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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 proapoptotic 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 apoptotically 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 the selective 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 Asp^{513} and Asp^{552} and one novel caspase-6 site at aa position Asp^{586} 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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[1] The abbreviations used are: HD, Huntington's disease; htt, huntingtin; aa, amino acid(s); z-VAD-fmk, z-Val-Ala-Asp (OMe)-CH_2F; z-DEVD-fmk, z-Asp(OCH_3)-Glu(OCH_3)-Val-Asp(OCH_3)-fmk; Ac-DEVD-CHO, Ac-Asp-Glu-Val-Asp-aldehyde; Ac-YVAD-CHO, Ac-Tyr-Val-Ala-Glu-aldyhyde; WT, wild type.

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 *Xba*I followed by religation to create pRC-CMV3949-15 and pRC-CMV3949-138. pCMV-lacZ and pRSV-lacZ were kindly provided by Drs. Paul Orban and Ite Laird-Offringa, 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 TAT TTG; constant 3′, CTG TTC CTC AGG GTA TTC CGT G; 1854F, CGG ACT CAG TGG CTC TGG CCA GCT G; 1854R, CAG CTG GCC AGA GCC ACT GAG TCC G; 1904F, GAT GAG GAG GCT ATC TTG AGC CAC AG; 1904R, CTG TGG CTC AAG ATA GCC TCC TCA TC; 1970F, GGA CCT GAA TGC TGG GAC CCA GGC C; 1970R, GGC CTG GGT CCC AGC ATT CAG GTC C; 2081F, GAC GGT ACC GCC AAC CAG TAT TTG GG; 2081R, CCC AAA TAC TGG TTG GCG GTA CCG TC; 2072F, GAA ATT GTG TTA GCC GGT ACC GAC AAC; and 2072R, GTT GTC GGT ACC GGC TAA CAC AAT TTC. 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 3 min, 30 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 products were pooled and further amplified using the constant primers, digested with *Bsu*36I and ligated to *Bsu*36I-digested pRC-CMV3949-15 or pRC-CMV3949–128. All mutations were confirmed by DNA sequencing. Full-length mutagenized constructs were generated by reinserting the previously deleted *Xba*I fragment.

Cell Culture and Protein Preparation—HEK 293T cells were cultured and transfected by calcium phosphate coprecipitation as described previously (21) to an efficiency of 80–95% as determined by β-galactosidase staining. After 24 h, cells were treated with 35 μM tamoxifen (Sigma). At time intervals after tamoxifen addition, cells were collected, washed with phosphate-buffered saline, and resuspended in 20 mM HEPES, pH 7.4, 5 mM $MgCl_2$, 0.5 mM EDTA, 0.01% (w/v) sucrose, 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml leupeptin, 10 μg/ml benzamidine, and 10 μg/ml aprotinin. For experiments with z-VAD-fmk or z-DEVD-fmk (Calbiochem), cells were preincubated with 50 μM of the desired inhibitor for 60 min prior to transfection until harvest.

HN33 hippocampal cells were cultured in Dulbecco's modified Eagle's medium (Canadian Life Technologies) with 10% fetal calf serum, 50 units/ml penicillin-streptomycin, 2 mM L-glutamine, and 1 mM sodium pyruvate and transfected at 60% confluency in 6-well dishes using Superfect (Qiagen) according to the manufacturer's instructions to an efficiency of 40% as determined by β-galactosidase staining. 24 h later, the medium was aspirated and replaced with serum-free growth medium for an additional 24 h. Cells were collected and resuspended in ice-cold cell lysis buffer (CLONTECH), incubated on ice for 10 min and centrifuged at 14,000 × *g* for 5 min.

Immortalized rat striatal cells (ST14A) were cultured at 33 °C in Dulbecco's modified Eagle's medium with 10% fetal calf serum, 50 units/ml penicillin-streptomycin, 2 mM L-glutamine, and 1 mM sodium pyruvate. Cells were plated onto coverslips and transfected at 60% confluency in 6-well dishes using LipofectAMINE (Canadian Life Technologies) according to the manufacturer's instructions to an efficiency of 30% as determined by β-galactosidase staining. 24 h later, the medium was replaced with serum-free growth medium for 30–45 min, after which coverslips were processed for immunofluoresence.

Western Blotting—Equal amounts of protein were separated on 7.5% SDS-polyacrylamide gel electrophoresis gels, electophoretically transferred to polyvinylidene fluoride membranes (Millipore), and probed with the N-terminal anti-htt antibody BKP1 directed against htt aa 1–17 (22) or Ab650, which recognizes htt aa 650–663 (23).

