Proteolytic Cleavage of Ataxin-7 by Caspase-7 Modulates Cellular Toxicity and Transcriptional Dysregulation*

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Spino­cerebellar ataxia type 7 (SCA7)4 is a polyglutamine (polyQ) disorder characterized by specific degeneration of cerebellar, brainstem, and retinal neurons. Although they share little sequence homology, proteins implicated in polyQ disorders have common properties beyond their characteristic polyQ tract. These include the production of proteolytic fragments, nuclear accumulation, and processing by caspases. Here we report that ataxin-7 is cleaved by caspase-7, and we map two putative caspase-7 cleavage sites to Asp residues at positions 266 and 344 of the ataxin-7 protein. Site-directed mutagenesis of these two caspase-7 cleavage sites in the polyQ-expanded form of ataxin-7 produces an ataxin-7 D266N/D344N protein that is resistant to caspase cleavage. Although ataxin-7 displays toxicity, forms nuclear aggregates, and represses transcription in human embryonic kidney 293T cells in a polyQ length-dependent manner, expression of the non-cleavable D266N/D344N form of polyQ-expanded ataxin-7 attenuates cell death, aggregate formation, and transcriptional interference. Expression of the caspase-7 truncation product of ataxin-7−69Q or -92Q, which removes the putative nuclear export signal and nuclear localization signals of ataxin-7, showed increased cellular toxicity. We also detected N-terminal polyQ-expanded ataxin-7 cleavage products in SCA7 transgenic mice similar in size to those generated by caspase-7 cleavage. In a SCA7 transgenic mouse model, recruitment of caspase-7 into the nucleus by polyQ-generated by caspase-7 cleavage products in SCA7 transgenic mice similar in size to those cleaved by caspase-7, and we map two putative caspase-7 cleavage sites to Asp residues at positions 266 and 344 of the ataxin-7 pro-
tive retinal dysfunction can be correlated with transcriptional dysregulation via an abnormal interaction of the polyQ-expanded form of ataxin-7 with the cone-rod homeobox protein (Crx) (25, 27). In two recently generated SCA7 transgenic mouse models, both Crx and other non-Crx regulated photoreceptor-specific genes are down-regulated (31, 32). SCA7 transgenic mice expressing full-length ataxin-7–92Q recapitulate the cone-rod dystrophy seen in humans by interfering with Crx, a photoreceptor specific transcription factor (25, 32). Mutations in human Crx cause retinal degeneration (33).

Although polyQ diseases have similar characteristics and pathogenicity aside from their polyQ tract, their proteins share little discernable sequence or functional homology. However, several clues suggest that shared pathways of toxicity exist. First, the diseases all have a highly cell-specific neurodegenerative phenotype despite widespread expression of the proteins. Second, cytoplasmic and nuclear inclusions are present in all of these diseases (34–36). Third, histological evidence shows cell death in these diseases is apoptotic (37–43). Fourth, many biochemical studies have shown that the gene products of Huntington disease (huntingtin), spinal bulbar muscular atrophy (C-terminal huntingtin) is a critical event in polyQ protein-mediated neurodegeneration (13, 45, 46).

Direct evidence of proteolytic cleavage of polyQ disease proteins in vivo by caspases comes from studies of rare early grade and presymptomatic Huntington disease human tissues as well as in brains from the yeast artificial chromosome transgenic animal model of Huntington disease using antibodies specific to caspase cleavage products of huntingtin (46, 47). Immuno-reactivity to neo-epitope antibodies reactive to the caspase-mediated apoptosis, the formation of inclusions, and disease pathogenesis (13, 45, 46).

Site-directed Mutagenesis—Site-directed mutagenesis was performed using the QuickChange kit (Stratagene, La Jolla, CA) according to the manufacturer’s instructions. Mutagenesis was performed using PCR primers D266N, 5’-ggagctttccagaatctgtgctcagcggctgcaagtgatccag-3’ and D344N, 5’-agaggttgtgcagcctaatccactacatcctgtggaagtc-3’. Primers were purchased from IDT (Coralville, IA). All constructs utilized for our studies were sequenced.

