Huntington’s disease (HD) is a neurodegenerative disorder caused by a polyglutamine (polyQ) tract expansion near the N terminus of huntingtin (Htt). Proteolytic processing of mutant Htt and abnormal calcium signaling may play a critical role in disease progression and pathogenesis. Recent work indicates that calpains may participate in the increased and/or altered patterns of Htt proteolysis leading to the selective toxicity observed in HD striatum. Here, we identify two calpain cleavage sites in Htt and show that mutation of these sites renders the polyQ expanded Htt less susceptible to proteolysis and aggregation, resulting in decreased toxicity in an in vitro cell culture model. In addition, we found that calpain- and caspase-derived Htt fragments preferentially accumulate in the nucleus without the requirement of further cleavage into smaller fragments. Calpain family members, calpain-1, -5, -7, and -10, have increased levels or are activated in HD tissue culture and transgenic mouse models, suggesting they may play a key role in Htt proteolysis and disease pathology. Interestingly, calpain-1, -5, -7, and -10 localize to the cytoplasm and the nucleus, whereas the activated forms of calpain-7 and -10 are found only in the nucleus. These results support the role of calpain-derived Htt fragmentation in HD and suggest that aberrant activation of calpains may play a role in HD pathogenesis.

Huntington’s disease (HD) is an autosomal-dominant neurodegenerative disease caused by a CAG expansion in the huntingtin gene (htt) (1), and is characterized by involuntary movements, personality changes, dementia, and early death (2). Huntington protein (Htt) is expressed throughout the central nervous system as well as non-neuronal cells, yet causes selective neuronal death in the striatum and cortex (3–6). The selective neurodegeneration found in HD may be linked to the expression of particular receptors and proteases in the affected striatal and cortical cells, but the molecular details of these events are not well characterized. One hypothesis that has received a great deal of attention is that production of N-terminal Htt fragments plays a key role in disease pathogenesis (7–10). Our recent studies have shown that calpain cleaves Htt at multiple sites to produce small N-terminal fragments (11). In addition, we have demonstrated that the active form of calpain is substantially increased in the caudate of HD patients suggesting that calpain-derived Htt fragments may contribute to the etiology of HD (11).

Recently, new insights have been gained as to how polyQ-expanded Htt may alter Ca2+ homeostasis at early stages of the disease, and these findings may be directly linked to excitotoxicity and the activation of the Ca2+-dependent proteases, the calpains (12–14). N-Methyl-D-aspartate receptor, subtype 2B, and metabotropic glutamate receptors class 1/5 are enriched in the striatum and play a role in the dysregulation of Ca2+ in HD. The polyQ-expanded form of Htt sensitizes N-methyl-D-aspartate receptors in the brain, leading to enhanced Ca2+ influx following receptor stimulation (12, 15, 16). The polyQ-expanded form of Htt sensitizes endoplasmic reticulum type 1 inositol (1,4,5)-trisphosphate receptor also leading to enhanced Ca2+ release following metabotropic glutamate receptors class 1/5 activation (14). Furthermore, the disease form of Htt has been implicated in dysfunctional mitochondrial Ca2+ buffering (17, 18).

We have shown that both calpains and caspases cleave Htt (9–11, 19–22). Far more is known about the proteolytic processing of Htt with caspases in vitro and in vivo (9, 10, 19–22). This is not surprising, because Htt was the first caspase substrate identified in a neurodegenerative disease, and the mechanism of action of the caspase family members has been studied extensively in the apoptotic cell death process (9, 23). However, little is understood about the role of calpain family members in cell death and neurodegeneration. Calpains were discovered over 40 years ago, and at least 15 distinct human family calpain members have been identified (24–26). At least 6 of the 9 family members found in the brain have not been characterized (25).

