Calpain Inhibition Is Sufficient to Suppress Aggregation of Polyglutamine-expanded Ataxin-3

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The formation of intraneuronal inclusions is a common feature of neurodegenerative polyglutamine disorders, including spinocerebellar ataxia type 3. The mechanism that triggers inclusion formation in these typically late onset diseases has remained elusive. However, there is increasing evidence that proteolytic fragments containing the expanded polyglutamine segment are critically required to initiate the aggregation process. We analyzed ataxin-3 proteolysis in neuroblastoma cells and in vitro and show that calcium-dependent calpain proteases generate aggregation-competent ataxin-3 fragments. Co-expression of the highly specific cellular calpain inhibitor pastatin abrogated fragmentation and the formation of inclusions in cells expressing pathological ataxin-3. These findings suggest a critical role of calpains in the pathogenesis of spinocerebellar ataxia type 3.

Spinocerebellar ataxia type 3 (SCA3), also known as Machado-Joseph disease (MJD), is a late onset neurodegenerative disease that is inherited in an autosomal-dominant fashion. Causative for SCA3 is an expansion of a CAG trinucleotide repeat in the MJD1 gene that is translated into an expanded polyglutamine (polyQ) segment in the corresponding ataxin-3 (AT3) protein (1). The polyQ tract in AT3 is normally 12 to 39 Gln long and expansion beyond 45 Gln results in disease. A unifying neuropathological feature of this and other polyQ diseases is the accumulation of protein deposits (inclusions) in a specific subset of neurons. These inclusions are mainly composed of the pathological polyQ protein but also contain molecular chaperones, transcription factors, ubiquitin, and components of the proteasome machinery (2, 3). Although these deposits are hallmarks of polyQ diseases, their precise contribution to pathogenesis and the mechanisms that trigger their formation late in life are not well understood.

Several hypotheses have been put forward to explain how polyQ disease proteins cause neuronal dysfunction and toxicity (reviewed in Ref. 4). The polyQ expansion confers a tendency to misfold, resulting in a toxic gain of function with aggregation and the formation of fibrillar inclusions (5). Early aggregation intermediates seem to play a critical role in pathogenesis (6–8).

Expression of full-length ataxin-3 or huntingtin is barely toxic to cells, regardless of the length of the polyQ stretch, whereas polyQ expanded fragments are cytotoxic and readily aggregate (1, 9–13). Likewise, transgenic mice expressing full-length polyQ proteins develop milder phenotypes compared with transgenic animals expressing polyQ-containing fragments of the respective disease proteins (e.g. Refs. 14–16). These observations gave rise to the “toxic fragment hypothesis,” which suggests that proteolytic production of polyQ-containing fragments is a prerequisite for the manifestation of polyQ diseases. The detection of a ~36-kDa polyQ fragment of AT3 in brain cells of mice transgenic for full-length AT3 with 71 Gln (~65 kDa) supports this notion in the case of SCA3. Appearance of this fragment was linked closely with neuropathological symptoms, and a similar fragment was observed in brain samples of SCA3 patients (17).

We have recently shown that the release of a polyQ-expanded fragment from full-length AT3 can initiate the aggregation cascade in neuroblastoma cells (8). These experiments indicated a critical role of proteolysis in the aggregation process, but the identity of the cellular protease(s) responsible for processing of AT3 has remained elusive.

Proteolytic processing of polyQ proteins by caspases, the mediators of apoptotic cell death (18–22), and by calcium-dependent calpains (20, 23–26), thought to be activated in the aging brain, has been reported in several model systems (27–29). For SCA3, evidence for a contribution of caspases to proteolysis under induced apoptotic conditions has also been provided (22), whereas a possible role of calpain in this process has remained unclear (12, 17).

Here we found that AT3 is proteolyzed in cell lysates in a calcium-dependent manner. The absence of caspase-3 activation under these conditions and inhibitor studies implicated the family of calpains as processing enzymes. Addition of recombinant calpain II increased the basal rate of AT3 fragmentation without changing the fragment pattern. In a limited proteolysis experiment with recombinant AT3 and calpain II, fragments similar in size to those observed in cells were obtained and identified by microsequencing. In mouse neuroblastoma (N2a) and human embryonic kidney (HEK293) cells we found that AT3 fragmentation is increased by the calcium ionophore ionomycin and suppressed by calpeptin. In cells

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expressing polyQ-expanded AT3, addition of calpeptin or coexpression of the cell-innate calpain inhibitor calpastatin also suppressed the formation of SDS-resistant aggregates. Based on these findings, calpains appear to have an important role in SCA3 pathology and thus represent promising targets for therapeutic intervention.

