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Amino-terminal fragments of huntingtin, which contain the expanded polyglutamine repeat, have been proposed to contribute to the pathology of Huntington's disease (HD). Data supporting this claim have been generated from patients with HD in which truncated amino-terminal fragments forming intranuclear inclusions have been observed, and from animal and cell-based models of HD where it has been demonstrated that truncated polyglutamine-containing fragments of htt are more toxic than full-length huntingtin. We report here the identification of a region within huntingtin, spanning from amino acids 63 to 111, that is cleaved in cultured cells to generate a fragment of similar size to those observed in patients with HD. Importantly, proteolytic cleavage within this region appears dependent upon the length of the polyglutamine repeat within huntingtin, with pathological polyglutamine repeat-containing huntingtin being more efficiently cleaved than huntingtin containing polyglutamine repeats of nonpathological size. © 2002 Elsevier Science (USA)

INTRODUCTION

Huntington's disease (HD) is a hereditable neurodegenerative disorder characterized by motor, psychiatric, and cognitive dysfunction (Gusella *et al.,* 1996; MacDonald, 1998; Vonsattel & DiFiglia, 1998). HD belongs to a class of neurodegenerative diseases caused by the expansion of a polyglutamine repeat in the affected protein (Ross *et al.,* 1998; Cummings & Zoghbi, 2000), which in HD resides within the amino-terminus of the affected protein huntingtin (htt). Polyglutamine diseases manifest when the repeat length expands beyond a certain size, which in HD is typi-

cally greater than 36 glutamines. Furthermore, there is a correlation among repeat length, disease severity, and age of onset, with earlier onset and more severe disease symptoms observed in patients with longer polyglutamine repeats (Furtado *et al.,* 1996; Brinkman *et al.,* 1997; Leavitt *et al.,* 1999; Rosenblatt *et al.,* 2001). These and other data support the role of the expanded polyglutamine repeat as the pathogenic moiety leading to neurodegeneration (MacDonald & Gusella, 1996).

Evidence from human HD patients, animal models and cell-based models of HD suggests that truncated polyglutamine-containing fragments are more toxic than full-length htt (DiFiglia *et al.,* 1997; Lunkes & Mandel, 1998; Saudou *et al.,* 1998; Li *et al.,* 2000). Additionally, many studies suggest that polyglu-

tamine-expanded htt is toxic within the nucleus (Saudou *et al.,* 1998; Peters *et al.,* 1999; Nucifora *et al.,* 2001). Within the nucleus truncated htt forms aggregates that are recognized only by antibodies that detect the amino-terminal region of htt (DiFiglia *et al.,* 1997; Lunkes and Mandel, 1998; Li *et al.,* 2000). Whether htt aggregates are intrinsically toxic or whether soluble htt is toxic is a source of controversy (Sisodia, 1998; Kuemmerle *et al.,* 1999). However, data supporting the toxicity of nuclear htt are compelling and have led to the toxic fragment hypothesis, which proposes that cleavage of htt is required for toxicity and that this toxicity is exerted by the expanded polyglutamine-containing amino-terminal fragment after its translocation to the nucleus (Leavitt *et al.,* 1999).

One of the mechanisms that have been proposed to explain the nuclear toxicity associated with expanded polyglutamine-containing proteins is that expanded polyglutamine is capable of sequestering molecules required for normal cellular function within the nucleus (Cummings *et al.,* 1998; Preisinger *et al.,* 1999; Cha, 2000; McCampbell *et al.,* 2000; Shimohata *et al.,* 2000). For instance, it was recently demonstrated that an amino-terminal fragment of htt could exert its toxicity by interfering with CBP-mediated transcription. These data indicated that polyglutamine-containing amino-terminal htt fragments sequester CBP, thereby blocking the ability of CBP to function in the transcription complex (Steffan *et al.,* 2000; Nucifora *et al.,* 2001). The proteases responsible for generating amino-terminal fragments of endogenously expressed htt are currently under investigation, and the identification of such proteases could lead to the development of HD therapeutics.