Western Blot Analysis—Ataxin-7 was extracted with Nonidet P-40 or modified radioimmune precipitation lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% SDS, 1% sodium deoxycholate, 1% Nonidet P-40). Cells from a 10-cm dish were washed in phosphate-buffered saline and lysed in 1 ml of Nonidet P-40 buffer (50 mM HEPES, 250 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, pH 7.4) with protease inhibitor Minicomp (Roche Applied Science) for 10 min and centrifuged at 15,000 × g. Alternatively, ataxin-7 was extracted in modified radioimmune precipitation lysis buffer. Proteins were resolved on NuPAGE Bis/Tris 10% gels (Invitrogen) according to the manufacturer's instructions and transferred to polyvinylidene difluoride (Bio-Rad) for 2 h at 300 mA. Membranes were blocked in 5% nonfat milk in Tris-buffered saline-Tween and probed with primary polyclonal antibodies to ataxin-7, antibody A (RAADDVRGEP), corresponding amino acids in the N terminus (1:1000) or polyclonal antibody K (1:1000) for 2 h at room temperature or 18 h at 4 °C. Alternatively, monoclonal 1C2 (Chemicon, Temecula, CA) was used to the polQ tract of ataxin-7 (1:2000) or polyclonal ataxin-7 (1:1000, Affinity BioReagents, PA1-749). Secondary anti-rabbit or antimouse antibody (1:3000, Amersham Biosciences) was applied for 45 min at room temperature, and ECL (Amersham Biosciences) was used for detection.

Cytotoxicity Assay—Cells were transiently transfected in 6-well dishes and treated with tamoxifen (35 μM, 4 h) to induce cell death. After 48 h cells were harvested by scraping and centrifugation at 500 × g for 10 min. Cells were lysed (Apoalert kit lysis buffer, Clontech, Palo Alto, CA) for 10 min, and the membrane fraction was pelleted by centrifugation at 15,000 × g for 2 min. Lysate containing 60 μg of total protein in a final volume of 20 μl was added to 100 μl of caspase reaction buffer (20 mM
**Caspase-7 Cleavage of Ataxin-7**

PIpes, pH 7.2, 100 mm NaCl, 1% CHAPS, 10% sucrose, 10 mM DTT) and 100 μM DEVD-aminofluoromethylcoumarin (Biolum, Plymouth Meeting, PA) in a 96-well dish. Reactions were followed using a Gemini SpectraMax fluorimeter plate reader using 400/505-nm excitation/emission wavelengths and SoftMaxPro analysis software (Molecular Devices, Sunnyvale, CA). The activity level of caspase-3 was taken from the linear portion of the reaction curve. To verify consistent expression levels, 20 μg of lysate was resolved by SDS-PAGE using 10% Bis/Tris prestained gels (Invitrogen) and transferred to polyvinylidene difluoride (Bio-Rad). Western blotting was performed using the ataxin-7 antibody K or Affinity BioReagents polyclonal ataxin-7 antibody (PA1-749; 1:500). Alternatively, 293T cells were transiently transfected to 8-well poly-D lysine- or collagen-coated slides (BD Biosciences). Cells were counterstained with Hoechst dye, fixed with 4% paraformaldehyde, and coveredslipped. Cell death was quantified by counting transfected cells and taking the percentage of dead cells per 200 transfected coverslipped. Cell death was determined by nuclear fragmentation and condensation seen with the Hoechst stain. Student’s t tests were performed.

**Subcellular Fractionation**—293T or COS-7 cells were transiently transfected and harvested at 48 h by scraping, and subcellular fractionation was performed using the NE-PER kit (Pierce). Nuclear and cytoplasmic fractionations were verified by immunoblotting with anti-poly(ADP-ribose) polymerase antibody (1:2500, Biomol) and monoclonal anti-tubulin antibody (1:500, Sigma).

**Coimmunoprecipitation**—293T cells were transiently transfected with ataxin-7 constructs and FLAG-tagged caspase-7, harvested at 48 h, and fractionated. Each lysate was pre-cleared with mouse IgG-agarose for 1 h at 4°C and then incubated with 25 μL ANTI-FLAG M2-agarose affinity gel (Sigma) overnight at 4°C. Proteins bound to the FLAG beads were washed 5 times (50 mM HEPES, 250 mM NaCl, 5 mM EDTA, 0.1% Nonidet P-40) eluted with NuPAGE® LDS sample buffer (Invitrogen) and resolved using 10% Bis/Tris prestained gels (Invitrogen). Western blotting was performed, and blots were probed with ataxin-7 antibody K (1:500), polyclonal anti-caspase-7 9492 antibody (1:1000, Cell Signaling, Beverly, MA) and monoclonal anti-tubulin antibody (1:500, Sigma).

