Inhibiting Caspase Cleavage of Huntingtin Reduces Toxicity and Aggregate Formation in Neuronal and Nonneuronal Cells*

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Huntington’s disease is a neurodegenerative disorder caused by CAG expansion that results in expansion of a polyglutamine tract at the extreme N terminus of huntingtin (htt). htt with polyglutamine expansion is pro-apoptotic in different cell types. Here, we show that caspase inhibitors diminish the toxicity of htt. Additionally, we define htt itself as an important caspase substrate by generating a site-directed htt mutant that is resistant to caspase-3 cleavage at positions 513 and 530 and to caspase-6 cleavage at position 586. In contrast to cleavable htt, caspase-resistant htt with an expanded polyglutamine tract has reduced toxicity in apoptosis-stressed neuronal and nonneuronal cells and forms aggregates at a much reduced frequency. These results suggest that inhibiting caspase cleavage of htt may therefore be of potential therapeutic benefit in Huntington’s disease.

Huntington’s disease (HD)1 is a progressive neurodegenerative disorder caused by polyglutamine expansion in the N terminus of htt (1). The cardinal neuropathological feature of HD is neuronal loss of γ-aminobutyric acid-ergic medium spiny neostriatal neurons and large projection neurons in cortical layers V and VI (2–4). The detection of DNA strand breaks in affected regions of HD patient brains (5–7) suggests that neurodegeneration occurs by an apoptotic mechanism and suggests that caspases could play an important role in HD.

Caspases are cysteine aspartic acid proteases that cleave specific target proteins during apoptotic death (8). We have previously shown that huntingtin is cleaved in apoptotic cells and by recombinant caspase-3 (9), and expression of truncated htt fragments with expanded polyglutamine repeats is known to be toxic to cells (10–14). These observations led to the development of the toxic fragment hypothesis (15), which postulates that proteolytic cleavage of htt liberates toxic fragments containing the expanded polyglutamine tract that are neurotoxic and that stimulate additional proteolytic activity.

Evidence of htt cleavage in HD includes the presence of N-terminal htt fragments in patient brains (16) as well as in yeast artificial chromosome transgenic mice that express full-length, expanded human htt (17). htt cleavage in the yeast artificial chromosome transgenic mice occurs in the cytoplasm, after which the N-terminal fragments are imported into the nucleus (17).

In vitro, htt is cleaved by caspase-3 at two sites yielding N-terminal fragments of 70 and 80 kDa for htt with 15 glutamines and 90 and 100 kDa for htt with 138 glutamines (9, 18). These fragments are also generated when htt is incubated with apoptotic extracts (9, 18) and accumulate from endogenous htt in apoptotic cells (19). Taken together, these results suggest that caspase-3 is likely to contribute to the generation of N-terminal htt fragments.

Further support for the toxic fragment hypothesis can be obtained by testing whether preventing the formation of N-terminal htt fragments lessens the toxicity of htt. Here we show abrogation of htt cleavage and diminishment of overall cytotoxicity in the presence of caspase inhibitors. This reduction in toxicity in the presence of caspase inhibitors could be due to general inhibition of proapoptotic caspase activity or by specifically preventing the caspase cleavage of htt. To test whether specifically inhibiting htt cleavage reduces toxicity, we first identified two caspase-3 sites at aa positions Asp513 and Asp552 and one novel caspase-6 site at aa position Asp596 and then generated mutated forms of htt that are resistant to caspase cleavage and contain either a normal or expanded polyglutamine tract. Neuronal and nonneuronal cells expressing caspase-resistant htt have reduced caspase activation during an apoptotic challenge and are less prone to aggregate formation compared with caspase-cleavable huntingtin. These results support the hypothesis that N-terminal cleavage products of htt enhance apoptotic death by accelerating the rate of

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† The abbreviations used are: HD, Huntington’s disease; htt, huntingtin; aa, amino acid(s); z-DEVD-fmk, z-Asp(OCH3)-Glu(OCH3)-Val-Asp(OCH3)-fmk; Ac-DEVD-tin; aa, amino acid(s); z-VAD-fmk, z-Val-Ala-Asp (OMe)-CH2F; CA69381.

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caspase-3 activation. Furthermore, these observations also suggest the possibility of novel therapeutic approaches for HD aimed at specifically blocking htt cleavage.