It has been demonstrated that htt is an *in vitro* substrate for caspase-3 and -6 and that cleavage at the caspase-3 sites occurs *in vivo* (Goldberg *et al.,* 1996; Lunkes & Mandel, 1998; Wellington *et al.,* 1998, 2000a). Furthermore, the caspase cleavage sites have been exactly determined and mutagenesis of those sites reduces the toxicity associated with expanded polyglutamine-containing htt in a cell-based model (Wellington *et al.,* 2000b). Smaller htt amino-terminal fragments (with an approximate size of 40–50 kDa) than those predicted to be generated by caspase cleavage were observed in patients with HD (DiFiglia *et al.,* 1997; Mende-Mueller *et al.,* 2001) and in an animal model of HD (Li *et al.,* 2000). Recently, it was shown that caspase-3-generated amino-terminal htt fragments were substrates for calpain-mediated cleavage, suggesting that cleavage of these fragments by calpain could account for the presence of the smaller amino-

terminal fragments observed in HD (Kim *et al.,* 2001). In this study we used cell-based models of htt cleavage to identify an amino-terminal fragment of similar size to those observed in HD patient samples and mapped the cleavage domain responsible for generating this fragment, as an initial step toward the identification of the protease responsible for this proteolytic event.

METHODS

Anti-huntingtin anti-peptide antibodies. To detect specifically the various regions of htt protein, we have produced peptide-specific antibodies in rabbits using the following peptides: HD-1, MATLE KLMKA FESLK SFC; HD-111, CNIVA QSVRN SPEFQ KLL; HD-170, CLELY KEIKK NGAPR SLR; HD-331, CVKDT SLKGS FGVTR KEM; HD-494, CHDII TEQPR SQHTL QAD; HD-560, CISDS SQTTT EGPDS AVT; HD-654, CEATE PGDQE NKPCR IKG; HD-654, CEATE PGDQE NKPCR IKG. In addition to the htt amino acids, the peptides included a cysteine residue at either the amino- or carboxy-terminus that was used for coupling the htt peptide to keyhole limpet hemocyanin. The resulting antisera were screened for titer and antibody specificity by ELISA and Western blot. The immunoglobulins were isolated by protein G column purification, and specific antibodies were further purified by affinity purification using peptide-specific columns. The mouse anti-polyglutamine monoclonal antibody 1C2 was purchased from Chemicon (Temecula, CA). The mouse anti-V5 antibody was purchased from Invitrogen (Carlsbad, CA).

Huntingtin expression constructs. The construction of full-length htt, N171, and N63 htt expression constructs has been described previously (Cooper *et al.,* 1998). The N171 construct was modified with a carboxy-terminal V5 epitope tag, which was accomplished by subcloning a *Bam*H1/blunted-*Cla*1 fragment that contained N171 into *Bam*H1/blunted-*Xba*1 pcDNA3.1 V5-hisA (Invitrogen). The N854 and N508 deletion constructs were generated from full-length HD constructs encoding either 23 or 68 glutamines. Subcloning a *Bam*H1/blunted-*Cla*1 fragment from full-length htt into *Bam*H1/*Eco*RV-digested pcDNA3.1 V5-hisA generated the N854 construct. The N508 expression construct was generated by PCR amplification of full-length htt. The following primers were used: 5′-CGG GAT CCG CCA TGG CGA CCC TGG AAA AG; 3′-GCG GCC GCG CCT GCA GTG TGT GCT GTG A. The 5′ primer incorporated a *Bam*H1 site

in its 5′ end and the 3′ primer incorporated a *Not*1 site in its 3′ end. The PCR product was digested with *Bam*H1 and *Not*1 and subcloned into similarly digested pcDNA3.1 V5-hisA. All htt expression constructs and glutamine lengths were confirmed by sequencing.

Huntington disease and control brain tissue extracts. Dr. Juan C. Troncoso of the HD Center Neuropathology Core at Johns Hopkins University graciously provided HD tissue. All tissues were isolated from the frontal cortex. HD tissue was obtained from Vonsattel grade 2 patients. The age and postmortem delay for the HD cases were as follows: Fig. 1B, lane 3, age 66 years PMD 12 h; lane 4, age 77 years PMD 20 h; and lane 5, age 73 years PMD 11 h. The age and postmortem delay for the control cases were as follows: Fig. 1B, lane 1, age 74 years PMD 4 h, lane 2, age 72 years PMD 20 h.

Control and HD brain tissue was homogenized on ice in lysis buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.7 μg/ml pepstatin, and protease inhibitor cocktail (Roche). The extracts were centrifuged at 4°C for 20 min at 20,000g. After centrifugation the supernatant was removed and the protein concentration was determined (BCA protein assay reagent; Pierce). Lysates were analyzed by Western blot as described below.