**In Vitro Translation and Caspase Cleavage**—Ataxin-7 constructs were in vitro translated using the TNT coupled reticulocyte lysate system (Promega, Madison, WI) according to manufacturer’s instructions in the presence of [35S] Methionine (PerkinElmer Life Sciences). 5 μL of in vitro translated reaction was incubated with caspasas (gift from the laboratory of Guy Salvesen) for 2 h at 37°C. Cleavage products were resolved using 10% Bis/Tris prestained gels (Invitrogen). Gels were dried and exposed to x-ray film.

**Confocal and Fluorescence Microscopy**—293T and COS-7 cells transfected with ataxin-7 constructs were mounted on poly-D-lysine- or collagen-coated slides (BD Biosciences), fixed, and coverslipped with Vectashield mounting medium with 4’,6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA). The slides were then visualized by confocal microscopy using a Nikon PCM-2000 laser confocal scanning microscope. Primary rat cerebellar granule neurons were transfected, fixed, and mounted. The cells were stained using ataxin-7 antibody “A” (1:100) and anti-MAP-2 antibody (1:100, Sigma). The cells were visualized by fluorescence microscopy using a Nikon TE300 inverted microscope. Images were taken with an Optronics digital camera and MagnaFire Software (Optronics MagnaFire, Goleta, CA). Polyclonal activated anti-caspase-7 (94915) from Cell Signaling (1:100) and polyclonal anti-ataxin-7 antibody K (1:500) were used for staining paraffin-embedded retinal transgenic tissue.

**Nucleofection of Primary Cerebellar Cultures**—Primary cerebellar cultures were prepared from 6-day-old Sprague-Dawley rat pups as previously described (51) with minor modifications. Briefly, cerebella from 6-day-old Sprague-Dawley rat pups were digested with 0.25% trypsin (Cell Grow). After neutralization with 10% serum, cells were triturated and centrifuged for 5 min at 800 g. The pellet was resuspended in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum and filtered through a 70-μm cell strainer. After washing the cells 3 times with the same media, the cells were resuspended in a density of 4 million cells/100 μl of Amaxa nucleofector solution (Amaxa Inc., Gaithersburg, MD) and electroporated according to the manufacturer’s specification. Cells were diluted with Dulbecco’s modified Eagle’s medium, 10% fetal bovine serum, and seeded onto polylysine-coated glass chamber slides (BD Biosciences) at a concentration corresponding to 2.5–3.5 × 10⁵ cells/cm². After a 30-min incubation, the medium was replaced with neurobasal A media containing 1 mm Glutamax-1, 24.5 mM KCl, and 2% B-27 (Invitrogen). The cultures were incubated at 37 °C in 95% air, 5% carbon dioxide with 95% humidity. Efficiency of transfection was between 75 and 85% by day 4 as estimated by GFP fluorescence.

**Transient Transfection and Luciferase Assays in 293T Cells**—Transient transfection experiments with bovine rhodopsin luciferase reporter (BR-225-luc), bCrx-pcDNA3.1/HisC, neural retinal leucine zipper protein, pCMV-SCA7 constructs, and dual luciferase assays were performed as described previously (25, 32).

**RESULTS**

**Ataxin-7 Protein**—The ataxin-7 protein, with the sequences relevant to this study (Fig. 1A), along with the various ataxin-7 constructs utilized or generated are shown (Fig. 1B). Ataxin-7 is an 892-amino acid protein with a glutamine repeat starting at amino acid position 30. Ataxin-7 contains a leucine-type NES in its N terminus (positions 340–349) (26), NLSs in its C terminus (positions 705 and 835) (25), and a functional phosphoprotein site (52). A highly conserved “zinc binding domain” between 311 and 406 of ataxin-7 may mediate its interaction with the SPT3-TAF9-ADA-GCNS acetyltransferase (STAGA) complex (29). The polyQ tract along with amino acids 1–66 of ataxin-7 is required for transcriptional interference with Crx (25, 32). The NLS at position 378 may mediate the import of ataxin-7 into the nucleus (25, 32).