**EXPERIMENTAL PROCEDURES**

**Expression Constructs**—FLAG- and Myc-tagged AT3, truncated C-terminal Myc-tagged expression constructs, and HisAT3Q22 were described previously (8). DNA was routinely prepared from *Escherichia coli* Sure cells (Stratagene). The pRK5 expression plasmid with human full-length calpastatin cDNA was a kind gift from Dr. Glenn C. Telling, University of Kentucky, Lexington (30).

**Protein Purification**—Protein purification was carried out by standard procedures as described previously (8). In brief, proteins were expressed in *E. coli* BL21 upon induction with 0.1 mM isopropyl β-D-thiogalactoside for 3 h at 30 °C. Collected cells were disrupted by lysozyme and sonication in buffer A (50 mM sodium-phosphate, pH 8, 100 mM NaCl, 10% glycerol, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, protease inhibitor mixture (Complete; Roche Applied Science)) containing 1% Triton X-100. The 30,000 × g supernatant was incubated with 3 ml of glutathione-agarose (Sigma) for 30 min at 4 °C. Beads were washed with buffer A/1M NaCl/1% Triton X-100 and eluted with buffer A containing 20 mM reduced glutathione followed by dialysis against buffer B (20 mM Tris, pH 8, 150 mM KCl, 0.1 mM EDTA, 10% glycerol, 0.1 mM dithiothreitol). His$_{6}$-tagged AT3 proteins were expressed in *E. coli* BL21 (DE3) pLysS and purified by standard procedures using Ni$^{2+}$-NTA (Qiagen) followed by anion exchange chromatography on Source Q columns (Amersham Biosciences). Purification of His$_{6}$-TBP was described elsewhere (7).

**Antibodies**—A polyclonal antiserum against rat AT3 was raised in rabbits by standard procedures and affinity-purified with GST-AT3Q22 (human sequence) cross-linked to glutathione-agarose (Sigma) for 30 min at 4 °C. Calpeptin (Calbiochem) dissolved in Me$_2$SO was applied to cells in concentrations of 1–5 μM in Optimum minimal Eagle’s medium (OptiMEM) (Invitrogen) together with 2 mM CaCl$_2$.

**Western Blot Analysis**—Western blot analysis was performed by standard procedures. Before probing membranes with different antibodies, bound antibodies were removed by incubation in stripping buffer (63 mM Tris/HCl, pH 6.7, 2% SDS, 100 mM β-mercaptoethanol) for 1 h at 50 °C followed by extensive washes in Tris-buffered saline.

**Preparation of N2a Postnuclear Supernatant**—Confluently grown N2a cells were washed with ice-cold 25 mM HEPES/KOH, pH 7.6, and incubated with 200 mM sucrose for 20 min on ice. After removing the sucrose solution, the swollen cells were collected in 250 mM sucrose, 3 mM imidazole, pH 7.4, and disrupted with a Dounce homogenizer (Glas-Col). A centrifugation at 1,000 × g for 10 min at 4 °C removed intact cells and nuclei. The remaining postnuclear supernatant (PNS) was adjusted to 20 mM Tris/HCl, pH 8, and 100 mM KCl. The protein concentration was determined with the Bio-Rad protein assay. Freshly prepared PNS containing 100 μg of protein was incubated with 50 ng of PreScission Protease (PP)-cleaved GST-AT3Q22 for 1 h at 30 °C shaking at 300 rpm. When indicated, 1 mM CaCl$_2$, 1 mM MgCl$_2$, 100 μM ALLN, 2 mM EGTA, 10 μM E-64, 15 μM pepstatin, 0.2 mM leupeptin, 1.5 mM aprotinin, 40–100 μM Z-VAD-FMK (benzyloxycarbonyl-VAD-fluoromethyl ketone), 40–100 μM Z-DEVD-FMK, 40–100 μM Ac-YVAD-CHO, or 20 μM MG132 were added. The ATP-regenerating system contained 2 mM ATP, 5 mM MgCl$_2$, 8 mM creatine phosphate (Roche Applied Science), and 50 μg/ml creatine kinase (rabbit muscle; Roche Applied Science). Recombinant calpain II was added together with an additional 1.5 mM MgCl$_2$.

**FACS Analysis**—Cells were processed for 1,2-bis-(2-amino-phenoxymethane)-N,N,N',N'-tetraacetic acid acetoxy methyl ester (BAPTA-AM; Molecular Probes) and annexin V/propidium iodide (Calbiochem) staining 48 h after transfection following the suppliers’ instructions and analyzed with a FACS Calibur instrument (BD Biosciences).

