Caspase Cleavage of Gene Products Associated with Triplet Expansion Disorders Generates Truncated Fragments Containing the Polyglutamine Tract*

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The neurodegenerative diseases Huntington disease, dentatorubropallidoluysian atrophy, spinocerebellar atrophy type 3, and spinal bulbar muscular atrophy are caused by expansion of a polyglutamine tract within their respective gene products. There is increasing evidence that generation of truncated proteins containing an expanded polyglutamine tract may be a key step in the pathogenesis of these disorders. We now report that, similar to huntingtin, atrophin-1, ataxin-3, and the androgen receptor are cleaved in apoptotic extracts. Furthermore, each of these proteins is cleaved by one or more purified caspases, cysteine proteases involved in apoptotic death. The CAG length does not modulate susceptibility to cleavage of any of the full-length proteins. Our results suggest that by generation of truncated polyglutamine-containing proteins, caspase cleavage may represent a common step in the pathogenesis of these neurodegenerative diseases.

Eight neurodegenerative disorders are caused by expansion of a CAG trinucleotide repeat encoding polyglutamine. This group of diseases includes Huntington’s disease (HD), dentatorubropallidoluysian atrophy (DRPLA), spinocerebellar ataxia type 3 (SCA-3), spinocerebellar ataxia types 1, 2, 6, and 7 (1–6). Remarkably, polyglutamine expansion results in disorders with numerous similarities, although the causative proteins of these diseases do not share structural homology outside of the CAG tract. For example, disease occurs only when the CAG length expands over a certain threshold (6–8). Also, each disorder has a distinct and selective pattern of neurodegeneration although the causative proteins are widely expressed (3, 6, 9). Because neurodegenerative illness results from a similar mutation in all of these diseases, they may share a similar pathogenesis resulting from polyglutamine expansion. We have previously hypothesized that neuronal toxicity may be a consequence of polyglutamine expansion per se, with the regional selectivity of degeneration conferred by amino acid sequences surrounding the polyglutamine tract (1, 10).

An apoptotic mode of cell death appears to be operative in HD, since brains of HD patients show increased levels of DNA strand breaks typical of apoptotic cells (11). Also, cells transfected with amino-terminal huntingtin constructs have an increased susceptibility to apoptosis (12). There is increasing evidence that truncation of polyglutamine-containing proteins may play an important role in the initiation or propagation of cellular toxicity in the triplet expansion diseases (12). Biochemical and immunohistochemical studies show amino-terminal huntingtin fragments in brain tissue from affected HD patients and not from controls (13). Brains from HD patients also contain neurons with intracellular aggregates of huntingtin that may develop from amino-terminal huntingtin fragments (13). Furthermore, transgenic animals expressing only exon 1 of the HD gene with long polyglutamine tracts develop a progressive neurological phenotype associated with intraneuronal intranuclear inclusions (14, 15). In vitro, cells transfected with amino-

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The abbreviations used are: HD, Huntington’s disease; DRPLA, dentatorubropallidoluysian atrophy; SCA-3, spinocerebellar ataxia type 3; Machado-Joseph disease; ICE, interleukin-converting enzyme; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; MEKK, mitogen-activated protein kinase kinase kinase; TBP, TATA-binding protein.

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terminal huntingtin constructs contain aggregates that are increased in frequency and size when huntingtin is truncated and the polyglutamine tract is expanded (12).

Intracellular aggregates are also observed in brains of SCA-3 patients and in an in vitro model of this disease (16). However, in vitro studies have shown that aggregates do not form unless cells are transfected with truncated ataxin-3 constructs (16). Similarly, truncation of atrophin-1 increases the frequency of aggregate formation in an in vitro model of DRPLA (17). The emerging principle from these studies is that one step in the pathogenic mechanism shared among these disorders is that truncation of the parent protein leads to the formation of intracellular aggregates that correlate with an increased susceptibility to apoptotic cell death.

It is important, therefore, to understand how proteins containing polyglutamine tracts may be proteolytically cleaved. Identification of key proteases involved in the truncation of polyglutamine-containing proteins could be important for the development of therapeutic agents that may be useful for each of the polyglutamine expansion disorders.

Thus far, it is known that huntingtin is a substrate for proteolytic cleavage by caspase-3 (18), one member of a large family of cysteine proteases that becomes activated as the cell initiates programmed cell death (19, 20). To date, 10 mammalian caspases have been identified, and they are classified into three major subfamilies known as ICE-like, activator, or effector caspases based on their sequence homology, function, and substrate specificity (21). However, this classification may not be absolute, since at least one caspase (caspase-6) has features of both activator and effector caspases (21).