The first calpains discovered, μ- and m-calpain (calpain-1, calpain-2, and calpain-4 (small regulatory subunit)), have been extensively studied and are distinguished by their differences in Ca2+ dependence, with μ-calpain requiring micromolar Ca2+ for activation whereas m-calpain requires millimolar levels.
Calpain Cleavage of Huntingtin

Cloning of Epitope-tagged Calpain-5 cDNA—RNA from 293T cells was extracted using TRIzol reagent (Invitrogen). Oligo(dT)-primed cDNA was generated using a Superscript cDNA kit (Invitrogen) according to the manufacturer’s instructions. Calpain-5 cDNA was amplified using primers CAPN5 F, 5'-GGGCAGCCGCCACCATGTTCTCG-3' and CAPN5-FLAG R 5'-CTTATCGTCGTCATCCTGTAATCGA-3'. The reverse primer contains codons encoding a FLAG epitope. Reaction mixtures contained 25 pmol of each primer, 0.5 mM dNTPs, 10 μl Pfu Turbo polymerase buffer, 5 μl of MgSO4 (Sigma), and 2.5 μl of Pfu Turbo DNA polymerase (Stratagene). Total reaction volume was 50 μl. Amplification parameters were denaturing at 94 °C for 1 min, 35 cycles of annealing at 56 °C for 30 s, and extension at 72 °C for 2 min. The PCR product was purified using a QIAquick PCR purification kit (Qiagen) and cloned into a pcDNA3.1/VS-His vector using a TOPO TA Expression Kit (Invitrogen).

Cell Cultures—Superfect reagent (Qiagen) was used for transient transfections of human embryonic kidney 293T cells with Htt constructs. Thapsigargin (500 nM, 2.5 μg/ml, Calbiochem) or 10 μg/ml of chloramphenicol was added 36 h following transfection, and cells were collected at 48 or 60 h post-transfection for analysis.

Western Analysis—293T cell pellets or dissected mouse tissue were lysed in Nonidet P-40 buffer (0.1% Nonidet P-40, 50 mM Hepes, pH 7.4, 10 mM NaCl, 5 mM EDTA, 0.5% Triton X-100, 0.5% sodium deoxycholate) for 20-40 min at 4 °C, then cleared by centrifugation. Protein levels were determined using the BCA assay (Pierce) for all Western blots. Immunoblots were developed with a peroxidase-conjugated secondary antibody (Vector). Enhanced chemiluminescence (ECL) was used as a liquid secondary antibody (Pierce). Immunoblots were documented using a Fluorchem FX imaging system (Alpha Innotech, San Leandro, CA).

Toxicity Measurements—Caspase activity was measured using the ApoAlert Caspase-3 Fluorescent Assay Kit (Clontech, Palo Alto, CA). Briefly, cells were lysed in Clontech cell lysis buffer for 10 min on ice. Following centrifugation to remove cell debris, lysate was added to reaction mixture containing 20 mM Pipes, pH 7.2, 100 mM NaCl, 1% Chaps, 10% sucrose, 10 mM diithiothreitol, and 100 μM D-7-amino-4 trifluoromethyl coumarin. Reactions were incubated at 37 °C and read at an excitation of 400 nm and an emission of 500 nm for 1 h. The rate of change in fluorescence within the linear range was normalized to protein levels to determine relative DEVD-ase activity. Protein levels were determined using the BCA assay (Pierce) for all experiments.