**Ataxin-7 Is a Substrate for Caspase-7**—A number of the polyQ disease proteins are caspase substrates, and cleavage products derived from them are thought to play a role in disease...
pathogenesis. Analysis of transgenic mouse models of SCA7 suggest that proteolytic cleavage products are generated from mutant ataxin-7, but the protease responsible for cleavage of ataxin-7 has not been identified (50). A number of caspase cleavage consensus sites can be found in the N-terminal region of ataxin-7 (Fig. 1A). To evaluate whether ataxin-7 is a caspase substrate, in vitro translated ataxin-7–10Q or ataxin-7–92Q were treated with purified recombinant caspase-1 through caspase-13 (caspase-11, -12, -13; data not shown). Ataxin-10Q and -92Q were cleaved by only one of the caspase family members tested; that is, caspase-7 (Fig. 2A). Cleavage of ataxin-7–10Q and -92Q produced three cleavage products (Fig. 2A, right panel). Two of the cleavage products migrated slower in the gel for ataxin-7–92Q (45 kDa (major) and 55 kDa (minor)) when compared with ataxin-7–10Q (30 and 40 kDa) and, therefore, must contain the N terminus of ataxin-7 since this region of the protein has the polyQ expansion. The other C-terminal cleavage products common to both the 10Q and 92Q forms fragment generated by cleavage at amino acid 344 (Fig. 2C). Fig. 2D demonstrates that production of ataxin-7–92Q proteins truncated at amino acid position 266 or 344 migrate at the same molecular weight as the cleavage products generated by treating full-length ataxin-7–92Q with caspase-7.

**Caspase-7 Cleavage of Ataxin-7** of the ataxin-7 protein migrated at an apparent molecular mass of 70 kDa (minor 60 kDa). The susceptibility of ataxin-7–10Q and ataxin-92Q to caspase-7 cleavage was also evaluated by measuring the rate and dose response of cleavage. Our in vitro translation cleavage assay indicated that the susceptibility of ataxin-7 to caspase-7 cleavage was independent of the polyQ expansion (data not shown).

**Analysis of the Ataxin-7 Caspase Cleavage Site**—Two putative caspase cleavage sites for ataxin-7 are clustered in the N terminus adjacent to and within a recently identified NES (Fig. 2B) (27). To confirm these cleavage sites, we mutated each site and treated in vitro translated ataxin-7 protein with caspase-7. Mutation of the caspase P1 residue in ataxin-7 from Asp-266 to Asn abolished the processing of in vitro translated ataxin-7–10Q (or -92Q) to generate the 30-kDa (45 kDa) cleavage product by caspase-7 (data not shown). Mutation from Asp-344 to Asn in ataxin-7 similarly blocked proteolytic cleavage to generate the 40-kDa (55 kDa) product (data not shown). As shown in Fig. 2C, mutations in both sites in ataxin-7–92Q (D266N/D344N, lanes 5 and 6) produced a protein completely resistant to cleavage by caspase-7 when compared with ataxin-7–92Q (lane 3 and 4). Interestingly, the cleavage product of ataxin-7 at amino acid 266 is far more abundant than the fragment generated by cleavage at amino acid 344 (Fig. 2C). Fig. 2D demonstrates that production of ataxin-7–92Q proteins truncated at amino acid position 266 or 344 migrate at the same molecular weight as the cleavage products generated by treating full-length ataxin-7–92Q with caspase-7.

**Evaluation of Cellular Toxicity and Caspase-resistant Ataxin-7 Constructs**—We evaluated the cytotoxicity of ataxin-7 in the context of the polyQ expansion. Transient transfection of human embryonic kidney 293T cells with expression constructs encoding ataxin-7–69Q-GFP or -92Q-GFP resulted in a repeat-dependent increase in cell death (15 and 24% for ataxin-7–69Q-GFP and 92Q-GFP; ***, p < 0.001) (Fig. 3A). Western blot analysis of ataxin-7 indicated that the expression levels of the proteins were equal (supplemental Fig. 1). Expression of GFP fusions of mutant ataxin-7 could induce cell death without the addition of cytotoxic agents.

Next, we assessed the effect of blocking caspase cleavage of mutant ataxin-7 on cellular toxicity in human embryonic kid-

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**FIGURE 1. Diagram of ataxin-7 constructs and cleavage sites.** A, potential ataxin-7 caspase cleavage sites and nuclear import and export signals (NLS or NES). Potential NLS lie at amino acids 378–393, 704–709, and 834–839 of ataxin-7. A NES in ataxin-7 is at one of the predicted caspase cleavage sites, 341–352. B, schematic of the ataxin-7 cDNA constructs utilized in these studies in either pcDNA3.1 or pSecTagA vectors. All constructs were also made as GFP fusions using pEGFP-N1 generating a GFP C-terminal tag in ataxin-7.
ney 293T cells. As shown in Fig. 3

SITE and enhanced caspase activity (Fig. 3C, data not shown). This suggests that ataxin-7 proteolytic cleavage at one site is influenced by cleavage at the other site.