**Limited Proteolysis and Peptide Sequencing**—Purified AT3 proteins (His-AT3Q22 or PP-cleaved GST-AT3Q22), recombinant TATA-binding protein (His-TPBPQ38), and rabbit muscle actin (Sigma) were incubated with recombinant rat calpain II (Calbiochem) in 20 mM HEPES/KOH, pH 7.6, 10 mM KCl, 1.5 mM MgCl$_2$, 1 mM dithiothreitol, and 1 mM CaCl$_2$ for 30 min at 22 °C. After SDS-PAGE, protein processing was analyzed by Western blotting or Edman N-terminal sequencing with phenylthiohydantoin chemistry and separation of amino acid derivatives by reverse phase high pressure liquid chromatography.
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about the cellular enzymes involved, we investigated the proteolysis of AT3 in PNS of N2a cell lysates (Fig. 1a). Addition of magnesium chloride or an ATP-regenerating system did not result in enhanced fragmentation of AT3. Lowering of the pH was similarly ineffective. In contrast, the addition of calcium chloride triggered the production of AT3 fragments (Fig. 1a). Procaspase-3 was not processed to the active p17 fragment, indicating that a caspase-mediated cell death cascade was not activated under these conditions (Fig. 1a). A similar CaCl2-dependent sensitivity of endogenous human AT3 was observed in HEK293 cells (Fig. 1b). We further tested a variety of protease inhibitors (Fig. 1c). While purified recombinant human AT3Q22 was not sensitive to CaCl2, the endogenous mouse protein contained in the PNS was hydrolyzed to generate mainly a ~29-kDa fragment upon addition of CaCl2 (Fig. 1c). Similarly, processing of the human protein into 20-, 24-, and 34-kDa fragments required PNS and was enhanced by the addition of CaCl2 (Fig. 1c). The calpain/proteasome inhibitors ALLN and MG132 (31) and the cysteine/serine protease inhibitor leupeptin inhibited the calcium-dependent cleavage of human AT3, whereas the cysteine protease inhibitor E-64 had only a weak effect. In contrast, the aspartyl protease inhibitor pepstatin and the serine protease inhibitor aprotinin did not inhibit cleavage of AT3. Hence, AT3 was likely processed by calcium-dependent cytoplasmic calpain proteases. In support of this conclusion, addition of recombinant calpain II resulted in a 1.8- to 3-fold increase in the production of the AT3 fragments at 16, 20, 24, and 34 kDa (Fig. 1d, compare lane 3 with lanes 5–8). To analyze a possible contribution of caspases in this process, PNS from AT3Q71-expressing cells were treated with CaCl2 and incubated with calpain and caspase inhibitors. Although ALLN inhibited the fragmentation of AT3, caspase inhibitors up to 100 μM were without effect (Fig. 1e). Thus, it

RESULTS

Calpain Inhibitors Attenuate Calcium-dependent Fragmentation of AT3 in Cell Lysates—AT3 is processed in mammalian cells by yet unknown proteases (8, 17). To obtain information