EXPERIMENTAL PROCEDURES

Plasmids—Full-length htt expression constructs (pRC-CMV10366-15 or -138) have been described (20). Expression plasmids truncated at htt nucleotide 3949 were prepared by digesting full-length pRC-CMV10366 containing 15 or 138 glutamines with XhoI followed by religation to create pRC-CMV3949-15 and pRC-CMV3949-138. pRC-CMVlacZ and pRSV-lacZ were kindly provided by Drs. Paul Orban and Ite Laird-Orrigin, respectively.

Mutagenesis—The P1 aspartate of each caspase site in htt was changed to alanine by polymerase chain-reaction-mediated mutagenesis using the following primers: constant 5′, CTG CTC ACC CTG AGG TAG TTG; constant 3′, CTG TTC CTC AGG GTA TTC CGT G; 1854F, CGG ACT CAG TGG CTC TGG CCA GCT G; 1854R, CAG CTC GGC AGA GCC ACT GAG TCC 1904F, 1904R, GAT GAG GAG GCT ATC TTG CCG G; 1970F, GGA CCT GAA TGG TAC GGC CAC GCA C; 1970R, GGC CTG GGT CCC ACC ATT CAG GTC C; 2081F, GAC GAG GCC GAC CATT TAG TTG; 2081R, CCC AAA TAC TAG TTG GGC ATC CCG TC; 2072F, GAA ATT GTG TTA GGC GAT GCC AAC; and 2072R, GTC CTT TGG GGA TAA CAG TAC ATT. Two mutagenic polymerase chain reactions were performed containing 10 ng of DNA, 1.5 µl of 1.25 mM dNTPs (Canadian Life Technologies), 5.0 µl of 10× PFU polymerase buffer (Stratagene), 1.0 µl of PFU polymerase (Stratagene), one mutagenic and one constant primer in 50 µl at 94 °C for 30 s, 65 °C for 30 s, and 72 °C for 45 s, and 7 min at 72 °C. Purified products were pooled and further amplified using the constant primers with 1.25 µl of 50× PCR buffer, 8 µl of 2.5 mM dNTPs, 1.25 µl of 50 mM MgCl2 (Promega), 0.5 µl of each primer, 5.0 µl of 50× Taq polymerase buffer, 1.0 µl of Taq polymerase, 2.5 µl of water, and 100 ng of DNA in a total volume of 50 µl. The PCR was performed using the following conditions: 35 cycles at 94 °C for 30 s, 65 °C for 30 s, and 72 °C for 45 s, and 7 min at 72 °C. Purified PCR products were sequenced using the appropriate primers and the ABI prism 373 DNA sequencer (Perkin Elmer Corp.).

Cleavage Assay—A C-terminal 10× GST-tagged htt fragment, containing a 3144-aa protein, was expressed in Escherichia coli and purified to apparent homogeneity using an antibody specific for the first 17 aa of htt (BKP1) shows a reduced rate of change of about 40% as determined by western blot analysis, which likely results from transfection or overexpression stress.

RESULTS

 htt Cleavage during an Apoptotic Challenge—HEK 293T cells were transiently transfected with full-length htt containing 15 or 138 glutamines (pRC-CMV10366-15 or pRC-CMV10366-138) and were harvested at intervals after treatment with 35 µM tamoxifen, which has previously been shown to result in apoptotic death of htt-expressing cells in a polynucleotide-dependent manner (25). Western blot analysis using an antibody specific for the first 17 aa of htt (BKP1) shows a 350-kDa band that represents intact htt as well as a 230-kDa processed fragment in untreated transfected cells (Fig. 1, A and B, h of tamoxifen) that likely results from transfection or overexpression stress.

Two major htt cleavage products (70 and 80 kDa for htt with 15 glutamines and 90 and 100 kDa for htt with 138 glutamines) are specifically generated 4–6 h after tamoxifen treatment (Fig. 1, A and B). Because similar products are generated from endogenous htt in apoptotic COS cells (19) and when htt is cleaved with purified caspase-3 (18), these products are consistent with cleavage at two caspase sites.

In vitro translation was performed using the TNT quick coupled rabbit reticulocyte system (Promega) as described (18). Cleavage assays using radiolabeled substrate protein and purified caspases or Jurkat extracts were performed as described (18). Caspase inhibitors Ac-DEVD-CHO and Ac-YVAD-CHO were purchased from Bachem (Switzerland). Caspases-3 and -6 were purified as described (8, 24).

Statistical Analysis—All statistical analyses were performed using one way analysis of variance with a Neuman-Keuls post test.
products consistent with N-terminal caspase cleavage 4–6 h after tamoxifen addition, as well as minor products that may represent proteolytic intermediates at earlier times (Fig. 1, C and D).