Immunofluorescence and Scoring of Aggregates—Transfected 293T or ST14A cells were processed for immunofluorescence as described previously (10, 11) using anti-htt MAB2166 (Chemicon). Immunofluorescence was viewed with a Zeiss Axioscope microscope and digitally captured with a charge coupled device camera (Princeton Instrument Inc.), and the images were colorized and overlapped using Eclipse (Empix Imaging Inc.). Control experiments in which the primary antibody was omitted did not show immunofluorescence. A sufficiently dilute concentration of primary antibody (1:1000) was used so that only transfected cells stained positively with the primary antibody. Aggregates were scored by examining transfected cells for the presence of aggregates, ensuring that at least 200 (for 293T) or 100 (for ST14A) transfected cells were scored per repetition of the experiment. Aggregate frequency was defined as the percentage of total transfected cells containing an aggregate of any size. Aggregates were defined as a clearly demarcated region of dense staining that was specific for the htt primary antibody, and aggregate frequency was calculated according to the following formula: aggregate frequency = (number of transfected cells containing an aggregate/total number of transfected cells) × 100%. Although we did not accurately measure the size of aggregates in 293T or ST14A cells, we noted that the moderate-to-large aggregates formed in 293T cells were roughly the size of the nucleoli and the small-to-moderate aggregates formed in ST14A cells tended to be between approximately 10–40% the size of the nucleoli.

Toxicity Measurements—Toxicity of the transfected htt constructs was measured using the WST assay (Roche Molecular Biochemicals) as described (11). Briefly, cells were seeded at a density of 5×10^4 cells into 96-well plates and transfected with htt or control constructs. At 24 h after transfection, 35 μM tamoxifen was added to the cells for 3 h, after which time a 1:10 dilution of the WST-1 reagent (Roche Molecular Biochemicals) was added and the incubation was continued for an additional hour. Following a total of 4 h of exposure to tamoxifen, release of formazan from mitochondria was quantified at 450 nm using an enzyme-linked immunosorbent assay plate reader (Dynatech Laboratories, Cantilly, VA), taking readings at 15-min intervals for 60 min to ensure linearity of the response.

DEVD-ase assays were performed with the ApoAlert fluorometric kit as specified (CLONTECH). 24 h after transfection, cells were apoptotically stressed by treatment with 35 μM tamoxifen for 2–12 h for 293T cells or exposure to serum-free medium for 24 h for HN33 cells. Cells were harvested by scraping and centrifugation followed by lysis in Cell Lysis Buffer (CLONTECH). Lysates were incubated on ice for 10 min followed by centrifugation to pellet cell debris. The protein concentration in the resulting supernatants was determined by Lowry assay, and DEVD-ase activity was quantitated by incubation with a reaction mix containing DEVD-AMC as specified (CLONTECH). Reactions were incubated at 37 °C and read at intervals at an excitation of 385 nm and emission of 510 nm. The rate of change in fluorescence within the linear range was normalized to protein concentration and expressed as relative levels. We observed in all experiments that the rate of change of fluorescence remained linear for at least one h, and the level of fluorescence at 1 h was used for analyses.

In Vitro Cleavage Assays—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.

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 polyglutamine-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*, 0 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-3 sites. Although recombinant caspase-1 cleaves htt *in vitro* (18), htt cleavage products consistent with cleavage at the caspase-1-specific sites are not observed in this model.

To facilitate further manipulations of the large htt cDNA (10366 base pairs encoding a 3144-aa protein), htt constructs truncated at aa 1212 (nucleotide 3949) that contained 15 or 138 glutamines were generated. These truncated 3949 constructs, like full-length htt, yield two major