Immunofluorescence—Transfected 293T cells were fixed in 2% parafomaldehyde for 20 min. Following blocking with 10% normal donkey serum, cells were incubated with monoclonal Htt 2166 (7 μg/ml, Chemicon) or polyclonal neoHtt 552 (0.05 μg/ml, Research Genetics), neoHtt 552 (0.05 μg/ml, Research Genetics), neoHtt 536 (Invitrogen); calpain antibodies: monoclonal calpain regulatory subunit 3083 (9 μg/ml, Chemicon), monoclonal calpain-1 (0.5 μg/ml, Sigma), monoclonal calpain-2 7533 (2 μg/ml, Santa Cruz Biotechnology), polyclonal calpain-5 RPI, polyclonal-7 RP2, polyclonal-10 RP1 (0.5 μg/ml, Trip Point Biologics), neo-epitope antibody specific for calpain-claved spectrin Ab38 (1 μg/ml) (44); or caspase antibodies: monoclonal caspase-2 3507 (5 μg/ml, Chemicon), polyclonal caspase-3 9662 (0.05 μg/ml, Cell Signaling), polyclonal caspase-6 AAP-106 (4 μg/ml, StressGen), polyclonal caspase-7 9492 (0.3 μg/ml, Chemicon), monoclonal caspase-12 C7611 (2 μg/ml, Sigma). Monoclonal β-tubulin T-02465 (1, 500, Sigma) and monoclonal poly-ADP-ribose polymerase (PARP, 1,3000, BIOMOL) antibodies were used to verify quality of fractionation. Immunoblots were developed with a peroxidase-conjugated secondary antibody and enhanced chemiluminescence (ECL).
RESULTS

Identification of Two Calpain Cleavage Sites in Htt—We have previously shown that calpains cleave Htt to produce three N-terminal cleavage products (11). In those studies, we found that incubation of in vitro translated pcMV-Htt15Q-(1–1212) with calpain-1 (μ-calpain) or -2 (m-calpain) produced 62-, 67-, and 72-kDa N-terminal products, two of which (67- and 72-kDa fragments) are in close proximity to the caspase-sensitive region of Htt (70–80 kDa, Fig. 1A). Both in cell culture and in vitro translation experiments, the major Htt calpain cleavage product appears at 72/92 kDa (15Q versus 138Q) and lies between the caspase-3 sites at amino acids 513 and 552 (Fig. 1, A and B). The exact site of cleavage in Htt and whether calpain cleavage modulates cellular toxicity of the polyQ-expanded form of Htt were not evaluated in our previous work (11). Therefore, we determined the site of calpain cleavage in Htt using deletion analysis and analyzed the cytotoxicity of these constructs in a cell culture model.

Deletion of predicted calpain recognition sequences was used to identify calpain cleavage sites in Htt. Although the primary amino acid sequences of calpain substrate cleavage sites have similar properties (i.e. small hydrophobic amino acids in P2 position), calpains also recognize the three-dimensional structure of their protein targets (26). This distinguishes them from the caspases, which only recognize conserved primary amino acid sequences. First, we evaluated whether deleting a Ser-Ser-Ser motif at amino acids 535–537 of Htt, an identified calpain cleavage site in protein kinase C (26), would alter proteolytic cleavage of Htt by calpains. Western blot analysis of Htt15Q-(1–1212) when expressed in 293T cells produced one prominent Htt cleavage product at 72 kDa along with three minor products at 67, 70, and 75 kDa (Fig. 1C). We found that Htt15Q-(1–1212)Δ535–537, when expressed in 293T cells, was resistant to calpain cleavage and eliminated production of the 72-kDa calpain-derived Htt fragment (Fig. 1, B and C). Confirming our results, in vitro translation of Htt15Q-(1–1212)Δ535–537 treated with recombinant calpain eliminated the production of the 72-kDa fragment. Next, we identified the calpain cleavage site in Htt that produces the 67-kDa fragment. We deleted a Leu-Thr-Ala motif at amino acids 468–470 of Htt, because this site is an identified calpain cleavage site in caspase-12 (46). We found that Htt15Q-(1–1212)Δ468–470, when expressed in 293T cells, was resistant to calpain cleavage and eliminated production of the 67-kDa calpain-derived Htt fragment (Fig. 1, B and C). Next, we evaluated the Htt constructs where both calpain sites were deleted. Expression of Htt15Q-(1–1212)Δ468–470Δ535–537 or Htt138Q-(1–1212)Δ468–470Δ535–537 in 293T cells eliminated both the 67/77 and 72/82-kDa calpain fragments (Figs. 1C, 4A, and 4B). The remaining minor Htt cleavage products at 70/80 and 75/85 kDa (Fig. 1C, 4A, and 4B) are caspase-derived (at amino acid 513 and 552 of Htt).