Broad Spectrum Caspase Inhibitors Prevent htt Cleavage and Diminish Toxicity—htt cleavage during a tamoxifen challenge was eliminated by either 50 μM z-VAD-fmk or 50 μM z-DEVD-fmk (Fig. 2A), demonstrating that htt cleavage is caspase-dependent in this model. In contrast, the MeSO-only diluent control was ineffective in preventing tamoxifen-induced htt cleavage. htt has previously been shown to be toxic in this model system (11), and toxicity is enhanced in the presence of an expanded polyglutamine tract. As measured by a modified MTT assay (11, 26, 27), z-VAD-fmk provided nearly complete protection from the toxic effects of transfected htt as well as pRSV-lacZ, reducing toxicity from 46.5 ± 1.2% to 1.1 ± 0.6% for 3949-15, from 78.6 ± 2.3% to 0.19 ± 0.8% for 3949-138, and from 21.7 ± 1.7% to 0.02 ± 0.01% for pRSV-lacZ (n = 6, p < 0.001 for each) (Fig. 2B). As well, z-DEVD-fmk eliminated the transfection-associated toxicity of pRSV-lacZ, reducing toxicity from 21.4 ± 0.8% to 0.1 ± 0.01% (n = 6, p < 0.001) and had a protective effect in cells expressing 3949-15 or 3949-138, reducing toxicity from 47.4 ± 1.4% to 30.7 ± 1.5% for 3949-15 (n = 6, p < 0.001) and from 78.1 ± 1.4% to 31.4 ± 1.4% for 3949-138 (n = 6, p < 0.001) (Fig. 2C). Because both inhibitors are irreversible and have different cellular permeabilities, it is not possible to conclude which caspases are relevant for toxicity in this system.

Caspase-3 Cleaves htt at D513 and D552—htt contains four potential caspase-3 sites (510DSVD513, 527DEED530, 549DLND552, and 586DGTD589; Fig. 3A), yet only two products are observed (Fig. 1). Caspase-3 has been shown to cleave htt at 510DSVD513 (8) (the underlined P1 glutamate residue) but not at 527DEED530, at least in the context of htt truncated at aa 548 (18). To identify the second caspase-3 site in htt and to confirm cleavage at 510DSVD513 in the presence of a larger htt fragment (extending to aa 1212), we generated double P1 mutants expressing htt with 15 or 138 glutamines, respectively (Fig. 4A). Asp513 and Asp552 are not cleaved by purified caspase-3, whereas the other combinations were cleaved at D513A, D552A, or D589A. Cleavage assays using radiolabeled htt containing wild-type htt sequence and each of these double mutants showed that one combination (Asp513 and Asp552) was not cleaved by purified caspase-3, whereas the other combinations were cleaved (Fig. 3B), indicating that htt is cleaved in vitro by caspase-3 solely at positions Asp513 and Asp552. These results were reproducible for 3949-138 (not shown), indicating that polyglutamine expansion did not change the caspase cleavage profile of htt.

A Novel Cleavage Product in Cells Expressing Caspase-resistant htt—Because purified caspase-3 did not cleave the double mutant D513A,D552A, we expected that it would also be resistant to cleavage in transfected cells. However, a novel tamoxifen-induced band was observed in cells expressing this double mutant that migrated at 90 or 115 kDa for htt with 15 or 138 glutamines, respectively (Fig. 4B, lanes 1–4), suggesting the existence of a cleavage event downstream of aa 552 by caspase-3 or a distinct protease.

To test for caspase-3 cleavage at an alternative site, we evaluated constructs with mutations at each of the four caspase-3 consensus sites in htt (510DSVD513, 527DEED530, 549DLND552, and 586DGTD589). Cleavage with the quadruple mutant generated cleavage products that were indistinguishable from those of the double mutant (Fig. 4B, lanes 5 and 6), suggesting that activation of a cryptic caspase-3 site is not a likely explanation for the origin of the 90- or 115-kDa fragments in cells expressing htt with 15 or 138 glutamines, respectively.