Because we have previously reported cleavage of huntingtin by caspase-3 (18), we wished to answer two questions relevant to extending this model to other polyglutamine expansion disorders. First, is huntingtin cleaved by other caspases, and if so, how does this cleavage compare with cleavage by caspase-3? Second, can caspases cleave other polyglutamine-containing proteins associated with neurodegeneration? We report here that, similar to huntingtin, the androgen receptor, atrophin-1, and ataxin-3 are also specifically cleaved by one or more caspases in vitro. In each case, a truncated protein fragment containing the polyglutamine tract is liberated by caspase cleavage. Caspase cleavage of these polyglutamine-containing proteins may thus represent a shared step in the pathogenesis of the CAG expansion disorders.

**EXPERIMENTAL PROCEDURES**

**Construction and Mutagenesis of Plasmids**

**Huntingtin**—The construction of the full-length human huntingtin expression construct has been previously described (22). The N-terminal huntingtin constructs, pCI1955–15 and pCI1955–128, were derived from pCMV1955–15 and pCMV1955–128, respectively (18). Site-directed mutagenesis was used to introduce a translational termination codon immediately after nucleotide position 1955 (amino acid 548) in huntingtin using the mutagenic primers 5'-GACC-CTGCCATGTGAGATCCTCTAGAG-3' for construction of 5'-GACC-CTGCCATGTGAGGTACCGAGCTC-3' for 1955–128. Mutagenesis was performed as recommended for the Transformer site-directed mutagenesis kit (CLONTECH). Following mutagenesis, huntingtin constructs were subcloned into the pCI-neo vector (Promega) for all subsequent experiments.

A C-terminal huntingtin expression construct encoding amino acids 585–3144 was prepared by excising a 8292-base pair KpnI–NotI fragment from pCMV10660 and inserting this fragment into pCDNA3. In vitro digestion with KpnI and NotI cleaved the vector and KpnI was used to digest the KpnI site of the resulting plasmid. Restoration of the correct translational reading frame was confirmed by sequence analysis.

Site-directed mutagenesis was used to identify a caspase-3 cleavage site within the first 548 amino acids of huntingtin. Huntington D513A was made using the mutagenic primer 5'-GACTCGAGTGGCCCTGCGCAGC-3'. Mutant D530A was made using the mutagenic primer 5'-GATGAGGAGGCTATCTTGAG-3'. Both primers were used simultaneously to generate the double mutant, D513A,D530A.

**Atrophin-1**—Clones 1 (in pCRII) and 6 (in pBluescript) as reported (23) were each digested with Accl and KpnI. The 6-kilobase pair fragment containing the 5-kilobase pair fragment was ligated into the EcoRI site of pCI1955–15, resulting in a construct (Atcon3) containing the complete atrophin-1 open reading frame with 22 consecutive CAG repeats. First strand cDNA from the liver of a patient with DRPLA (61 consecutive CAG repeats) was amplified across the repeat, with the final product including an Accl site at base pair 1350 (numbered according to GenBank accession number U22851). The 5-kilobase pair fragment containing the repeat was ligated into pCRII (Invitrogen) and digested with Accl and NarI. Atcon3 was similarly digested, and the 495-base pair Accl-NarI fragment containing the expanded repeat was ligated into Atcon3, creating At61, a clone with a full-length atrophin-1 cDNA containing 61 consecutive CAG repeats. This was subsequently ligated into the vector pcDNA3 (Invitrogen) after digestion with KpnI and XhoI.

**Ataxin-3**—The ataxin-3 constructs with 35 or 79 glutamines have been previously described (24). These constructs contain a hemagglutinin tag on the N-terminal end to facilitate detection of the protein in cells.

**Androgen Receptor**—The construction of the expression plasmids encoding androgen receptors with 12 or 50 glutamines in their polyglutamine tract has been fully described (25).

**Preparation of Extracts**

Extracts from D8 osteosarcoma cells were prepared as described previously (25). Jurkat cells were induced to undergo apoptosis by treatment with 10 μg/ml camptothecin for 4 h at 37 °C. Cells were centrifuged at 1000 × g for 15 min at 4 °C, washed once with cold phosphate-buffered saline, and resuspended in 105 cells/ml of lysis buffer (10 mM Hepes/KOH, pH 7.0, 10% (w/v) sucrose, 2 mM EDTA, 0.1% (w/v) CHAPS, 5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml leupeptin, 10 μg/ml pepstatin A, and 10 μg/ml antipain). After incubation on ice for 15 min, the lysates were centrifuged at 1000 × g for 10 min at 4 °C, and the supernatants were further centrifuged at 10,000 × g for 10 min at 4 °C. The resulting supernatant was aliquoted and stored at -80 °C.