To validate the specificity of our deletion analysis, we produced deletions in Htt at other potential calpain cleavage sites, which were only four amino acids from the identified calpain cleavage sites (amino acid 469 and 536). We found these deletions had no effect on calpain proteolysis of Htt. Deletion of a
third potential calpain cleavage motif (Ser-Ser-Ser; amino acids 464–466) did not influence production of the 67-kDa calpain-derived Htt fragment (Fig. 1A) when expressed in 293T cells. Deletion of a potential calpain site (Val-Ser-Ala; amino acids 539–541) did not influence production of the 72-kDa calpain-derived Htt fragment. Therefore, deletion of other potential calpain cleavage sites immediately adjacent to the identified Leu-Thr-Ala site (amino acids 468–470) or Ser-Ser-Ser (amino acids 535–537) did not affect calpain cleavage of Htt and further supports the specificity of these deletion mutants and the ability to successfully utilize them in studying calpain proteolysis and toxicity of Htt. Finally, we evaluated whether deletion of these calpain cleavage sites would affect caspase cleavage of Htt since these sites are in close proximity to one another (Fig. 1A). Treatment of these calpain-resistant constructs Htt15Q(—1–1212) Δ468–470, Htt15Q(—1–1212) Δ535–537 and Htt15Q(—1–1212) Δ468–470 Δ535–537 with recombinant caspase (data not shown) and production of caspase-derived Htt fragments when these calpain-resistant constructs are expressed in culture (see Fig. 4) demonstrated they were still susceptible to caspase cleavage.

The calpain cleavage site at amino acid 536 of Htt was confirmed by producing a neoepitope antibody to this specific Htt calpain cleavage product (Fig. 1D). When Htt15Q(—1–1212) is cleaved with low levels of calpain-2 (0–0.3 U/μl), the neoHtt 536 antibody detected increasing amounts of the N-terminal 536 amino acid Htt fragment being produced. A Htt stop construct (Htt15Q(—1–536)) which expresses Htt protein from amino acids 1 to 536 was used as a positive control and ran at the same molecular weight as the identified cleavage product.

We were not able to identify the calpain cleavage site responsible for producing the 62-kDa N-terminal Htt fragment. A Val-Leu-Ser motif at amino acids 436 to 438 and a Ser-Arg-Lys motif at amino acids 438 to 440 were two of the potential calpain cleavage sites investigated and neither of these deletions eliminated production of the 62-kDa calpain-derived Htt fragment (data not shown). Since the 62-kDa N-terminal cleavage product represents a minor calpain cleavage product of Htt in cell culture (often not detected), identification of this site was not pursued further.

Calpain-resistant Htt Mutants Reduce Cellular Toxicity in an In Vitro Cell Culture Model—In our previous work we evaluated the proteolysis and toxicity of Htt in tissue culture models under conditions that activated caspase-dependent cell death (21). It is not clear whether this pathway of cell death activation is relevant in vivo since recent evidence suggests that altered Ca²⁺ homeostasis plays an important role in HD pathogenesis and progression (47). Therefore, we established a simple cell culture model to evaluate the cleavage of Htt by both caspasases and calpains under conditions that modulate intracellular Ca²⁺ levels (11). 293T cells overexpressing wild-type, caspase-resistant, calpain-resistant or calpain/caspase-resistant Htt were treated with thapsigargin, a pro-apoptotic agent that increases intracellular Ca²⁺ levels through inhibition of the endoplasmic reticulum Ca²⁺/Mg²⁺-ATPase (Figs. 1–4, and 6).