Increased Cytotoxicity Correlates with Production of the N-terminal Fragment of Ataxin-7—Because mutation of the caspase cleavage sites reduced cellular toxicity of mutant ataxin-7, we then examined if fragments derived from mutant ataxin-7 were cytotoxic. Proteolytic cleavage as well as the location of the NLSs relative to the polyQ tract in a protein can influence its cytotoxicity. Previous studies of the polyQ disease protein atrophin-1 suggest that retention of NLS or NES sequences in a proteolytic cleavage fragment may modulate the toxicity of the derived fragment (53). This led us to evaluate the cytotoxicity of the caspase-derived fragments of ataxin-7, fragments containing the NLS, and full-length protein. To perform this experiment, we created N-terminal truncation-GFP fusion ataxin-7 constructs ending at amino acids 239, 460, and 645 (Fig. 3D). Expression of the short N-terminal fragment ending at amino acid 239 had the greatest cytotoxicity (Fig. 3D), suggesting that fragments derived from caspase cleavage of ataxin-7–92Q are highly toxic.

Caspase Non-cleavable Ataxin-7 Yields Reduced Inclusion Formation in Cerebellar Granule Neurons—One hallmark of polyQ diseases is the formation of inclusions from mutant proteins that are misfolded or resistant to degradation by the proteasome. For SCA7, nuclear inclusions are stained with antibodies against epitopes close to the polyQ tract but not against the C terminus (49). This suggests cleavage of the full-length protein is involved in the formation of inclusion; therefore, we evaluated if caspase cleavage modulated the formation of nuclear inclusions. Immunofluorescence analysis of transiently transfected 293T cells or COS-7 cells was carried out. Confocal micrographs of ataxin-7–10Q-GFP, ataxin-7–69Q-GFP, and ataxin-7–92Q D266N/D344N-GFP expressed in 293T cells counterstained with propidium iodide were obtained. Expression of ataxin-7–10Q-GFP resulted in diffuse nuclear localization, whereas ataxin-7–69Q-GFP led to the formation of densely stained nuclei with small nuclear inclusions and altered localization (Fig. 4). Expression of caspase-resistant

FIGURE 2. Recombinant caspase-7 cleaves in vitro translated ataxin-7 at Asp-266 and Asp-344. A, treatment of ataxin-7–10Q and ataxin-7–92Q with recombinant caspase-7 demonstrates ataxin-7 is proteolytically cleaved by caspase-7. Caspase-11, -12, and -13 were also tested and did not cleave ataxin-7. B, ataxin-7 potential caspase cleavage sites lie at amino acid 266 and 344 of ataxin-7. C, mutagenesis at Asp-266 and -344, and subsequent in vitro translation of altered ataxin-7–92Q construct demonstrates removal of caspase cleavage sites. D, in vitro translation of truncated ataxin-7–92Q constructs at amino acids 266 and 344 demonstrates cleavage products are the same size as those generated by treating full-length ataxin-7–92Q-translated construct with recombinant caspase-7. The preferred site of caspase-7 cleavage in ataxin-7 appears to be at amino acid 266 in vitro.

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ataxin-7–69Q D266N/D344N-GFP reduced the formation of nuclear inclusions in both 293T and COS-7 cells, and the nuclear staining was more diffuse (Fig. 4, from 48 ± 6% inclusions to 30 ± 4%).