**In Vitro Cleavage Assays**

**Preparation of Substrate Proteins**—Coupled in vitro transcription/translation was performed using the Promega TNT reticulocyte or wheat germ systems as recommended by the manufacturer, using between 0.5 and 1 μg of Qiagen or cesium chloride-prepared cDNA and including [35S]methionine in the reaction mixture for incorporation into the synthesized polypeptide. Radioactively labeled polypeptides were resolved by fractionation on SDS-polyacrylamide gels and detected as described previously (18). The SDS-PAGE conditions were as follows: 7.5 or 5% polyacrylamide for 35S-huntingtin and 35S-atrophin-1, 7.5 or 12% for 35S-androgen receptor, 12% for 35S-ataxin-3, and 10% for 35S-poly(ADP-ribose) polymerase and 35S-labeled TATA-binding protein (35S-TBP). The reaction product from each translation was included in each assay after incubation in cleavage buffer only, as a negative control (Uncleaved lanes in Figs. 2–5).

**In Extracts**—Equal amounts of apoptotic or nonapoptotic extracts (50 μg of protein) were preincubated with cleavage buffer (50 mM Hepes/KOH (pH 7.0), 10% sucrose, 0.2 mM EDTA, 0.1% (w/v) CHAPS, 5 mM dithiothreitol, 24.5-μl reaction volume) or with cleavage buffer containing 10 mM Ac-DEV-D-CHO or 10 mM Ac-YVAD-CHO for 30 min at 37 °C. Cleavage reactions (n = 3) were initiated by the addition of radiolabeled substrate protein (0.5–1.0 μl) to the extract, and incubation was continued for an additional 3 h at 37 °C. Proteolytic fragments were resolved by SDS-PAGE and detected as described (18).

**With Purified Caspases**—In vitro cleavage assays were performed essentially as described (18). Radiolabeled substrate protein (0.5–1.0 μl) was incubated at 37 °C in cleavage buffer along with purified caspase at different concentrations and times. Recombinant caspases were generated as described previously (21, 27–29). Cleavage products were resolved by SDS-PAGE and detected as described (18). Reactions were performed at least in triplicate.

**Kinetic Evaluation of Cleavage In Vitro**—Measurements (n = 3) of kcat/Km values for caspase cleavage of substrates were performed exactly as described previously (18). Proteolytic cleavage was quantified by laser densitometry.
RESULTS

Cleavage of Human Huntingtin, Atrophin-1, Ataxin-3, and the Androgen Receptor in Apoptotic Extracts—Caspases cleave proteins following an essential aspartate residue (30). Combinatorial and synthetic approaches have been used to define the optimal tetrapeptide consensus cleavage sites for each of the known caspase families as (I/V/L)E\_X\_D for the activator caspases, DE\_X\_D for the effector caspases, and (W/L)EHD for the ICE-like caspases, where \( \text{X} \) is any amino acid (21). As shown in Fig. 1, human huntingtin, atrophin-1, androgen receptor, and ataxin-3 all contain potential sites for caspase cleavage. Interestingly, the predicted caspase cleavage sites in these proteins sometimes occur in clusters.

To determine whether human huntingtin, atrophin-1, ataxin-3, and the androgen receptor were cleaved in extracts, in vitro translated proteins were incubated with cytoplasmic extracts prepared from proliferating (nonapoptotic) or postconfluent (apoptotic) D8 osteosarcoma cells. Extracts prepared from postconfluent D8 osteosarcoma cells are known to express high levels of caspase-3 (26) and have been used in previous studies on huntingtin cleavage (18). The reversible competitive tetrapeptide aldehyde inhibitors Ac-DEVD-CHO and Ac-YVAD-CHO inhibit the effector and ICE-like caspases, respectively (25). After first confirming that inhibition of purified caspase-3 or caspase-1 could be achieved under the experimental conditions used, these tetrapeptide inhibitors were used to determine the class of caspases responsible for specific cleavage events in extracts.

In vitro translation of huntingtin truncated at amino acid 548 produced proteins of 80 and 110 kDa for proteins containing 15 and 128 glutamines, respectively (Fig. 2A). Truncated huntingtin is cleaved in apoptotic D8 extracts, yielding a 67-kDa fragment for a construct with 15 glutamines and a 98-kDa fragment for a construct with 128 glutamines (Fig. 2A). These sizes are consistent with our previous observation that the caspase-3 cleavage product of this construct with 44 glutamines migrates at 80 kDa (18). Cleavage of both constructs was inhibited by preincubation of the extract with Ac-DEVD-
CHO but not Ac-YVAD-CHO, indicating that these cleavage products probably result from the activity of an effector caspase (Fig. 2A).