Treatment with thapsigargin (2.5 or 10.0 μM) increased levels of the 28-kDa precursor and 21-kDa active form of calpain by 2- and 1.9-fold, respectively, when compared with control in 293T cells (Fig. 2A, n = 3). Thapsigargin-treated cells generated increased levels of Htt cleavage products relative to untreated cells (Fig. 2B, n = 3), which are predominantly calpain-derived. Thapsigargin treatment also increased caspase-2/3 cleavage at amino acid 513 of Htt (Htt15Q513, Fig. 2B) but represented less than 5% of the accumulated cleavage products. Overexpressing caspase-resistant Htt constructs (Htt15Q/Htt138Q(—1–1212) D513A, D530A, D552A, D586A, and D589A) (Fig. 2B) confirmed that the majority of the Htt cleavage products appear to be calpain-derived. The apparent reduction of calpain/caspase-derived Htt138Q(—1–1212) cleavage products relative to Htt15Q(—1–1212) is likely due to insoluble material accumulating in the stacking gel for the polyQ-expanded form of Htt (Fig. 2B, top panel).

Because we established cell culture conditions that modulate Ca²⁺ homeostasis and mapped the calpain cleavage sites in
Calpain Cleavage of Huntingtin

Aggregation in an in Vitro Cell Culture Model—Currently, three proteolytic cleavage pathways have been reported for Htt 

in vivo that may influence cellular toxicity and aggregation. These include cleavage of Htt by caspases (9, 21), calpains (11, 48), and an unknown aspartic endopeptidase (49). Whether these proteolytic pathways act independently or sequentially is currently not known. Because we generated calpain-resistant Htt constructs, we tested whether cleavage at these Htt calpain sites was required for aggregation and the production of smaller N-terminal fragments.

Calpain-resistant Htt138Q-(1–1212) protein also showed a reduction in Htt aggregation relative to Htt138Q-(1–1212) (Fig. 3B). Elimination of calpain-sensitive region(s) $\Delta$468–470 $\Delta$535–537 from Htt138Q-(1–1212) not only eliminated production of the specific calpain-derived cleavage products but led to a reduction in further proteolysis of Htt (4.2-fold decrease by densitometry). Most notably, a reduction in the amount of lower molecular weight N-terminal Htt cleavage products was observed when the calpain-resistant form of Htt138Q-(1–1212) was expressed, which may represent the aspartic endopeptidase-cleaved N-terminal Htt product described previously (Fig. 3B) (49).

The calpain-resistant Htt138Q-(1–1212) protein also showed a reduction in Htt aggregation relative to Htt138Q-(1–1212) (Fig. 3, B and C, n = 3). 293T cells overexpressing calpain-resistant Htt138Q-(1–1212) showed a 3.7-fold decrease in the amount of Htt immunoreactive proteins left in the stacking gel (Fig. 3B) by densitometry, as well as a decrease in the number and size of cytoplasmic/nuclear Htt-containing aggregates observed in intact cells (Fig. 3C) relative to Htt138Q-(1–1212). In 293T cells overexpressing Htt138Q-(1–1212), large, single perinuclear aggregates were found, whereas these aggregates were absent from 293T cells overexpressing the calpain-resistant Htt138Q-(1–1212) construct (Fig. 3C). Because these structures have been associated with late-stage aggregation and HD pathology, it supports the idea that the calpain-resistant Htt138Q-(1–1212) construct is less toxic than Htt138Q-(1–1212). It should also be noted that similar decreases in proteolytic cleavage products and aggregates were also observed with other Htt antibodies, including a second monoclonal N-terminal Htt antibody (EM48; 5374, Chemicon) and monoclonal Htt 2166.

Calpain and Caspase Cleavage Products of Htt Preferentially Localize to the Nuclear Compartment—The presence of polyQ-expanded fragments in the nucleus has been correlated with cellular cytotoxicity in HD, as well as other polyQ diseases (10, 20, 50–55). Cellular toxicity of N-terminal Htt fragments can be enhanced by addition of a nuclear localization signal and decreased by addition of a nuclear export signal (NES) (53). Indeed, Htt contains a naturally occurring NES, suggesting full-length Htt may shuttle back and forth between the cytosol and nucleus (56). The majority of Htt is cytosolic (>95%) suggesting the importance of the NES signal in maintaining the subcellular distribution of Htt (57, 58). Many studies have also suggested Htt must be truncated to 50 kDa or less for N-terminal fragments to preferentially accumulate in the nucleus, implying caspase/calpain-derived fragments may not redistribute to the nucleus unless further truncated (10, 20, 52). These conclusions were based mainly on immunofluorescence studies evaluating Htt aggregates and not subcellular fractionation. Clearly, it is of interest to determine whether caspase/calpain-derived fragments can redistribute from the cytoplasm to the nucleus. In addition, a small percentage of full-length Htt exists in the nucleus, and therefore it is also important to establish whether cleavage can occur in this subcellular compartment.