FIGURE 1. Proteolysis of AT3 in cell-free lysates is stimulated by external calcium. a, PreScission Protease (PP)-cleaved GST-AT3Q22 was incubated with N2a postnuclear supernatant (PNS) and protease inhibitor mixture (Complete), CaCl2, MgCl2, and ATP-regenerating system, respectively, for 1 h at 30 °C. Alternatively, a low pH was adjusted. Upper panel, samples were subjected to Western blot analysis with anti-AT3 antibody. Arrowheads indicate calcium-induced AT3 fragments. Lower panel, the same Western blot membrane was probed for caspase-3 activation. b, HEK293T cell lysate was incubated with protease inhibitor mixture (Complete), CaCl2, or CaCl2/ALLN. Upper panel, Western blot analysis (anti-AT3). Arrowheads indicate calcium-induced AT3 fragments. Lower panel, the same Western blot membrane was probed with a caspase-3 antibody. c, PP-cleaved human GST-AT3Q22 (H) was incubated alone (lanes 1–2) or with N2a PNS (lanes 3–12) containing endogenous AT3 (M) for 1 h at 30 °C. Alternatively, N2a PNS was incubated alone (lanes 13–14). CaCl2, and various protease inhibitors (ALLN, EGTA, E-64, pepstatin, leupeptin, aprotinin, MG132) were added as indicated. SDS-PAGE followed by Western blot analysis (anti-AT3). Lowercase letters indicate fragments derived from the human (h) or mouse (m) AT3 protein. d, N2a PNS was incubated alone (lane 1) or with PP-cleaved GST-AT3Q22 (lanes 2–8) for 1 h at 30 °C. CaCl2, the calpain inhibitor ALLN, or increasing amounts of calpain II were added as indicated. Samples were subjected to a Western blot analysis (anti-AT3). Arrowheads with lowercase letters indicate fragment bands derived from human (h) or mouse (m) AT3. Fragment bands in lanes 3, 5, 6, 7, and 8 were quantified. Results are listed in the box below. The intensities of the respective fragments in lane 3 (PNS + GST-AT3Q22 + CaCl2) were set as 100%. e, postnuclear supernatants were prepared from AT3Q71-expressing N2a cells and incubated with or without 1 mM CaCl2, 50 μM ALLN, and the indicated amounts of caspase inhibitors for 1 h at 30 °C. Lysates were subjected to Western blotting and detection with anti-AT3 antibody.
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FIGURE 2. Identification of calpain II cleavage sites in AT3 in vitro. a, His-AT3Q22 was incubated alone (lane 1) or with CaCl2, and increasing amounts of calpain II for 30 min at 22 °C as indicated. Alternatively, calpain II was incubated alone (lane 5). The samples were subjected to SDS-PAGE followed by transfer to a polyvinylidene difluoride membrane and Coomassie staining. AT3 fragments were excised from the membrane and identified by Edman sequencing. b, human His-AT3Q22, human His-TBPQ38, or rabbit muscle actin (150 ng each) was incubated alone or with increasing amounts of calpain II for 30 min at 22 °C. Amounts of uncleaved proteins were determined by Western blot analysis with anti-AT3, anti-TBP, or anti-actin antibodies, respectively, followed by densitometry.

appears likely that AT3 cleavage occurred by calpains contained in N2a postnuclear supernatants.

Identification of Calpain II Cleavage Sites in AT3 in Vitro—To obtain information about calpain-sensitive regions within AT3, we set up an in vitro assay with recombinant calpain II. Upon incubation with increasing amounts of calpain II in a limited proteolysis assay, several AT3 fragments were detected by SDS-PAGE and Coomassie staining (Fig. 2a). The N-terminal sequences of the most abundant and stable fragments were determined by microsequencing (Fig. 2a, box). AT3Q22 was cleaved by calpain II in the regions around amino acid residues 60, 200, and 260. An additional cleavage site was found C-terminal to the polyQ stretch (residue 318). Interestingly, fragments of polyQ-expanded AT3 processed from positions 260 are highly prone to aggregation in vitro (8) and in living cells (1, 8).

To determine the susceptibility of AT3 toward calpain cleavage, we compared the sensitivity of AT3 to increasing concentrations of calpain II with that of the established calpain substrate actin (32) and of TATA-binding protein (TBP), another polyQ protein. AT3 was highly calpain-sensitive, with as little as 6 ng of calpain II being sufficient for 50% degradation of 150 ng of AT3 within 30 min at 22 °C, whereas degradation of 150 ng of TBP required ~4 times more protease. Actin (150 ng) was stable under these conditions up to 50 ng of calpain. These data suggest a high susceptibility of AT3 toward calpain cleavage, consistent with the high sensitivity of AT3 toward calcium-induced proteolysis in PNS.

Ionomycin Increases Fragmentation of AT3 in Living Cells—The experiments carried out so far showed that AT3 is sensitive against calpain proteolysis in vitro. To investigate AT3 processing in vivo, cleavage of AT3 was analyzed in neuroblastoma N2a cells upon stimulation or inhibition of calpains.

Ionomycin is an ion carrier that increases the cytoplasmic level of Ca2+ ions and thereby activates intracellular calpains (33). In N2a cells treated with ionomycin for a sublethal period, the amount of endogenous mouse AT3 and of recombinantly expressed polyQ-expanded AT3Q71 were reduced and fragmentation was increased compared with carrier-treated control cells (Fig. 3a, lanes 1 and 2). The cell-permeable calpain inhibitor calpeptin abolished this effect, indicating the involvement of calpains in AT3 processing (Fig. 3a, lanes 3–5). Although incubation of cells with ionomycin for longer than 1.5 h resulted in toxicity, incubation with calpeptin for several days did not cause overt toxicity. Under these conditions, calpeptin strongly inhibited the formation of SDS-resistant aggregates in cells expressing AT3Q71 without affecting the apparent steady state levels of the protein, as determined by Western blotting with anti-polyQ antibody (Fig. 3b). This suggests that endogenous calpain proteolysis either affects only a small subpopulation of AT3Q71 or is limited to a subset of AT3Q71-expressing cells. Interestingly, in ionomycin-treated cells the polyQ-expanded AT3 was not more sensitive to calpain cleavage than the endogenous mouse AT3 containing 6 Gln (Fig. 3a).
in the brains of transgenic animals expressing polyQ-expanded AT3.

