA) into this pGEX-6P-1-AT3 fragment. The construct, AT3Q71ΔUIM, is a FLAG- and myctagged AT3 with a deletion of residues 221–256. AT3Q71T contains a TEV recognition sequence (ENLYFQG) inserted between residues 260 and 261. The construct, His-AT3Q22, contains a TEV recognition sequence (ENLYFQG) inserted for 1–16 h. Proteins for recruitment analyses (0.1–0.5 mM) were added at the beginning of the reaction. An equal volume of 4% SDS/100 mM DTT was added, and the aggregation reaction was stopped by heating for 5 min at 95°C. Several dilutions (corresponding to 5–75 pmol of GST-fusion protein) were filtered through a cellulose acetate membrane (0.2 μm pores, Schleicher & Schuell) (32). Aggregates retained on the membrane were detected with anti-myc or anti-FLAG antibody and the ECF-system (Amersham).

Cell culture
Mouse neuroblastoma cells (N2a) were maintained in Dulbecco’s modified Eagle’s medium (Biochrom AG) supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco). For maintenance of responsive N2a cells (Tet-Off), Tet-system approved FCS (BD Biosciences Clontech) and 0.5 mg/ml G418 (Clontech) was used. To switch off expression from pTRE-plasmids, 1 μg/ml doxycycline was added to the medium. For transient transfections, 70% confluent cells on 35 mm dishes were transfected with 2 μg of DNA using Lipofectamine PLUS reagents (Invitrogen). For the preparation of lysates, cells were washed once with PBS, scraped in lysis buffer [1 × TBS containing 0.2% Triton X-100, 10 mM MgCl2 and protease inhibitor cocktail (Complete, Roche)] and incubated with Benzonase (Merck, 40 U/cm3 plate) for 1 h at 4°C. Afterwards, the lysates were adjusted to 2% SDS/50 mM DTT, followed by heating at 95°C for 5 min. Filter assays were performed as described earlier. For immunofluorescence imaging, cells transfected on glass cover slips were fixed with ice-cold methanol followed by incubation with primary and secondary antibodies (20 μg/ml each) for 1 h at room temperature. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) during incubation with the secondary antibody. Epifluorescence microscopy was performed on a Zeiss Axiovert 200M.

FRET measurements
Thiol-reactive probes, 1,5-IAEDANS and IANBD ester (Molecular Probes) were the donor–acceptor pair for intramolecular FRET measurements. His-AT3Q22 (400 μM) was incubated for 2 h at 4°C with 2.5-fold molar excess of IAEDANS added from DMF. The unbound dye was removed by dialysis and the IAEDANS-labeled protein was incubated with 3.5-fold molar excess of IANBD. Labeling was quantified using the molar extinction coefficients of AT3 at 280 nm (3.3 × 10^4 M^-1 cm^-1), of IAEDANS at 336 nm (5.7 × 10^3 M^-1 cm^-1) and of IANBD at 472 nm (2.3 × 10^4 M^-1 cm^-1). As determined by tryptic digestion and mass spectrometry, one molecule of IAEDANS was attached to Cys14 and one molecule of IANBD to Cys172 per molecule of AT3. IAEDANS (donor) fluorescence changes were measured in an aggregation reaction of 1 μM GST–257cQ71 and 0.1–0.2 μM doubly labeled His-AT3Q22 in buffer C. The relative quantum yield of the donor (Qd) was determined by comparing the integrated emission spectrum (excitation 360 nm) with that of the reference quinine bisulfate in 0.1 M H2SO4 and normalized to the same absorbance at 360 nm. The quantum yield of the standard was 0.55. FRET efficiency was calculated from the decrease of