Independent htt Cleavage by Caspase-3 and Caspase-6—Caspase-3 cleavage at D586 in htt was not detected in 293T cells unless caspase-3 cleavage at Asp513 and Asp552 was blocked. To determine whether caspase-6 cleaves htt independently of caspase-3, we used an antibody specific for htt residues C-terminal to the caspase-6 cleavage site (Ab650). C-terminal fragments resulting from cleavage of the wild type and caspase-3-resistant htt are equivalent in size, indicating that caspase-6 cleaves htt independent of caspase-3 (Fig. 5D). Additionally, N-terminal products detected by the N-terminal antibody BKPI in cells expressing caspase-6-resistant htt are indistinguishable from those of the wild type construct (Fig. 5D), showing that caspase-6 cleaves htt independent of caspase-6. Furthermore, these results provide support for the retention of the normal tertiary structure of htt bearing these mutations because cleavage by caspase-6 is not affected by mutation of the nearby caspase-6 site and vice versa.

Caspase Activation Is Reduced by Caspase-resistant htt—The toxic fragment hypothesis suggests that N-terminal htt fragments enhance toxicity from 47.4.

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FIG. 1. htt cleavage during apoptotic cell death. HEK 293T cells were transiently transfected with 10366-15 (A), 10366-138 (B), 3949-15 (C), and 3949-138 (D) and treated with tamoxifen. Samples were harvested at the indicated time points, separated on SDS-polyacrylamide gel electrophoresis gels, and analyzed by Western blot using the N-terminal htt antibody BKPI.
cell death by accelerating the activation of proteolytic enzymes including caspases by as yet undefined mechanisms. We directly tested this hypothesis by measuring DEVD-ase activity in transiently transfected cells exposed to an apoptotic stress (Fig. 6A). Mock transfected HEK 293T cells exposed to 35\(\mu\)M tamoxifen for 12 h show a mild increase in DEVD-ase activity, demonstrating that this dose of tamoxifen has a slight toxic effect that occurs gradually. Transfected cells are sensitized to tamoxifen exposure as measured by a significantly increased rate of DEVD-ase activation over 12 h compared to mock-transfected cells (\(p<0.001\) for each for both pCIneo or pCMVlacZ versus mock at 4, 8, and 12 h, \(n=4\)). This sensitization presumably occurs because of the stresses involved in exposure to the calcium-phosphate mixture or protein overexpression in this cell background.

Cells expressing cleavable full-length htt have significantly elevated rates of DEVD-ase activation that is modulated by polyglutamine length. DEVD-ase activity becomes significantly elevated in cells expressing 10366-138 WT by 2 h (\(p<0.001\), \(n=4\)) and is markedly elevated in cells expressing cleavable htt with 15 or 138 glutamines by 4, 8, and 12 h (\(p<0.001\) for each, \(n=4\)) compared with mock or control transfected cells. These time course results suggest that the presence of N-terminal htt fragments that become detectable on a Western blot between 2 and 4 h (Fig. 1) result in an accelerated rate of caspase activation that is particularly enhanced for expanded htt. By 12 h, DEVD-ase activity in cells expressing 10366-138 WT is lowered, presumably because of the decay of caspase activity that occurs after cell death.

In contrast, cells expressing uncleavable full-length htt have significantly reduced relative DEVD-ase activities compared with cleavable htt (\(p<0.001\) for each at 4, 8, and 12 h, \(n=4\)). The rate of tamoxifen-induced caspase activation in cells expressing uncleavable htt resembles that of tamoxifen-treated control cells transfected with the empty vector or pCMVlacZ.

A similar reduction in toxicity was observed in neuronal cells expressing uncleavable htt as compared with cleavable htt. DEVD-ase activity assays performed on transfected hippocampal HN33 cells exposed to serum-free medium for 24 h showed that both full-length htt with 15 or 138 glutamines resulted in a significant increase in DEVD-ase activity when compared with the empty vector control, with the effect being greater for 138 versus 15 glutamines (pCIneo = 1.0 ± 0.04 versus 10366-15 = 1.36 ± 0.02, \(n=3\), \(p<0.01\); pCIneo versus 10366-138 = 1.67 ± 0.04, \(n=4\), \(p<0.001\)) (Fig. 6B). In contrast, DEVD-ase activities in cells expressing caspase-resistant htt with 15 or 138 glu-
DISCUSSION

In this manuscript we provide several lines of evidence supporting the toxic fragment hypothesis. Inhibition of htt cleavage by general caspase inhibitors reduces overall toxicity as measured by a MTT assay. These results suggest that caspases contribute to overall toxicity but do not define whether this is due to general inhibition of caspases or whether htt itself is a critical caspase substrate. Using sequential mutagenesis, we show that caspase cleavage of htt per se has an important role in apoptosis because caspase activation, aggregate formation, and toxicity are significantly reduced in neuronal and nonneuronal cells expressing caspase-resistant htt. These experiments suggest a role for htt as a caspase substrate in the pathogenesis of HD and provide support for the ability of N-terminal polyglutamine-containing caspase cleavage products of htt to amplify or accelerate caspase activation.