In vitro translation of atrophin-1 generated proteins of 180 and 210 kDa for constructs with 22 and 61 glutamines, respectively (Fig. 2B). The smaller molecular weight band visible in the Uncoled lanes is likely due to internal initiation or premature termination products generated during in vitro protein synthesis. A specific predominant product of 130 or 135 kDa was generated when atrophin-1 with 22 or 61 glutamines, respectively, was incubated with apoptotic D8 osteosarcoma extract (Fig. 2B). These cleavage products were nearly completely inhibited by Ac-DEVD-CHO but not by Ac-YVAD-CHO, demonstrating that they too are likely to result from the activ-

![Fig. 2. Cleavage in apoptotic extracts.](image)
ity of an effector caspase. Small amounts of cleavage product were observed after incubation in the nonapoptotic extract, which may be due to self-activation of caspases during extract preparation or to additional proteases active against atrophin-1.

In vitro translation of ataxin-3 produced a predominant 36- or 40-kDa protein containing 35 or 79 glutamines, respectively (Fig. 2C). The several smaller molecular weight bands visible in the Uncleaved lanes most likely represent internal initiation or premature termination products produced during in vitro protein synthesis. The specific cleavage products that appear after incubation with the apoptotic extract and that were absent in the uncleaved preparation are indicated with arrows (Fig. 2C). Cleavage of ataxin-3 is inefficient in D8 extracts, resulting in small amounts of fragments migrating at 14 and 18 kDa for constructs with 35 and 79 glutamines, respectively. Interestingly, neither Ac-DEVD-CHO nor Ac-YVAD-CHO inhibited formation of these products (Fig. 2C), suggesting that proteases other than effector or ICE-like caspases may generate these products in D8 osteosarcoma extracts.

In vitro translation of the androgen receptor produced a 95- or 100-kDa protein containing 12 and 50 glutamines, respectively. The androgen receptor was resistant to cleavage in apoptotic and control osteosarcoma extracts (Fig. 2D). However, the androgen receptor was cleaved in apoptotic Jurkat extracts, yielding a major fragment of 87 kDa (Fig. 2E) for both normal and expanded alleles, as well as several smaller fragments that are not clearly resolved. The 87-kDa fragment does not contain the polyglutamine tract (Fig. 3A) for both normal and expanded alleles. As a control for a polyglutamine-containing protein, we also tested the ability of TBP to be cleaved in apoptotic extracts, since TBP contains a tract of 38 glutamines (31). TBP remained intact in both control and apoptotic D8 extracts (Fig. 2F).

The results from cleavage and inhibition assays in cell extracts are summarized in Table I. Of the substrates tested, atrophin-1 is cleaved most efficiently in D8 extracts, followed by huntingtin and ataxin-3. The androgen receptor was not cleaved by extracts prepared from D8 osteosarcoma cells but was cleaved efficiently in Jurkat apoptotic extracts.

Cleavage of Huntingtin in Vitro by Purified Caspases—In an effort to assess which caspases were active in these apoptotic extracts and were cleaving these gene products, we next compared the ability of individual purified caspases to cleave huntingtin, atrophin-1, ataxin-3, and the androgen receptor in vitro. We used caspase-3 and caspase-7 as representatives of the effector group of caspases, caspase-8 as representative of an activator caspase, and caspase-1 as a representative of an ICE-like caspase (21). All enzymes used in these experiments were normalized to identical molar concentrations to facilitate meaningful comparisons between their cleavage efficiencies.

Huntingtin contains two predicted caspase-3 cleavage sites within the N-terminal construct truncated at amino acid 548 (Fig. 1A). When incubated with caspase-3, products of 67 and 90 kDa were produced from constructs with 15 and 128 glutamines, respectively (Fig. 3A), similar to those seen with the apoptotic extracts (Fig. 2A). Incubation with caspase-1 resulted in the liberation of 52- and 67-kDa fragments for the construct with 15 glutamines, whereas products of 79 and 90 kDa were generated from the construct with 128 glutamines (Fig. 3A). Neither caspase-7 nor caspase-8 cleaved within the first 548 amino acids of huntingtin (Fig. 3A). In vitro translation of full-length huntingtin generates several products ranging from 348 kDa, corresponding to intact full-length huntingtin, to 120 kDa, corresponding to synthesis artifacts due to premature translation termination or internal translation initiation of this large protein (Uncleaved lanes in Fig. 3B). Two major fragments containing the polyglutamine tract were liberated from full-length huntingtin (348 kDa) by incubation with caspase-3, migrating at 67 and 80 kDa for full-length huntingtin with 15 CAG repeats and at 98 and 110 kDa for full-length huntingtin with 128 CAG repeats (Fig. 3B). Purified caspase-1 cleaves full-length huntingtin into a major 67-kDa fragment and a minor 52-kDa fragment for the construct with 15 glutamines, whereas a major product of 90 kDa and a minor product of 79 kDa were generated from the construct with 128 glutamines. Neither purified caspase-7 nor caspase-8 generated significant products from full-length huntingtin (Fig. 3B).