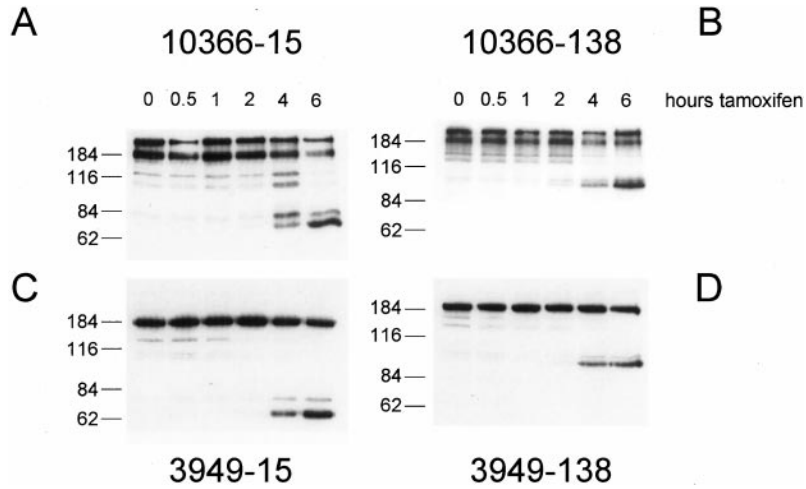


FIG. 1. **htt cleavage during apoptotic cell death.** HEK 293T cells were transiently transfected with 10366-15 (*A*), 10366-138 (*B*), 3949-15 (*C*), and 3949-138 (*D*) and treated with tamoxifen. Samples were harvested at the indicated time points, separated on SDS-polyacrylamide gel electrophoresis gels, and analyzed by Western blot using the N-terminal htt antibody BKP1.

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. 2*A*), demonstrating that htt cleavage is caspase-dependent in this model. In contrast, the Me_2SO-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. 2*B*). 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. 2*C*). 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 (^{510}DSVD^{513}, ^{527}DEED^{530}, ^{549}DLND^{552}, and ^{586}DGTD^{589}; Fig. 3*A*), yet only two products are observed (Fig. 1). Caspase-3 has been shown to cleave htt at ^{510}DSV<u>D</u>^{513} (after the underlined P1 aspartate residue) but not at ^{527}DEED^{530}, 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 ^{510}DSVD^{513} in the context of a larger htt fragment (extending to aa 1212), we generated double P1 aspartate to alanine mutations in 3949-15 and 3949-138, where one mutation was constant at D513A and the second mutation was generated at D530A, D552A, or D589A. Cleavage assays using radiolabeled proteins containing the wild type sequence and each of these double mutants showed that one combination (Asp^{513} and Asp^{552}) was not cleaved by purified caspase-3, whereas the other combinations were (Fig. 3*B*), indicating that htt is cleaved *in vitro* by caspase-3 solely at positions Asp^{513} and Asp^{552}. 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-3-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. 4*B*, *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 (^{510}DSVD^{513}, ^{527}DEED^{530}, ^{549}DLND^{552}, and ^{586}DGTD^{589}). Transfection with the quadruple mutant generated cleavage products that were indistinguishable from those of the double mutant (Fig. 4*B*, *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.

Generation of Caspase-resistant htt—A group III caspase consensus site, ^{583}IVLD^{586} (Fig. 4*A*) was then identified in htt. To determine whether cleavage at ^{583}IVLD^{586} generated the 90- or 115-kDa products, quintuple mutants with modifications of all four caspase-3 sites as well as the group III site (D513A, D530A, D552A, D586A, and D589A), each with 15 or 138 glutamines, were generated. Cleavage products were essentially undetectable in 293T cells expressing these quintuple mutants after tamoxifen treatment (Fig. 4*B*, *lanes 7* and *8*), showing that htt is also cleaved at ^{583}IVLD^{586}, possibly by a group III caspase, during an apoptotic challenge. In addition, full-length huntingtin bearing mutations at aa 513, 530, 552, 586, and 589 also failed to be cleaved in transfected 293T cells during an apoptotic challenge, demonstrating that there are no caspase sites within the htt C-terminal region that are recognized or accessible when htt is expressed in cells (Fig. 4*C*).

Caspase-6 Cleaves htt at Asp^{586}—Additional experiments confirmed cleavage of htt by caspases other than caspase-3. Three products were observed when radiolabeled full-length htt is incubated in apoptotic Jurkat extracts, two of which result from caspase-3 cleavage because they are inhibited by preincubation with 10 nM Ac-DEVD-CHO and comigrate with caspase-3-specific 70- and 80-kDa products (Fig. 5*A*). The 90-kDa product, however, is not inhibited either by Ac-DEVD-CHO or 10 nM Ac-YVAD-CHO (Fig. 5*A*). Analyses of the htt mutants in Jurkat extracts confirms that the double and quadruple mutations eliminate the Ac-DEVD-CHO-inhibitable products but fail to prevent formation of the 90-kDa band (Fig. 5*B*). In contrast, Jurkat apoptotic extracts fail to cleave the quintuple mutant, confirming that the D586A mutation identifies a non-caspase-3 site in htt. These results were confirmed using htt with 138 glutamines (not shown).