We also evaluated whether aggregate formation was modulated by caspase cleavage in primary cerebellar granule neuron cultures. Transcriptional Dysregulation and Proteolytic Cleavage of Ataxin-7—One of the more compelling mechanisms for retinal degeneration and neuroselectivity in SCA7 is interference with the transcriptional function of Crx (32). In previous work Crx transcriptional interference by the polyQ-expanded ataxin-7 is mediated by a direct interaction with Crx and requires the polyQ tract in the N terminus (25). One question relevant to the ability of mutant ataxin-7 to mediate transcriptional interference is whether fragments derived from proteolysis play a role in this process. This may be particularly important given the age-dependent accumulation of ataxin-7–92Q fragments in the SCA7 transgenic mice as well as in other polyQ disease transgenic models. To evaluate this question, we tested the ability of normal ataxin-7–10Q, ataxin-7–69Q, and the non-cleavable ataxin-7–69Q D266N/D344N to cause transcriptional interference using a rhodopsin promoter-luciferase reporter construct (BR-225-luc). As previously published, in the presence of Crx and neural retinal leucine zipper protein, the full-length ataxin-7–69Q produces a marked decrease in transcriptional activation of the reporter construct, whereas ataxin-7–10Q does not (Fig. 5). Interestingly, expression of the full-length ataxin-7–69Q D266N/D344N non-cleavable mutant does not cause the same magnitude of transcriptional interference as ataxin-7–69Q (Fig. 5), suggesting that ataxin-7 proteolysis may contribute to the SCA7 “transcriptionopathy” (32).

Caspase-7 Cleavage of Ataxin-7—Immunofluorescence analysis of cerebellar cultures transfected with mutant ataxin-7 69Q or the non-cleavable ataxin-7 69Q mutant D226N D344N version indicated that whereas ataxin-7–69Q forms distinct nuclear inclusions, ataxin-7–69Q D266N/D344N does not (Fig. 4C).

Caspase-7 Is Recruited into Inclusions and Activated by Ataxin-7—Next, we evaluated whether caspase-7 is activated by mutant ataxin-7 using immunofluorescence. Activation and recruitment of caspases have been reported for a number of polyQ disease proteins (13, 19, 45, 48). COS-7
cells were co-transfected with GFP-tagged ataxin-7 constructs and FLAG-tagged caspase-7, and recruitment of caspase-7 by polyQ-expanded ataxin-7 was observed (Fig. 6A). In the nucleus ataxin-7 co-localizes with caspase-7 in the soluble fraction as well as in inclusions. We found that recruitment of caspase-7 and formation of co-localizing inclusions are reduced in the caspase non-cleavable mutant (Fig. 6A). Western blot analysis of the nuclear fraction probed with antibody against caspase-7 demonstrates a repeat-dependent activation of caspase-7 and an increase in nuclear localization (see supplemental Fig. 3). Expression of the caspase-resistant ataxin-69Q D266N/D344N attenuates the activation of caspase-7 (Fig. 6B). In accord with previous reports on caspase-7 we found the majority of this protease was present in the cytoplasm (90%, see supplemental Fig. 3) (57).

We documented that physical interaction of specific caspase family members correlated with the caspase family member responsible for cleavage of the polyQ protein. For example, physical interaction of huntingtin with caspase-2, -6, and -7 and subsequent cleavage of huntingtin by these caspases has been noted (19). Therefore, we evaluated whether ataxin-7 co-immunoprecipitated with caspase-7 and if this was repeat-dependent. Anti-FLAG pull-down of caspase-7 resulted in a repeat-dependent interaction with ataxin-7. Particularly of note was the increased interaction of ataxin-7–69Q with caspase-7 when compared with ataxin-7–10Q (Fig. 6C).

To evaluate if activation and recruitment of caspase-7 is relevant to disease pathogenesis, we obtained retinal sections from the MoPrP-SCA7–92Q transgenic mouse model (32) and checked for the presence of activated caspase-7 in the photoreceptor cells destined to degenerate. Using an antibody to activated caspase-7, immunostaining of retina from SCA7–92Q mice demonstrated the presence of activated...
Caspase-7 in the nucleus (Fig. 7A). Activated caspase-7 was not detected in SCA7–24Q transgenic mice, however (Fig. 7A).

Proteolytic Cleavage Fragments Detected in SCA7 Transgenic Mice Are Similar in Size to Caspase-7 Cleavage Products—Previous characterization of the SCA7 transgenic models has demonstrated the production of proteolytic cleavage products (50, 54). We evaluated whether the fragments are similar in size to the caspase cleavage products of ataxin-7–92Q. In SCA7 transgenic mice, a 45-kDa proteolytic fragment of ataxin-7–92Q was detected with the polyQ-specific 1C2 antibody, and the amount of this fragment increases with age (Fig. 7B). The size of the fragment from the SCA7 mouse was compared with recombinant full-length ataxin-7–92Q treated with caspase-7 and identical migration on SDS-polyacrylamide gels was noted (Fig. 7B), suggesting that the cleavage product found in SCA7 transgenic mice may be due to caspase-mediated proteolysis at position 266. The size concordance of the in vitro caspase-cleaved ataxin-7 with the in vivo ataxin-7 truncation fragment supports the physiological importance of the cleavage sites that we report in this study.