To test for specific cleavage events in the C-terminal portion of huntingtin, we developed an expression construct that extended from amino acid 585 to 3144 of huntingtin. Both caspase-1 and caspase-3 cleaved inefficiently within the C-terminal portion of huntingtin, each generating a fragment of 55 kDa (Fig. 3C). Also consistent with results presented above, significant cleavage was not observed with either caspase-7 or caspase-8 (Fig. 3C).

In summary, huntingtin has at least two caspase-3 sites, one of which is within the first 548 amino acids and one additional site distal to amino acid 548. In addition, caspase-1 cleaves huntingtin at a major and a minor cleavage site within the N-terminal 548 amino acids. The C-terminal caspase-3 and caspase-1 sites within the C-terminal portion of huntingtin are used far less efficiently than the N-terminal sites.

Identification of a Specific Caspase Cleavage Site in Huntingtin—Although there are two potential caspase-3 cleavage sites in huntingtin prior to amino acid 548 (a DSLD at amino acid 513 and a DEED at amino acid 530), only one of them appears to be used (Fig. 3A). To determine which of the sites is utilized by caspase-3, we changed each of the potential P1 aspartic acid residues to alanine by site-directed mutagenesis. In these experiments, we constructed single mutants (D513A and D530A) as well as a double mutant (D513A,D530A) that were in vitro translated and subjected to cleavage in vitro with purified caspase-3 and caspase-1. Caspase-3 cleavage was prevented specifically and only by the D513A mutation, whereas the D530A mutation had no effect on cleavage by caspase-3 (Fig. 4). This result clearly indicates that the caspase-3 site within the first 548 residues of huntingtin is at amino acid position 513. Neither of the single mutants (D513A or D530A) nor the double

### Table I

| Protein      | Normal (15 CAG) | Expanded (128 CAG) | Normal (22 CAG) | Expanded (61 CAG) | Normal (35 CAG) | Expanded (78 CAG) | Normal (12 CAG) | Expanded (56 CAG) |
|--------------|-----------------|--------------------|-----------------|-------------------|-----------------|------------------|-----------------|------------------|
| Huntingtin   |                 |                    |                 |                   |                 |                  |                 |                  |
| Poly-Q fragment size: D8 | 67 kDa | 98 kDa | 130 kDa | 135 kDa | 14 kDa | 18 kDa | None | None |
| Inhibited by Ac-DEVD-CHO | Yes | Yes | Yes | Yes | No | No | ND | ND |
| Inhibited by Ac-YVAD-CHO | No | No | No | No | No | No | ND | ND |
| Poly-Q fragment size: Jurkat | ND | ND | ND | ND | ND | ND | 18 kDa<sup>b</sup> | 35 kDa<sup>b</sup> |

<sup>a</sup> ND, not done.

<sup>b</sup> Presumed size based on non-polyglutamine-containing fragment of 87 kDa.
In Vitro Cleavage of Atrophin-1, Ataxin-3, and the Androgen Receptor, but Not TBP, by Purified Caspases—All four caspases tested cleaved atrophin-1 in vitro (Fig. 5A). The cleavage products generated by each enzyme were 130 and 135 kDa for constructs with 22 and 61 glutamines, respectively, consistent with the cleavage products generated in D8 apoptotic extracts. Because the fragment is itself large, the change in mobility resulting from the presence of the polyglutamine tract in this fragment is relatively minor with the electrophoretic conditions used.

Although many bands are evident in the uncleaved control of ataxin-3 (Fig. 5B, lane 1), fragments of 14 kDa for ataxin-3 with 35 CAG repeats and 18 kDa for ataxin-3 with 79 CAG repeats were produced with either caspase-3 or caspase-8 (Fig. 5B). However, generation of these ataxin-3 cleavage products required 3 h of incubation at 37 °C with 10 mM caspase, whereas products were observed after 1 h of incubation for the other proteins analyzed in this study. These results suggest that relative to huntingtin, atrophin-1, and the androgen receptor, ataxin-3 is cleaved far less efficiently with either caspase-1 or caspase-3. No detectable cleavage products were observed when ataxin-3 was incubated with either caspase-7 or caspase-8 (Fig. 5B). The sizes of the cleavage products generated by caspase-3 or caspase-1 are consistent with the fragments produced upon incubation of ataxin-3 with osteosarcoma apoptotic extract. However, because the cleavage fragments produced by osteosarcoma extracts were not inhibited by Ac-DEVD-CHO or Ac-YVAD-CHO, these results suggest that while caspase-1 and caspase-3 can cleave ataxin-3, additional proteases are also able to do so.