To address whether Htt calpain/caspase cleavage products

Htt, we were now able to evaluate whether calpain cleavage of polyQ-expanded Htt influenced toxicity, proteolysis, and aggregation. First, we expressed the calpain-resistant Htt138Q-(1–1212) constructs in 293T cells and evaluated cytotoxicity. Expression of the polyQ-expanded form of Htt138Q-(1–1212) in the thapsigargin-stimulated tissue culture model (500 nm, 24 h), enhanced cytotoxicity 3.2-fold as measured by DEVD-ase activity following thapsigargin treatment (500 nm, 24 h) and probed with N-terminal monoclonal Htt antibody (BKP1). C, immunofluorescent detection of Htt aggregates in 293T cells overexpressing Htt138Q-(1–1212) and calpain-resistant Htt138Q-(1–1212). Probed with Htt 2166 antibody (n = 3). Arrows indicate the large perinuclear aggregates formed in 293T cells.
enter the nucleus, cytoplasmic/nuclear fractionation of 293T cells overexpressing Htt15/138Q-(1–1212), calpain-resistant Htt15/138Q-(1–1212), and calpain/caspase-resistant Htt15/138Q-(1–1212) was performed. As in previous experiments, cells were treated with thapsigargin (500 nM, 24 h) to maximize production of calpain-derived Htt fragments. Full-length endogenous Htt was predominately excluded from the nucleus with only 1–5% in the nuclear fraction (Fig. 4A, n = 3). Interestingly, significant amounts of the overexpressed Htt15/138Q-(1–1212) constructs were observed in the nucleus as well as the cytoplasm. This is consistent with the absence of the Htt NES located at amino acids 2397–2406 (Fig. 4A, n = 3) in these proteins. In addition, we found calpain and caspase Htt cleavage products accumulate in the nucleus (Fig. 4, A and B). Although the 72/82-kDa calpain-derived Htt cleavage product (arrowheads, Fig. 4A) was present in the cytoplasmic and nuclear fractions, the 67/77-kDa calpain-derived Htt cleavage product (arrowheads, Fig. 4B) was primarily localized to the nuclear fraction.

We have recently produced and characterized Htt antibodies that specifically recognize the caspase cleavage products of Htt at amino acid 513 and 552 (22). Using the neoHtt 513 antibody, the caspase-3-derived Htt cleavage product at amino acid 513 was identified primarily in the nuclear fraction (asterisk, Fig. 4A, and data not shown). Utilizing the neoHtt 552 antibody, similar results were observed for the caspase-2/3-derived Htt cleavage product at amino acid 552 by subcellular fractionation experiments (arrowheads, Fig. 4C). Complete fractionation was confirmed using antibodies to the cytoplasmic protein, β-tubulin, and the nuclear protein, poly(ADP-ribose)polymerase (PARP) (Fig. 4A, lower panels). Immunofluorescence studies using the neoHtt 552 antibody also demonstrate the nuclear localization of the Htt cleavage product at amino acid 552 along with accumulation of this fragment into perinuclear aggregates (Fig. 4D). Our studies do not address whether cleavage of Htt occurs in the nucleus or cytoplasm.