Caspases-6, -8, -9, and -10 cleave at (I/V/L)*XX*D sites (8). Although htt is not cleaved by caspase-8 (18), htt is a robust substrate for caspase-6 (Fig. 5, *B* and *C*). Furthermore, caspase-6 cleavage occurs in the presence of caspase-3 resistant htt (double and quadruple mutants) but not when htt contains an additional modification of position Asp^{586} (quintuple mutant) (Fig. 5*C*). These results suggest that caspase-6 may contribute to htt cleavage *in vivo*, although we have not excluded the possibility that caspases-9 and -10 may also do so. Identical results were obtained using htt with 138 glutamines (not shown).

Independent htt Cleavage by Caspase-3 and Caspase-6—Caspase-6 cleavage at D586 in htt was not detected in 293T cells unless caspase-3 cleavage at Asp^{513} and Asp^{530} was blocked. To determine whether caspase-6 cleaves htt independent 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. 5*D*). Additionally, N-terminal products detected by the N-terminal antibody BKP1 in cells expressing caspase-6-resistant htt are indistinguishable from those of the wild type construct (Fig. 5*D*), showing that caspase-3 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-3 is not affected by mutagenesis 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

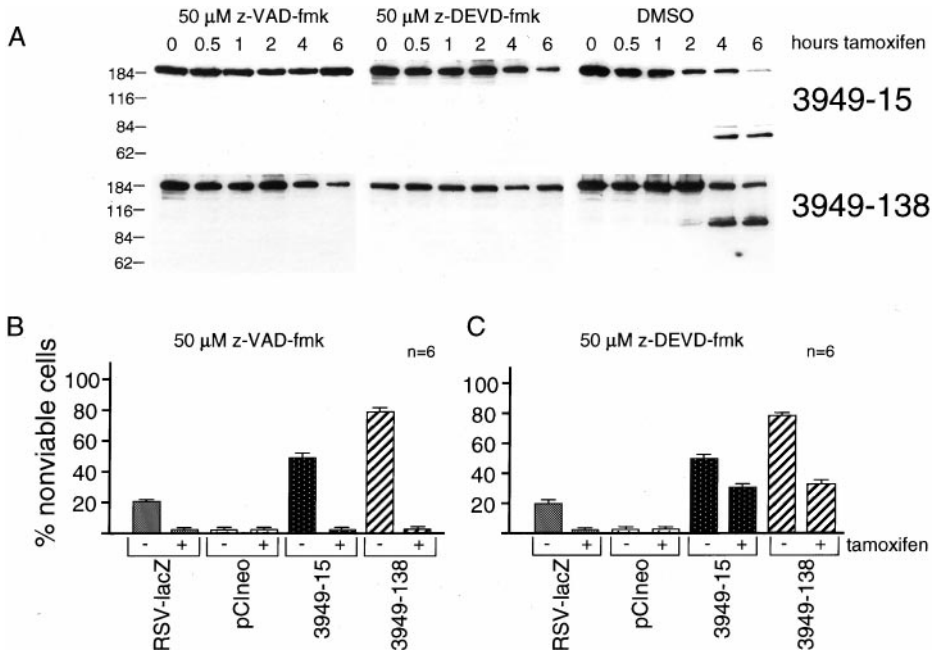


FIG. 2. **Caspase inhibitors prevent htt cleavage and toxicity.** *A*, HEK 293T cells were treated with z-VAD-fmk, z-DEVD-fmk, or Me_2SO only from 60 min prior to transfection until samples were harvested. Cells were transfected with 3949-15 or 3949-138, treated with tamoxifen, and harvested at the indicated time points. Cleavage was assessed on Western blots using BKP1. *B* and *C*, WST assay of HEK 293T cells treated with 50 μM z-VAD-fmk (*B*) or 50 μM z-DEVD-fmk (*C*). Cells were transfected with pRSV-lacZ, pCIneo, 3949-15, or 3949-138. *Bars* represent the average of six independent assays of six replicates each.