In this experiment, in vitro synthesis of the androgen receptor produced spurious bands migrating below the intact 95- or 100-kDa products for alleles with 12 or 50 glutamines (Uncleaved lanes in Fig. 5C). The androgen receptor was cleaved into several fragments by more than one caspase. For example, caspase-3 cleaves the androgen receptor into products of 87, 60, 47, and 18 kDa for a construct with 12 glutamines, and 87, 80, 47, and 34 (arrow) kDa for a construct with 50 glutamines (Fig. 5C). Because two bands are constant in size and two have mobility shifts when the CAG length is increased, these results suggest that the androgen receptor is cleaved at two sites by caspase-3. Cleavage at one site would generate a constant C-terminal 87-kDa fragment plus an N-terminal polyglutamine-containing fragment of 18 or 34 kDa, and cleavage at the second site would generate a constant 47-kDa fragment plus a polyglutamine-containing fragment of 60 or 80 kDa for alleles with 12 and 50 glutamines, respectively. Because the androgen receptor sequence was predicted to contain only one caspase-1 site and caspase-8 (Fig. 5B), it is possible that at least one caspase-3 site is cleaved at the C-terminal portion of the receptor at amino acid 514 (D514A), position 530 (D530A), or both positions (D513A,D530A). The normal construct (WT) and the three mutagenized constructs were translated in vitro and tested for cleavage by 10 mM caspase-3 (A) or 10 mM caspase-1 (B). 35S-radiolabeled protein was incubated in cleavage buffer only (–) or with the indicated enzyme (+) at 37 °C for 1 h followed by electrophoresis through 5% SDS-polyacrylamide gels.

![Figure 3](image3.png)  **Fig. 3. Cleavage of huntingtin in vitro with purified caspases.** 35S-Labeled protein generated by in vitro translation was electrophoresed after incubation in buffer only (Uncleaved) or after incubation with 10 mM purified caspase-1, caspase-3, caspase-7, or caspase-8 as indicated, for 1 h at 37 °C. The arrows represent specific cleavage products. A, cleavage of the N-terminal portion of huntingtin (amino acids (aa) 1–548); B, cleavage of full-length huntingtin; C, cleavage of the C-terminal portion of huntingtin (amino acids 585–3144).

![Figure 4](image4.png)  **Fig. 4. Determination of the caspase-3 cleavage site within huntingtin truncated at amino acid 548.** Site-directed mutagenesis was used to change the essential P1 aspartate residue to alanine at amino acid position 513 (D513A), position 530 (D530A), or both positions (D513A,D530A). The normal construct (WT) and the three mutagenized constructs were translated in vitro and tested for cleavage by 10 mM caspase-3 (A) or 10 mM caspase-1 (B). 35S-radiolabeled protein was incubated in cleavage buffer only (–) or with the indicated enzyme (+) at 37 °C for 1 h followed by electrophoresis through 5% SDS-polyacrylamide gels.

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zyme/substrate combination (Table II). For example, although caspase-3 and caspase-7 have indistinguishable specificities toward tetrapeptide substrates (21), these caspases displayed different activities toward the proteins tested here. Caspase-7 is more active against atrophin-1 than caspase-3. However, caspase-7 is completely unable to cleave huntingtin and ataxin-3, while caspase-3 cleaves both proteins. In addition, caspase-3 cleaves the androgen receptor efficiently at either of two sites, yet caspase-7 cleaves very inefficiently and only at the more amino-terminal site. These results suggest that substrate recognition by caspases can be markedly influenced by the tertiary structure of the substrate.

Kinetic Evaluation of Cleavage—Analysis of cleavage kinetics shows that huntingtin truncated at amino acid 548 is cleaved significantly more efficiently by caspase-3 than by caspase-1 in vitro (Fig. 6A). Interestingly, atrophin-1 appears to be a robust substrate for both caspase-3 and -7 (Fig. 6B), with caspase-7 being slightly more efficient. In contrast, both caspase-1 and caspase-8 are much less efficient at cleaving atrophin-1. Relative to atrophin-1 and huntingtin, the androgen receptor is cleaved considerably less efficiently by caspase-3 (Fig. 6C). We found no differences in cleavage efficiency between the two caspase-3 sites in the androgen receptor (data not shown). Ataxin-3 was not included in this analysis because of its relative resistance to cleavage by caspase-3.