DISCUSSION

SCA7 is characterized by cone-rod dystrophy retinal degeneration and cerebellar degeneration and is caused by a polyglutamine expansion in the N terminus of the protein, ataxin-7. The specificity of retinal degeneration is likely attributable to the unique protein sequence and domains of ataxin-7 surrounding the polyQ stretch. Indeed, characterization of the ataxin-7 protein revealed a polyQ-dependent interaction with the cone-rod homeobox protein (Crx), a photoreceptor specific transcriptional factor (25, 33). In SCA7 knock-in and transgenic mice, retinal-specific genes are down-regulated and contribute to disease progression (25, 32, 33). Ataxin-7 is also a member of the STAGA complex. Ataxin-7 contains three conserved blocks, and the second block contains a highly conserved “zinc binding motif” between residues 311 and 406 of ataxin-7 (31). This region of the ataxin-7 protein may be required for its interaction with GCN5 histone acetyltransferase of the STAGA complex.
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One factor that is likely to contribute to neurodegeneration of specific neuronal populations is how proteins with polyglutamine expansions are proteolytically processed and by which proteases. This is particularly applicable to SCA7 where both transgenic models and SCA7 patients accumulate proteolytic cleavage fragments of polyglutamine-expanded ataxin-7 (50). Questions relevant to the proteolysis of ataxin-7 and to selective neurodegeneration in the context of the unique protein sequence and domains of ataxin-7 are: 1) Where are the sites of proteolytic cleavage in the protein with respect to known functional domains of the protein? 2) Does cleavage disrupt the function of the protein? 3) Are polyQ-containing cleavage products more neurotoxic than the full-length protein? 4) Does cleavage of the protein alter subcellular localization? 5) Is cleavage polyQ repeat-dependent? 6) Do the polyQ fragment accumulate with age? 7) Are proteases specifically activated or expressed in the regions of retinal or cerebellar degeneration?

To explore some of these questions and gain further insight into the role of proteolysis in SCA7, we evaluated whether caspases where involved in the proteolytic cleavage of ataxin-7. We found 1) that ataxin-7 is a substrate for caspase-7-mediated proteolysis in vitro and in cell culture, 2) caspase-7 cleavage of polyQ expanded ataxin-7 promotes toxicity in vitro, 3) caspase-7 cleavage of ataxin-7–69Q promotes the formation of inclusions in granule neurons, 4) cleavage of polyQ-expanded ataxin-7 promotes transcriptional repression, 5) cleavage of ataxin-7 by caspase-7 will disrupt nuclear export causing mutant ataxin-7 N-terminal fragments to accumulate in the nucleus (27), 6) caspase-7 is recruited into the nucleus and activated by polyQ expanded ataxin-7, and 7) the caspase-7-generated cleavage product of ataxin-7 is similar in size to the cleavage product found in a transgenic SCA7 mouse model.

Beyond showing that ataxin-7 is a substrate for caspases, we used site-directed mutagenesis to identify two caspase-7 cleavage sites in ataxin-7, localizing them to amino acids 266 and 344. We found that the caspase-non-cleavable polyQ-expanded ataxin-7 has reduced cytotoxicity, transcription repression, and aggregate formation when compared with caspase-cleavable polyQ-expanded ataxin-7. This extends our findings with other polyQ expansion disease proteins, namely, huntingtin, androgen receptor, ataxin-3, and atrophin-1, where we evaluated the caspase-resistant forms of these polyQ expanded proteins and found the cleavage promotes cytotoxicity and aggregation (45, 55). Our work suggests that proteolysis of these proteins is required to generate toxic conformers and that proteolysis plays a central role in polyQ disease pathogenesis, findings with potentially important therapeutic implications.