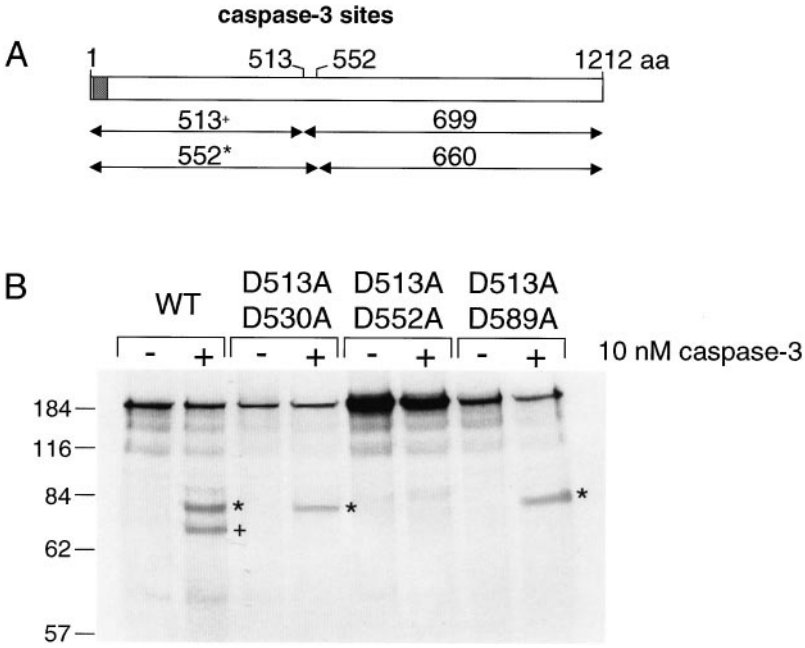


FIG. 3. **Identification of the caspase-3 cleavage sites in htt.** *A*, expected product sizes generated upon cleavage at position 513 or 552. *B*, radiolabeled wild type htt 3949-15 and 3949-15 and mutagenized derivatives were incubated in cleavage buffer alone (−) or in cleavage buffer with 10 nM of purified caspase-3 (+) for 1 h at 37 °C. Reaction products were visualized by autoradiography. Because of the altered mobility by SDS-polyacrylamide gel electrophoresis of polyglutamine-containing fragments, the two N-terminal fragments (+ and *) partially overlap the two C-terminal fragments. The minor band visible at 80 kDa when 3949-15 wild type is cleaved with caspase-3 is the C-terminal remnant detected by autoradiography.

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. 6*A*). Mock transfected HEK 293T cells exposed to 35 μM 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. 6*B*). In contrast, DEVD-ase activities in cells expressing caspase-resistant htt with 15 or 138 glu-

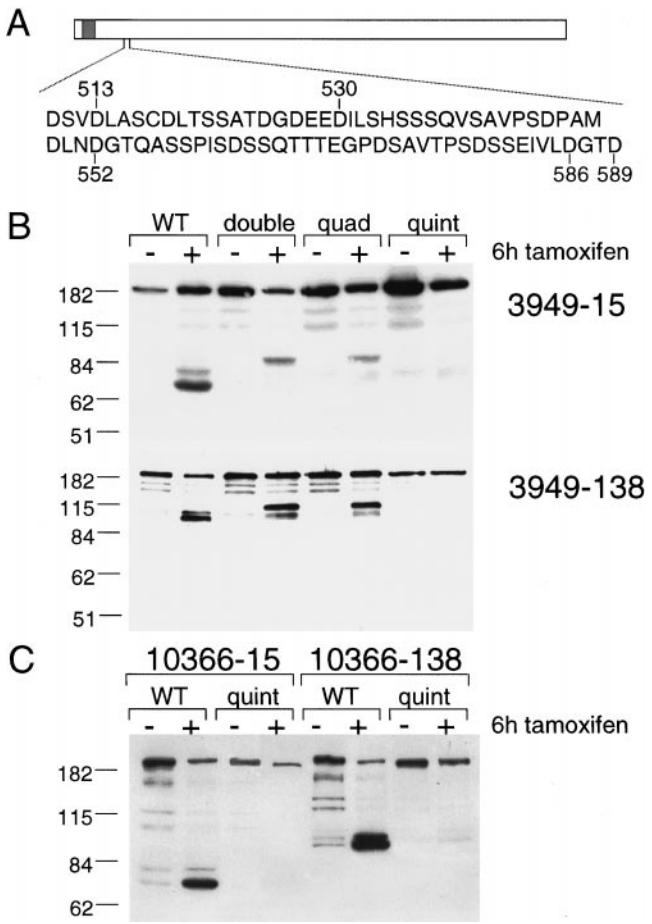


FIG. 4. **Generation of caspase-resistant htt.** *A*, diagram of htt (*open bar*) showing the position of the polyglutamine tract (*shaded box*) and the sequence of the cluster of caspase consensus sites. *B*, HEK293T cells were transfected with 3949-15 or 3949-138 with the wild type caspase cleavage sites (*WT*) or mutations at D513A,D552A (*double*), D513A,D530A,D552A,D589A (quad), or D513A,D530A,D552A,D586A-,D589A (*quint*). *C*, HEK293T cells were transfected with 10366-15 or 10366-138 with the wild type caspase cleavage sites (*WT*) or mutations at D513A,D530A,D552A,D586A,D589A (*quint*). Cells were harvested in the absence of tamoxifen (−) or treated with tamoxifen for 6 h (+) and analyzed by Western blot using BKP1.

tamines were indistinguishable from the empty vector or pRSV-lacZ controls (pCIneo = 1.0 ± 0.04, pRSV-lacZ = 1.02 ± 0.07, 10366-15 quint = 1.02 ± 0.04, 10366-138 quint = 1.04 ± 0.05, $n = 4$, $p > 0.05$).