We next compared the relative caspase-3 cleavage efficiencies of huntingtin, atrophin, and the androgen receptor to poly(ADP-ribose) polymerase, a well characterized caspase-3 substrate (26) (Fig. 7). These results show that atrophin-1 is cleaved nearly as efficiently as poly(ADP-ribose) polymerase by caspase-3, whereas huntingtin and the androgen receptor are cleaved with decreasing efficiencies.

Length of the Polyglutamine Tract Does Not Modulate Cleavage Efficiency in the Full-length Protein—We previously reported that the CAG length in truncated huntingtin influenced susceptibility to cleavage by caspase-3 in the context of a truncated construct (18). However, it is important to test for CAG modulation of cleavage in the full-length protein, which more closely parallels the biologically relevant gene product. Therefore, we tested for CAG modulation of cleavage in all of the intact proteins by measuring the amount of cleavage product generated over time. The results of these experiments showed that CAG length did not modulate the in vitro cleavage efficiency for any of the full-length proteins tested (Fig. 8, A–C). However, because these in vitro studies were performed in the absence of proteins known to interact with huntingtin, atrophin-1, or the androgen receptor, we cannot exclude the possibility that interacting proteins may influence the rate or specificity of caspase cleavage in vivo.

DISCUSSION

In this study, we show that human huntingtin, atrophin-1, ataxin-3, and the androgen receptor are each a specific caspase substrate for one or more caspases. Further, cleavage is specific to these proteins. Cleavage occurs in apoptotic extracts and with purified recombinant caspases. In contrast, another polyglutamine-containing protein, TBP, is neither cleaved in apoptotic extracts nor cleaved by purified caspases. The finding that polyglutamine-containing proteins involved in neurodegenerative diseases are all specific caspase substrates suggests that caspase cleavage of these proteins may be an important common mechanism for generating truncated toxic fragments of these proteins.

Huntingtin was the first protein identified as a caspase substrate that is specifically involved in a neurodegenerative disease. Since then, effector caspases have also been shown to cleave the familial Alzheimer’s gene products, presenilins 1 and 2 (32, 33). By demonstrating that atrophin-1, ataxin-3, and androgen receptor are also caspase substrates, the number of caspase substrates directly implicated in neurodegenerative diseases has increased to six.

We have proposed that the pathological mechanism opera-
tive in HD may include the production of a toxic fragment containing the polyglutamine tract (1, 10, 18). The results reported here suggest the possibility of extending this toxic fragment hypothesis to include DRPLA, spinal bulbar muscular atrophy, and SCA-3. For each of these diseases, the toxic fragment hypothesis suggests that basal caspase activity, particularly in aging cells, may be sufficient to generate small amounts of cleavage product. If this cleavage product is indeed toxic to neurons, then accumulation of this toxic fragment would add further stress to the cell, resulting in additional caspase activation, additional cleavage, and eventual cell death. Because cells expressing activated caspses are often thought to be within the execution phase of cell death, the toxic fragment hypothesis raises the question of how caspases may also be responsible for initiating the cell death program for the polyglutamine expansion disorders. Recently, the proenzyme form of caspase-3 has been found to have low levels of catalytic activity that are approximately 60-fold lower than that of the activated enzyme. 3 S. Roy and D. W. Nicholson, unpublished observations.

| Table II | Relative efficiency of cleavage by purified caspases |
|----------|-----------------------------------------------------|
|          | Huntingtin | Atrophin-1 | Ataxin-3 | Androgen receptor | TBP |
| Caspase-1 | +++        | +++        | +        | ++               | -   |
| Caspase-3 | +++        | +++        | +        | ++               | -   |
| Caspase-7 | -          | +++        | +        | -                | -   |
| Caspase-8 | -          | +++        | -        | ++               | -   |

3 S. Roy and D. W. Nicholson, unpublished observations.

**Fig. 6.** Kinetic evaluation of cleavage of huntingtin truncated at amino acid 548 (A), atrophin-1 (B), and androgen receptor (C). 35S-Radiolabeled proteins were incubated with various concentrations of the indicated caspase (0–10 μM) for 1 h at 37 °C. Products were resolved by SDS-PAGE, quantitated by laser densitometry, and expressed as percentage cleaved. The values presented represent the mean and S.E. of at least three independent experiments.
affected individual, sufficient amounts of toxic polyglutamine-containing protein fragments may be produced from this basal caspase activity to tip the balance toward commitment to cell death and concomitant neurodegeneration.