DISCUSSION

We found through a series of in vitro and in vivo experiments that AT3 is a sensitive substrate of calcium-dependent calpain proteases. PNS from N2a cells induced proteolysis of AT3 upon addition of calcium in the absence of apparent caspase-3 activation (Fig. 1). AT3 fragmentation was inhibited by the calcium chelator EGTA, by the calpain inhibitor ALLN, and the cysteine protease inhibitor E-64 and leupeptin, and by the proteasome inhibitor MG132 that also inhibits calpains (31) (Fig. 1c), whereas caspase inhibitors were without effect (Fig. 1e). Sequence motifs within AT3 that are recognized by calpain II were identified by microsequencing. In agreement with findings by Tompa et al., these motifs are predominantly located in unstructured regions (34). The recognition sequence around amino acid residue 260 in AT3 is of particular interest because cleavage of polyQ-expanded AT3 in this region is known to produce highly aggregation-prone fragments of ~32 kDa that can initiate the aggregation process and recruit full-length AT3 into co-aggregates (8). The production of these polyQ-containing fragments was increased upon ionomycin treatment of AT3Q71-expressing cells, whereas fragmentation and aggregation were inhibited by calpeptin (Fig. 3a). Importantly, the identification of calpain as processing enzyme was corroborated by experiments in which the highly specific innate calpain inhibitor calpastatin was co-expressed with polyQ-expanded AT3. Under these conditions, the generation of polyQ-containing fragments (Fig. 4a) and their aggregation (Fig. 4b) was almost completely suppressed. Based on these findings, we suggest that the cellular calpastatin/calpain system is a promising target for therapeutic intervention in SCA3.

A variety of chronic neurodegenerative conditions, including amyotrophic lateral sclerosis (35), Parkinson disease (36), and Alzheimer disease (28) are accompanied by altered levels of calpains and/or its inhibitor calpastatin (reviewed in Ref. 37). Furthermore, evidence for a dysregulation of cellular calcium homeostasis in polyQ diseases has been provided for Huntington disease (24) and SCA1 (38). In the latter case, down-regulation of proteins involved in glutamate metabolism and in calcium signaling and homeostasis was shown to precede pathological symptoms in a transgenic mouse model. Early down-regulation of the Ca^{2+}-binding proteins parvalbumin and calbindin has been demonstrated in a similar model (39). These observations would suggest that calpains can act on polyQ proteins early in disease and without being strongly activated as a result of an apoptotic program. In fact, in a late onset mouse model of Huntington disease and in postmortem brains of affected individuals, pathogenesis was shown to occur independently from necrotic or apoptotic cell death (40). In accordance with these findings, we found no effect of caspase inhibitors (Fig. 1e) and we did not observe signs of necrosis or the induction of apoptotic cell death upon expression of AT3 in our cell culture model (supplemental Fig. S3). A basal, low level activity of calpains might thus be responsible for the constitutive production of AT3 fragments that may eventually exceed a critical concentration and become cytotoxic. However, this

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FIGURE 4. Cleavage and aggregation of AT3 is inhibited by the specific calpain inhibitor calpastatin. a, HEK293T cells were transiently transfected with pcDNA3.1-AT3Q71 and with pRK5-calpastatin and pcDNA3.1-lacZ (β-galactosidase) plasmids, respectively, and cultured for 24–72 h. Cell lysates were subjected to Western blot analysis with anti-polyQ antibody. Lysates of non-transfected HEK293T cells (lane 1) and recombinant PP-cleaved GST-257cQ71 (right panel) were analyzed as controls. Arrowheads indicate AT3 fragments that were not detected in lysates from cells transfected with calpastatin. b, HEK293T cells were transfected as in panel a followed by lyse preparation and filter trap analysis 72 h post-transfection to detect SDS-resistant AT3Q71 aggregates.
process may be accelerated in individual neurons following excitotoxic stimuli that transiently disturb intracellular Ca\(^{2+}\) levels. Additionally, cellular systems that normally cope with the load of aberrant molecular species, such as molecular chaperones or the ubiquitin proteasome system, may become overwhelmed, thereby facilitating disease manifestation. Further work on animal models will be required to clarify these issues.

Acknowledgment—We thank Ramunas M. Vabalas for valuable help with the FACS analysis and critical reading of the manuscript.

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