Most Calpain Family Members Localize to and Are Activated in the Nuclear Compartment—Although proteolysis of cytoplasmic and nuclear substrates by caspase family members has been extensively studied, our knowledge of calpain cleavage events is limited. Three of the fifteen calpain family members identified, calpain-1, calpain-2, and calpain-4 (small regulatory subunit), have been well characterized. Three other calpain family members, calpain-5, calpain-7, and calpain-10, are highly expressed in the brain tissue (41) and therefore may play a role in Ca2+-mediated HD pathogenesis. In addition, mRNA levels of calpains-5, -7, and -10 are significantly altered in the R6/2 HD mouse brains relative control tissue2 making them promising candidates for further investigation.

To further understand which calpain and caspase isoforms cleave Htt and in what subcellular compartment cleavage could occur, Western blot analysis of cytosolic/nuclear fractions from 293T cells were probed with relevant calpain and caspase antibodies to determine subcellular localization of the active and inactive forms of these proteases (Fig. 5, n = 3). Western

Fig. 4. Nuclear accumulation of calpain- and caspase-derived Htt fragments. Htt localization was analyzed using cytoplasmic/nuclear fractionation and Western analysis. A, 293T cells overexpressing Htt15Q-(1–1212), Htt138Q-(1–1212), calpain-resistant Htt138Q-(1–1212)(Δcp), or calpain/caspase-resistant Htt138Q-(1–1212) (ΔcpΔcs) were treated with thapsigargin (500 nM, 24 h) and fractionated (C = cytoplasmic; n = nuclear). Western blots were probing with N-terminal monoclonal Htt antibody (BKP1), β-tubulin or PARP (n = 4). Arrowheads represent the 72/82-kDa calpain-derived Htt fragment at amino acid 513 indicated by an asterisk. B, increased exposure of BKP1 blot in A shows the 67/77-kDa calpain-derived Htt fragment at amino acid 469

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analysis showed that both inactive (arrow) and active (arrowhead) forms of calpain-2 as well as the calpain regulatory subunit were primarily localized to the cytoplasmic fraction (Fig. 5A). Calpain-7 and -10 showed the inactive form in both nuclear and cytoplasmic compartments and the active form exclusively in the nuclear compartment (Fig. 5A). Both inactive and active forms of calpain-5 were found in the cytosol and nucleus (Fig. 5A).

Caspases included in these studies were those previously identified as cleaving Htt, as well as caspase-12, which is activated following induction of endoplasmic reticulum stress (46). The inactive form of caspase-2 was localized to the nucleus and cytosol, whereas the active form was limited to the cytosolic compartment (Fig. 5B). The majority of inactive forms of caspase-3, caspase-6, and caspase-7 was localized to the cytosolic fraction (Fig. 5B). We found less activation of caspases in the thapsigargin treatment model utilized in these studies relative to the tamoxifen model used to activate caspases in previous studies (21).

Next we tested whether transcription and/or translation of calpains was induced by thapsigargin treatment in our cell culture system. Semi-quantitative PCR studies revealed that calpain-5, -7, and -10 transcripts are increased following thapsigargin treatment relative to control (Fig. 6A). Correlating with increased transcription was an increase in calpain-5, -7, and -10 protein levels along with activation of calpain family members (Fig. 6B). These results are consistent with increased calpain message, protein levels, and activation of calpains under altered Ca^{2+} homeostasis. Next, we assessed whether the increase and activation of calpain family members detected in our simple cell culture model was relevant in vivo by evaluating these proteases in an HD knock-in mouse model.

**Increased Proteolysis in an HD Knock-in Mouse Model Correlates with Calpain Activation**—To analyze the contribution of calpain activation and proteolysis in HD in vivo, we utilized an HD knock-in mouse model expressing mouse Htt with 150 polyQ repeats (59). In our studies we utilized both heterozygotes Htt^{150Q/7Q} and homozygotes Htt^{150Q/150Q} allowing us to analyze gene dosage effects on proteolysis and activation of calpains. The HD transgenic mouse has late-onset behavioral and neuropathological abnormalities consistent with HD (59).