A major question arising from this study is how the fragments liberated by cleavage may be linked to cell death. One possibility is that the protein fragment liberated by caspase cleavage may facilitate the formation of insoluble aggregates that are toxic to cells. Since aggregates that have been observed in in vitro models appear to require truncation of the parent protein to be readily formed (12). For example, cells transfected with huntingtin truncated at amino acid 548 (corresponding to the predicted caspase-3 cleavage fragment) with 128 glutamines develop perinuclear aggregates, and these cells have an increased susceptibility to apoptosis (12). Additionally, recent studies on SCA-3 have shown that a high proportion of cells transfected with a truncated ataxin-3 fragment containing an expanded polyglutamine tract are apoptotic in contrast to cells transfected with a full-length construct (16, 24).

Although the relationship between protein truncation and aggregate formation has not been formally demonstrated in vivo, HD patients demonstrate distinct intranuclear aggregates (14). In addition, Western analyses revealed the presence of small N-terminal huntingtin fragments in the brains of HD patients (14). Although the huntingtin fragments observed in Western analyses of HD patient brains are smaller than the huntingtin cleavage products reported in this study, it is possible that further proteolytic processing of caspase cleavage products, perhaps ubiquitin-mediated, may further truncate the larger cleaved product (34). Alternatively, it is possible that smaller huntingtin fragments may be generated by other proteases.

We did not find evidence supporting CAG modulation of cleavage for any of the full-length proteins tested in this study. These results suggest that the expanded polyglutamine tract may not influence susceptibility to caspase cleavage. Rather, the expanded polyglutamine may exert its toxic effects through the cleavage product where generation of a truncated fragment is toxic in the presence of polyglutamine expansion but not in its absence. Alternatively, it is possible that the polyglutamine length may affect the rate of truncation in vivo, perhaps by way of interacting proteins.

Formal proof of the relationship between cleavage and toxicity has recently emanated from in vitro studies of (SBMA). In cells expressing the androgen receptor, apoptosis is triggered through a mechanism that involves the production of a caspase-3 cleavage product. However, in vitro mutagenesis of the N-terminal caspase-3 cleavage site blocks cleavage in transfected cells and significantly lessens cell death. This finding shows that formation of an androgen receptor fragment...
by caspase-3 clearly plays a central role in cellular toxicity. Importantly, these results suggest that inhibiting caspase cleavage of a single substrate can significantly mitigate cell death in this system.

Inhibiting cell death by preventing cleavage of a single substrate has also been shown recently for mitogen-activated protein kinase kinase kinase (MEKK) in its role in mediating anoikis (apoptosis as a result of detachment of cells from a substrate). MEKK was recently shown to be cleaved by caspasas during anoikis (35). Importantly, blocking caspase cleavage of MEKK protects cells from anoikis (35). This finding supports the hypothesis that the active kinase generated by caspase cleavage of MEKK may be acting as a toxic fragment. Because MEKK contributes substantially to signal transduction pathways that mediate anoikis, it was postulated that MEKK may be involved in a positive feedback loop such that basal caspase activity activates MEKK, which activates more caspase activity and leads to cell death (35). This loop model is strikingly similar to the toxic fragment hypothesis proposed for HD (1, 10).

Although our results so far favor models in which entry to the toxic fragment feedback loop is through caspase-mediated cleavage of proteins containing expanded polyglutamine tracts, this does not exclude the possibility that other proteases may play a crucial role in the processing of these proteins. If so, alternative routes leading to production of a toxic fragment may be possible. Furthermore, once the fragment is generated, the enhanced toxicity due to the expanded polyglutamine may be sufficient to activate caspase activity and as such amplify the production of further toxic fragments.

The finding that huntingtin, atrophin-1, ataxin-3, and the androgen receptor are each caspase substrates establishes the possibility that these four unique diseases have common elements in their pathogenesis, namely cleavage by caspasas. However, these findings do not fully explain the specificity of cell loss for each disease. The different efficiencies by which huntingtin, atrophin-1, the androgen receptor, and ataxin-3 are cleaved in vitro by caspases raises the possibility that if cleavage is also observed in vivo, then specific neuronal loss may be mediated through cell-specific caspase expression. Assessment of regional and cellular caspase expression in the brain will help to address this possibility. Alternative explanations for selective neurodegeneration include models that propose a selective vulnerability of cells to the effects of the cleavage products produced. Additionally, the cleavage products themselves may have selective interactions with other proteins that may be quite different from those of the intact protein.

The finding that huntingtin, atrophin-1, ataxin-3, and the androgen receptor are cleaved by caspases provides the possibility for the development of therapies for several polyglutamine expansion diseases. For example, caspases can be inhibited by synthetic tetrapeptides or antiapoptotic proteins such as members of the Bcl2 family, baculovirus p35, and several others (36). Therefore, if caspase cleavage of polyglutamine-containing proteins is found to be an important step in the pathogenesis in vivo, inhibitors of caspase activity may be useful for the treatment of this class of disorders.

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