To date, we have thus analyzed caspase cleavage for five of the nine polyQ repeat disease proteins. There are 14 different mammalian caspases that are separated into three separate functional groups. Notably, ataxin-7 is cleaved by caspase-7 and not the other caspase family members. This is important because multiple family members target huntingtin (caspase-2, -3, -6, -7), ataxin-3 (caspase-1, -3), atrophin-1 (caspase-2, -3, -6, -7, -9), and androgen receptor (caspase-1, -3, -7) (15, 55). Whether these proteolytic enzymes are required in polyQ disease progression in these different disorders is currently not known. Because the action of only one protease, caspase-7, is implicated in SCA7 disease pathology, SCA7 may represent a particularly simple and, therefore, appealing model for understanding the role of proteolysis in polyQ expansion disease. Caspase-7 is an executioner caspase that directly causes morphological changes by cleaving various death substrates. Caspase-7 targets a number of nuclear substrates, including the first caspase substrate ever discovered, namely poly(ADP-ribose) polymerase (56). In vitro experiments demonstrate that poly(ADP-ribose) polymerase cleavage by caspase-7, but not by caspase-3, is stimulated by auto-modification by long and branched poly(ADP-ribose), and cell lines deficient in caspase-7 do not cleave poly(ADP-ribose) polymerase (56).
Interestingly, the prodomain of caspase-7 inhibits its nuclear translocation and apoptosis-inducing activity. This nuclear localization is dependent on the presence of a basic tetrapeptide that is conserved in mammalian and *Xenopus* ataxin-7 and is located downstream of a cleavage site between a prodomain and a catalytic protease domain (57). The basic tetrapeptide may also be involved in the activation of caspase-7 (58). Our studies suggest that the active form of caspase-7 cleaves ataxin-7 in the nucleus rather than in the cytoplasm and, therefore, may disrupt STAGA complex function in the nucleus.

One pathological mechanism proposed for SCA7 is the dysregulation of transcription, and we propose that production of toxic fragments of the polyQ-expanded form of ataxin-7 may contribute to this process. In accordance with the role of proteolysis in transcriptional dysregulation, we found that the caspase non-cleavable form of ataxin-7–69Q D266N/D344N was able to interfere with Crx-mediated transcription activation. Because cleavage of ataxin-7 requires caspase-7, it is possible that regional expression and activation of caspase-7 in the cerebellum and retina may contribute to cell-specific neurodegeneration. Our finding that activated caspase-7 is found in the retina of the MoPrP-SCA7–92Q transgenic mice supports such a model.

One important finding from our studies is that ataxin-7 binding to caspase-7 is polyQ repeat-dependent. Previous work from our laboratory has shown that huntingtin interaction with caspase-2 is repeat-dependent (40, 44). Our studies suggest that different polyQ repeat proteins may have different interactions with and be cleaved by distinct caspase family members. Confoocal microscopy demonstrates that the polyQ-expanded form of ataxin-7 specifically recruits caspase-7 into the nucleus in *vivo*, where it is activated. Caspase activation is likely facilitated by the enhanced interaction of the polyQ form of ataxin-7 and caspase-7 as determined by co-immunoprecipitation studies. These studies suggest a role for cleavage of ataxin-7 by caspase-7 and suggest proteolytic processing by caspase-7 is likely to account for the *in vivo* truncation of ataxin-7, contributing to transcriptional interference with Crx, and permit the formation of inclusions.

The ataxin-7 caspase cleavage sites that we identified are clustered at amino acids 266 and 344, an area that contains a NES (26). Ataxin-7 is found both in the cytoplasm and in the nucleus (3). Ataxin-7 shuttling between these two cellular compartments may control the nuclear levels of ataxin-7 and regulate the activity of the STAGA complex. Cleavage of ataxin-7 by caspase-7 would disrupt export of ataxin-7 of the nucleus leading to accumulation of toxic ataxin-7 N-terminal fragments, potentially impairing STAGA complex function. Disruption of nuclear export for huntingtin and atrophin-1 by proteolysis has also been proposed to result in the accumulation of nuclear fragments and toxicity (53, 59), although for these two proteins the cleavage site is not adjacent to the NES.

Our work suggests that caspase-7 may play a role in ataxin-7 proteolysis and SCA7 pathology. Therefore, inhibition of this protease may be therapeutically important. Many of the recently developed small molecule inhibitors of caspases inhibit other protease family members such as calpains. However, a different approach has been initiated to inhibit caspases with small molecules (fragment-based drug discovery method (Tethering)) that does not target the active site of the enzyme but rather an allosteric site of caspase-7 preventing its activation (60). Small interfering RNA against caspase-7 delivered by adeno viral or adeno-associated virus vectors may also be useful in treating the retinal degeneration in SCA7. Future efforts to inhibit the association of caspases and their target substrates may similarly prove fruitful. Further studies will be necessary to determine whether inhibition of caspase cleavage will be beneficial in an *in vivo* setting.

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