These results show that caspase-resistant htt with an expanded polyglutamine tract is less toxic than cleavable htt and that the acceleration of caspase activation because of the presence of N-terminal htt fragments is specifically prevented in cells expressing uncleavable htt. Furthermore, these findings suggest that htt containing an expanded polyglutamine tract can be rendered to a toxicity similar to that of β-galactosidase by mutating the caspase cleavage sites in htt. Taken together, these data suggest that caspase cleavage of htt actively contributes to apoptotic progression in HD by generating toxic N-terminal fragments that accelerate additional caspase activation in a vicious cycle.

Aggregate Formation Is Inhibited in Cells Expressing Caspase-resistant htt—As an additional index of toxicity, we evaluated the importance of caspase cleavage of htt to the formation of intracellular aggregates, which have been shown to correlate with toxicity in a number of *in vitro* model systems (10–14). Immunofluorescence in transfected 293T cells showed that tamoxifen-treated cells expressing cleavable 3949-138 formed aggregates at a frequency of approximately 12%, as defined by the percentage of transfected cells that contained a visible aggregate of any size (Fig. 7). Aggregates in 293T cells were exclusively cytoplasmic and moderate-to-large in size (Fig. 7*A*), in keeping with our previous demonstrations that htt fragments larger than 548 aa (corresponding to 1955 nucleotides) do not enter the nucleus (11). Aggregate frequency was not significantly altered in cells expressing caspase-3-resistant 3949-138 (double or quadruple mutations) ($n = 2$, $p > 0.05$) or caspase-6-resistant 3949-138 (the D586A mutation alone) ($n = 2$, $p > 0.05$) (Fig. 7*B*). In contrast, aggregate frequency was significantly reduced to approximately 3% in tamoxifen-treated cells expressing caspase-3- and -6-resistant 3949-138 ($n = 2$, $p < 0.001$). These results show that aggregates can be formed by either the caspase-3 or caspase-6 htt cleavage products 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. 7*C*). 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. 7*D*). 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.

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 an 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 in htt reduces this effect has important therapeutic implications. It may be possible to design agents that specifically prevent the cleavage of htt but do not indiscriminately interfere with the general cleavage activity of caspases toward other caspases or other important cellular substrates. Such agents may prevent the accelerated death of susceptible neurons in HD while allowing appropriate apoptotic death to proceed in other cells.

These observations suggest that caspase-3 may have an important role in the pathogenesis of HD, although additional caspases may also contribute to cell death. For example, caspase-8, which does not cleave htt (18), is recruited to and activated by polyglutamine-containing aggregates including those in HD patient brains (28). Additionally, the neurological phenotype in mice expressing expanded htt exon 1 is reduced when crossed with mice expressing dominant negative caspase-1 (29). These studies suggest that additional caspases may also contribute to the pathogenesis of HD.

It is remarkable that htt has three tightly clustered caspase cleavage sites and that two different caspases from distinct caspase families are capable of cleaving htt. This redundancy suggests that caspase cleavage of htt may have functional