Behavioral deficits are found in gait, cage activity, and rotarod analysis. The neuropathological abnormalities described are nuclear accumulation of Htt and enhanced glial fibrillary acidic protein immunoreactivity in the striatum. We found increased calpain activity and Htt fragmentation in the cortex and striatum of this HD transgenic model (Fig. 7). Of particular significance is the gene dosage effect seen with a neo-epitope antibody to the calpain-derived cleavage product of spectrin (44) and a calpain-5 antibody in the striatum, with homozygous mice, Htt^{150Q/150Q}, having the highest levels relative to heterozygotes, Htt^{150Q/7Q}, or controls (Fig. 7B). A 2.2-fold increase in the spectrin cleavage product and a 4.8-fold increase in calpain-5 were found in the striatum of homozygous Htt^{150Q/150Q} relative to control mice as determined by densitometry. Distinct calpain family members were also altered in the cortex of the homozygotes Htt^{150Q/150Q} relative to control mice as determined by densitometry. Distinct calpain family members were also altered in the cortex of the homozygotes Htt^{150Q/150Q} and heterozygotes Htt^{150Q/7Q} when compared with age-matched controls (Fig. 7C). Comparison of calpain-1 (1.5-fold), -7 (1.5-fold), and -10 (1.7-fold) expression in the cortex showed a gene dosage effect, with the homozygotes, Htt^{150Q/150Q}, having the highest levels when compared with the heterozygotes, Htt^{150Q/7Q}, or controls. Analysis was carried out at 11 months of age when these mice have impaired motor performance (59).

**DISCUSSION**

We used numerous approaches to examine the role calpains play in the proteolysis of Htt in HD cell culture and transgenic...
mouse models. One pathological mechanism proposed for HD is that production of toxic fragments of the polyQ-expanded form of Htt contributes to HD pathology and progression (7–10). To continue our investigation of the cleavage pathways involved in Htt proteolysis, we used deletion mutagenesis to identify two calpain sites in Htt at amino acids 469 and 536. These calpain sites are clustered between amino acids 468 and 537, an area that overlaps with caspase cleavage sites in Htt (amino acids 513–586), suggesting this area of Htt is highly susceptible to proteolytic cleavage (Fig. 1A). We found expression of the calpain-resistant polyQ-expanded Htt reduced cytotoxicity, proteolysis, and aggregate formation when compared with polyQ-expanded Htt during Ca²⁺ dysregulation. In earlier work, we evaluated the caspase-resistant forms of the polyQ-expanded Htt under conditions that stimulate caspase activation but not necessarily Ca²⁺ dysregulation (21). In fact, here, we found that the caspase-resistant form of Htt has more calpain cleavage products accumulating under conditions of Ca²⁺ dysregulation. Ca²⁺ dysregulation is an early event in HD pathogenesis and therefore Ca²⁺-stimulated activation of calpains may be particularly important in Htt proteolysis and represent a more relevant in vitro model.
Currently, three proteolytic cleavage pathways have been reported for Htt in vitro that may influence cellular toxicity and aggregation. These include cleavage of Htt by caspases (9, 21), calpains (11, 48), and an unknown aspartic endoproteinase (49). Whether these proteolytic pathways are independent of each other or act in a sequential process is currently not known. Analysis of the calpain-resistant Htt constructs demonstrates that cleavage at the calpain sites is required for the production for smaller N-terminal fragments consistent with a sequential cleavage model. We found that both calpains and caspases could cleave Htt independently of each other. Calpain-resistant Htt was cleaved by caspases and conversely caspase-resistant Htt was cleaved by calpains. As noted the caspase-resistant form of Htt produced more calpain cleavage products of Htt (Fig. 2F). In addition, calpain-resistant polyQ-expanded Htt generated more caspase cleavage products (Fig. 4C). Our current studies do not address the order of activation of the protease family members in HD.

Although it is not yet known the relative contribution of calpain, caspases, or other proteases to HD progression, we determined that a number of uncharacterized calpain family members may be increased and activated in HD tissue culture and transgenic mouse models. We found that thapsigargin, an inhibitor of the endoplasmic reticulum Ca2+-ATPase, in-...
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