Although it is not yet known which caspases or other proteases may be responsible for contributing to htt cleavage in vivo, our results suggest that caspase-3 and caspase-6 may play key roles in generating toxic N-terminal htt fragments. Specifically, we have determined that the N-terminal htt fragments generated upon caspase cleavage of htt are associated with further caspase-3 activation. In addition, the activation of caspase-3 is markedly inhibited in the presence of a noncleavable form of huntingtin. The observation that cleavable htt, particularly when containing an expanded polyglutamine tract, accelerates caspase-3 activation and that mutating the caspase cleavage sites and that inhibiting htt cleavage by both caspase-3 and caspase-6 reduces aggregates in parallel with toxicity.

Aggregates in striatal ST14A cells were also exclusively cytoplasmic but smaller than those observed in 293T cells, presumably because of the lower level of htt expression in ST14A compared with 293T cells (Fig. 7C). Although striatal ST14A cells expressing cleavable 3949-138 formed aggregates at a frequency of 7.1 ± 2.0% in cells assayed 24 h after transfection. This increased to 14.2 ± 2.6% after 30–45 min in serum-free medium, providing support for an increase in aggregate formation in response to a toxic stimulus (Fig. 7D). By contrast, ST14A cells expressing uncleavable 3949-138 formed aggregates at a reduced frequency of 2.6 ± 0.4 and 3.1 ± 0.8%, before and after serum withdrawal, respectively (n = 6, p < 0.01). Inhibiting caspase cleavage of htt therefore reduces aggregate formation in serum-starved neuronal cells.

The N-terminal polyglutamine-containing caspase cleavage products of htt to amplify or accelerate caspase activation.
significance such as to separate or inactivate functional domains, which is a common theme for many caspase substrates (reviewed in Ref. 30). For htt, it is interesting that a redistribution of N-terminal htt fragments to the nucleus has been implicated in toxicity in patients and in animal models (16, 31–33) and in some but not all in vitro models (13, 27). In yeast artificial chromosome transgenic animals expressing full-length htt, we have identified N-terminal htt fragments traversing the nuclear pore (17), showing that proteolytic cleavage has to precede nuclear entry. Furthermore, nuclear entry of N-terminal htt fragments would permanently disrupt interactions with its cytoplasmic binding proteins, some of which have binding affinities that are modulated by polyglutamine length (22, 34–36) and are hypothesized to contribute to pathogenesis.
when their interactions with htt are altered.

A number of caspase substrates appear to have active roles in apoptosis. In addition, preventing their cleavage alters their influence on cell death. For example, caspase-resistant lamin protects cells from chromatin condensation and nuclear shrinkage (37). Death triggered by CD95 activation is delayed in cells expressing caspase-resistant poly(ADP-ribose) polymerase (38). Additionally, the antiapoptotic properties of the presenilin-2 C-terminal fragment are enhanced in the context of a caspase-resistant form (39). Amyloid precursor protein is cleaved by caspses during apoptotic cell death (40, 41), and mutation of the caspase cleavage site in amyloid precursor protein blocks cleavage in the presence of an apoptotic stress (39), although it is not yet known whether this is sufficient to inhibit toxicity.

It is striking that six of seven pathogenic polyglutamine-containing proteins are substrates for caspase cleavage. Huntingtonin, atrophin-1, the androgen receptor, ataxin-3, ataxin-7, ataxin-6, and ataxin-2 are cleaved by caspases in vitro and in apoptotic extracts (Ref. 18 and data not shown). That so many polyglutamine-containing proteins are substrates for caspase cleavage is suggestive that caspase cleavage of these proteins may represent a common step in several neurodegenerative disorders caused by polyglutamine expansion. Furthermore, several of these polyglutamine-containing proteins are emerging as having an active role in the progression of cell death. We have recently shown that toxicity and aggregate formation are reduced in cells expressing caspase-resistant mutant forms of the androgen receptor (25) or atrophin-1 (26), which when containing expanded polyglutamine tracts cause the diseases spinal bulbar muscular atrophy or dentatorubralpallidoluysian atrophy, respectively. With the generation of a caspase-resistant mutant form of huntingtin, we provide further support for the generality of caspase cleavage of polyglutamine-containing proteins as an important step in the onset or progression of this group of diseases. These results form the basis for additional investigations of inhibition of proteolytic cleavage as potential approaches to therapy for HD and other polyglutamine disorders.

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