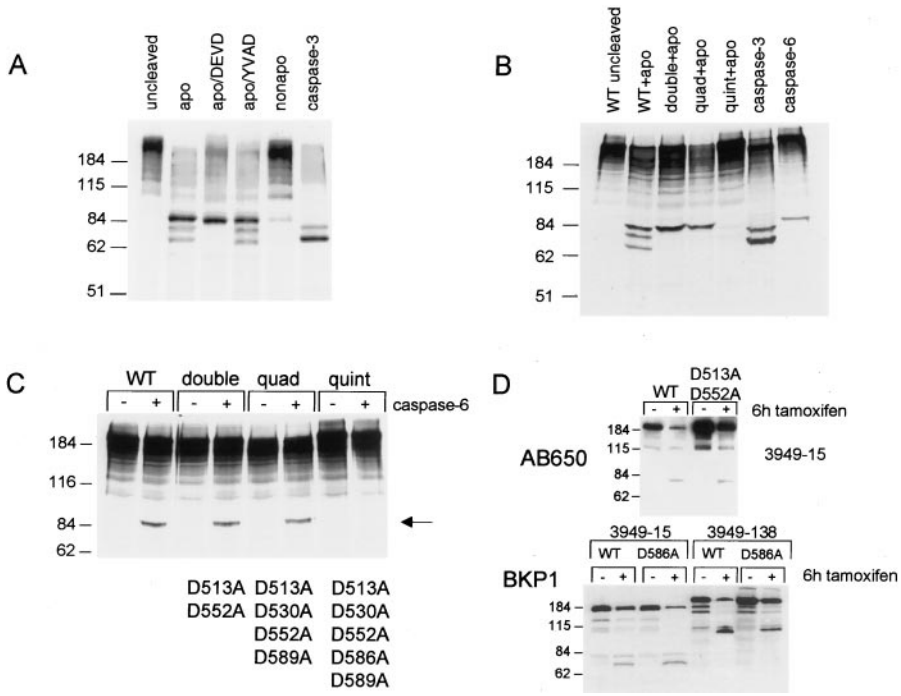


FIG. 5. **Non-caspase-3 cleavage of htt.** *A*, radiolabeled full-length htt with 15 glutamines and wild type caspase sites was incubated for 1 h at 37 °C in cleavage buffer (*uncleaved*), in cleavage buffer with Jurkat apoptotic extract (*apo*), Ac-DEVD-CHO-preincubated apoptotic extract (*apo/DEVD*), or Ac-YVAD-CHO-preincubated apoptotic extract (*apo/YVAD*), with nonapoptotic extract (*nonapo*), or with 10 nM purified caspase-3 (*caspase-3*). *B*, radiolabeled full-length htt with 15 glutamines and wild type caspase sites was incubated in cleavage buffer (*uncleaved*), with cleavage buffer with apoptotic Jurkat extract (*WT+apo*), 10 nM purified caspase-3 (*caspase-3*), or 5 nM purified caspase-6 (*caspase-6*). Radiolabeled full-length htt with mutations at D513A,D552A (*double*), D513A,D530A,D552A,D589A (*quad*), or D513A,D530A,D552A,D586A,D589A (*quint*) were incubated with cleavage buffer and Jurkat apoptotic extract at 37 °C for 1 h. *C*, radiolabeled full-length htt with 15 glutamines with wild type caspase sites (*WT*) or mutations at aa positions D513A,D552A (*double*), D513A,D530A,D552A,D589A (*quad*), or D513A,D530A,D552A,D586A,D589A (*quint*) were incubated in cleavage buffer (−) or in cleavage buffer with 5 nM of caspase-6 for 1 h at 37 °C. *D*, Western blot of untreated (−) or tamoxifen-treated (+) HEK 293T cells transfected with wild type (*WT*), caspase-3-resistant (*D513A,D552A*), or caspase-6-resistant (*D586A*) 3949-15 using Ab650 or BKP1.