Protein purification
GST–AT3 fusion proteins were expressed in E. coli BL21 upon induction with 0.1 mM isopropyl β-d-thiogalactoside (IPTG) for 4 h at 30°C. Collected cells were resuspended in buffer A [50 mM Na-Phosphate (pH 8), 100 mM NaCl, 10% glycerol, 1 mM dithiothreitol (DTT), 1 mM PMSF, protease inhibitor cocktail (Complete, Roche)] plus 1% Triton X-100 and disrupted by lysozyme and sonication. After centrifugation at 30 000 g, the supernatant was incubated with 3 ml glutathione–agarose (Sigma, Santa Cruz, USA) for 30 min at 4°C. The beads were washed with buffer A/1 mM NaCl/1% Triton X-100 and eluted with buffer A containing 20 mM reduced glutathione. The purified protein was dialyzed against buffer B (20 mM Tris (pH 8), 150 mM KCl, 0.1 mM EDTA, 10% glycerol, 0.1 mM DTT). His-tagged AT3 proteins were expressed in E. coli BL21 (DE3) pLysS and purified by standard procedures using Ni2+–NTA (Qiagen) and the ECF-system (Amersham). Antibodies
The monoclonal (clone M2) and polyclonal anti-FLAG antibodies, the anti-myc antibody (clone 9E10) and the antipolyQ antibody (clone 1C2) are available from Sigma and Chemicon, respectively. A polyclonal antiserum against AT3 at 280 nm (3.3 × 10^4 M^-1 cm^-1) and of IANBD at 472 nm (2.3 × 10^4 M^-1 cm^-1). As determined by tryptic digestion and mass spectrometry, one molecule of IAEDANS was attached to Cys14 and one molecule of IANBD to Cys172 per molecule of AT3. IAEDANS (donor) fluorescence changes were measured in an aggregation reaction of 1 μM GST–257cQ71 and 0.1–0.2 μM doubly labeled His-AT3Q22 in buffer C. The relative quantum yield of the donor (Qd) was determined by comparing the integrated emission spectrum (excitation 360 nm) with that of the reference quinine bisulfate in 0.1 M H2SO4 and normalized to the same absorbance at 360 nm. The quantum yield of the standard was 0.55. FRET efficiency was calculated from the decrease of

In vitro aggregation reactions and filter assay
Up to 3 μM GST–AT3 fusion protein was incubated with 48 U/ml PP (Amersham) in buffer C (20 mM Tris pH 8, 150 mM KCl) at 30°C with shaking, on an orbital shaker (Thermomixer comfort, Eppendorf AG Hamburg), at 300 r.p.m. for 1–16 h. Proteins for recruitment analyses (0.1–0.5 μM) were added at the beginning of the reaction. An equal volume of 4% SDS/100 mM DTT was added, and the aggregation reaction was stopped by heating for 5 min at 95°C. Several dilutions (corresponding to 5–75 pmol of GST-fusion protein) were filtered through a cellulose acetate membrane (0.2 μm pores, Schleicher & Schuell) (32). Aggregates retained on the membrane were detected with anti-myc or anti-FLAG antibody and the ECF-system (Amersham).
the donor quantum yield ($Q_D$) in the presence of the acceptor ($Q_{DA}$). The average ($r$) and the critical distance ($R_0$) of the donor–acceptor pair were calculated, assuming random orientation of the two chromophores, according to Förster’s theory (47). In an additional experiment, IAE/DANS fluorescence changes of singly labeled AT3Q22 upon 257cQ71 aggregation or upon denaturation with guanidinium hydrochloride were measured.

**Western blotting**

Proteins were separated by SDS–PAGE and blotted onto nitrocellulose membranes followed by immunodetection using the ECL or ECF system (Amersham). For quantitative analysis, a LAS-3000 imager (Fujifilm Image Reader) or a Fuji FLA-2000 phosphoimager was used.

**Dissociation of aggregates with formic acid**

Cell lysates prepared as described earlier were adjusted to 2% SDS and centrifuged at 400 000 g for 1 h. Pellets were resuspended in 100% formic acid (36) and incubated for 3 h at 37°C with shaking, on an orbital shaker (Thermomixer comfort, Eppendorf AG Hamburg), at 700 r.p.m. The samples were vacuum-dried and resuspended in SDS-sample buffer, heated at 95°C for 5 min and applied to SDS–PAGE and western blotting.

**Affinity purification of aggregates with Ni-NTA**

For isolation of *in vitro* formed aggregates, the aggregation reactions containing 3 μM GST–257cQ71 protein and 0.5 μM His-AT3Q22 or 0.5 μM GST–AT3Q22 were carried out for 16 h. Then 50 μL Ni-NTA agarose beads (Qiagen) pre-equilibrated in buffer C were added for 30 min at room temperature. After five washes with buffer D (20 mM Tris (pH 8), 2% SDS, 1 mM NaCl) containing 6 M urea for 1 h, bound complexes were eluted with buffer D plus 200 mM imidazole and used for the filter assay or centrifuged at 400 000 g for 1 h. The pellet fraction was prepared for western blotting by treatment with formic acid as described earlier.

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG Online.

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**Conflict of Interest statement.** None declared.

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