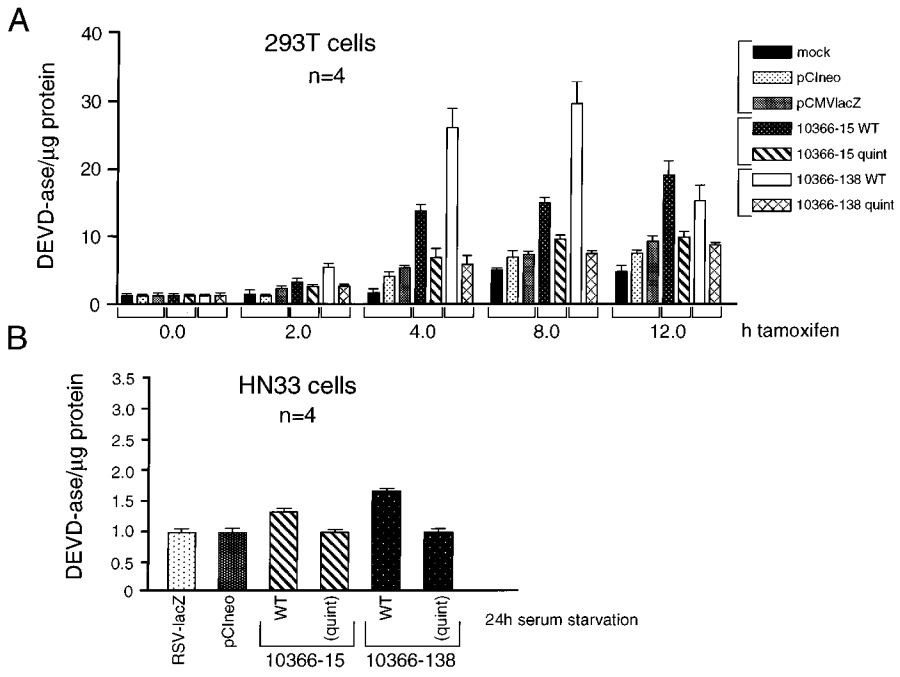
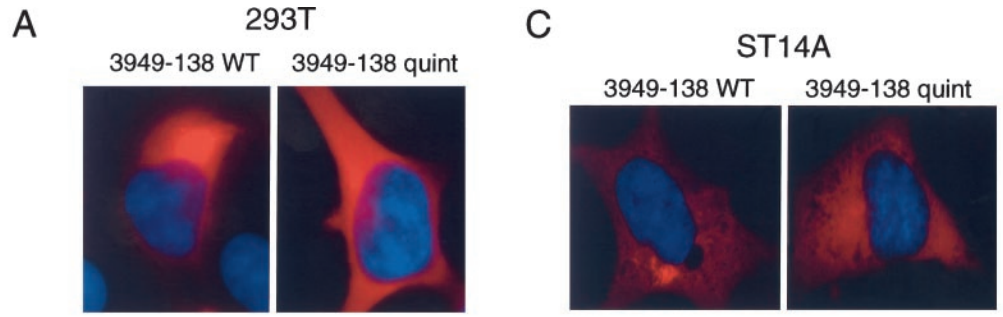


FIG. 6. **Reduced toxicity of caspase-resistant htt.** DEVD-ase assay of HEK 293T cells (*A*) or HN33 hippocampal cells (*B*) transfected with pRSV-lacZ, pCI-neo, full-length cleavable htt (*WT*), or caspase-3- and -6-resistant htt (D513A-,D530A,D552A,D586A,D589A (*quint*)). DEVD-ase activity was measured after 2, 4, 8, and 12 h of exposure to 35 µM tamoxifen (*A*) or 24 h of exposure to serum-free medium (*B*). Values are averaged from four independent trials.

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



B

Aggregate frequency in 293T cells
n=3

	- tam	+tam
WT	0.3 (0.15)	12.8 (0.5)
3-res (double)	0.1 (0.08)	14.2 (4.0)
3-res (quad)	0.3 (0.10)	17.1 (3.7)
6-res	0.3 (0.15)	11.0 (0.6)
3&6-res (quint)	0.05 (0.03)	2.8 (0.9)

D

Aggregate frequency in ST14A cells
n=6

	+serum	- serum
WT	7.13 (2.04)	14.2 (2.57)
quint	2.60 (0.45)	3.12 (0.78)

FIG. 7. **Aggregate frequency is reduced in cells expressing caspase-resistant htt.** *A*, immunofluorescence of HEK 293T cells transfected with wild type htt (*3949-138 WT*) or caspase-resistant htt (*3949-138 quint*) following tamoxifen treatment. htt (*red*) was detected with monoclonal antibody 2166 (Chemicon), and the nuclei are stained *blue* with DAPI. *B*, quantitation of aggregates in 293T cells expressing wild type htt (*WT*), caspase-3-resistant htt (D513A,D552A (*double*)) or D513A,D530A,D552A,D589A (*quad*), caspase-6-resistant htt (*D586A*), or caspase-3 and -6-resistant htt (D513A,D530A,D552A,D586A,D589A (*quint*)). Over 200 transfected untreated (−) or tamoxifen-treated (+) cells were scored in each repetition ($n = 3$). *C*, immunofluorescence of ST14A cells transfected with wild type htt (*3949-138 WT*) or caspase-resistant htt (*3949-138 quint*) following 24 h of serum starvation. htt (*red*) was detected with monoclonal antibody 2166 (Chemicon), and the nuclei are stained *blue* with DAPI. *D*, quantitation of aggregates formed in ST14A cells expressing cleavable 3949-138 (*WT*) or caspase-3- and -6-resistant htt. Between 100 and 200 transfected untreated (−) or serum-starved (+) cells were scored for aggregates in each repetition of the experiment ($n = 6$).

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 caspases 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 (40), 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. Huntingtin, 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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Inhibiting Caspase Cleavage of Huntingtin Reduces Toxicity and Aggregate Formation in Neuronal and Nonneuronal Cells

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