Cell culture and transfection. The X57 and X58 cell lines (cloned mouse striatal cell lines) were obtained from Prof. Alfred Heller (University of Chicago) and their generation and culture conditions have been described (Wainwright *et al.,* 1995; Kim *et al.,* 1999). NG108-15 cells were obtained from ATCC (ATCC HB-12317, Manassas, VA) and were cultured under described conditions (Lunkes & Mandel, 1998). Human embryonic kidney cells (293T) were maintained in DMEM (Gibco BRL, Grand Island, NY) supplemented with 5% fetal bovine serum and penicillin/streptomycin. *In vitro* transfections were performed with lipofectamine 2000 (Gibco BRL) or Fugene 6 (Roche) following the manufacturers protocol. Mock transfections omitted plasmid DNA and were performed as control. Cells were harvested 48 h after transfection and analyzed by Western blotting.

Western blotting. Transfected cells were harvested on ice in phosphate-buffered saline (PBS) and centrifuged. Cell pellets were resuspended in appropriate volume of lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.7 μg/ml pepstatin, protease inhibitor cocktail (Roche)) and sonicated. Lysis in boiling SDS load-
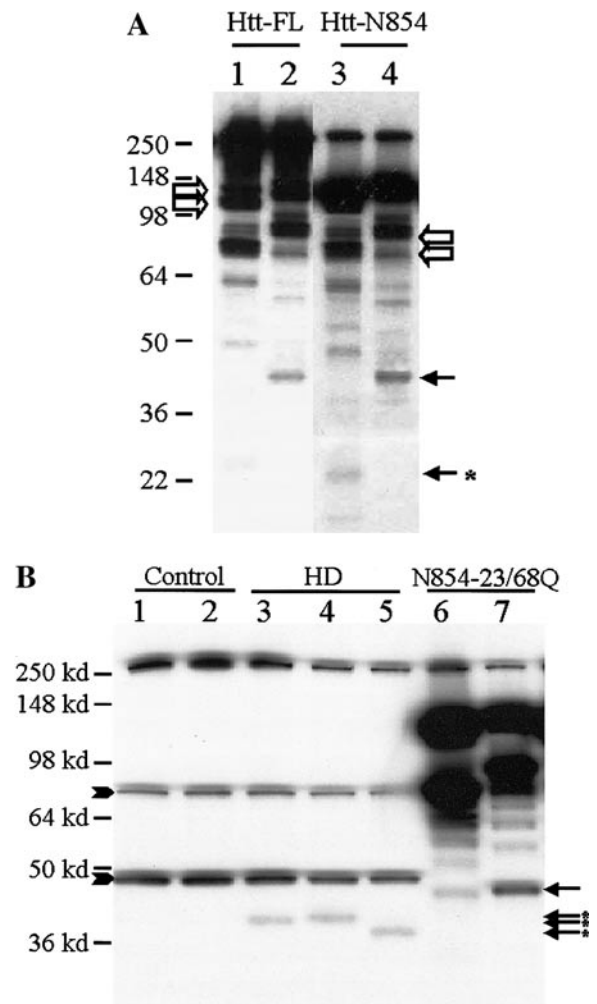

FIG. 1. Proteolytic cleavage of huntingtin (htt). Western blots were probed with the HD-1 antibody that recognizes the amino-terminus of htt. (A) Amino-terminal fragments of htt generated from htt transiently transfected 293T cells. Lane 1, full-length-23Q htt; lane 2, full-length-68Q htt; lane 3, N854-23Q; lane 4, N854-68Q. Open arrows right: Amino-terminal fragment likely generated by caspase cleavage of htt. Open arrows left: Amino-terminal fragments generated by cleavage at a site carboxy-terminal to the predicted caspase cleavage sites. Arrow: 45-kDa amino-terminal fragment that is generated in a polyglutamine repeat-dependent manner. Arrow asterisk: The corresponding 25-kDa amino-terminal fragment from 23 glutamine-containing htt. (B) Htt amino-terminal fragments from control (lanes 1 and 2) or HD tissue (lane 3, 43/19Q; lane 4, 40/15Q; and lane 5, 42/22Q) compared to N854-23/68Q transiently transfected 293T cell extracts. Amino-terminal fragments of approximately 40 kDa (asterisk arrow) were observed in extracts of HD tissue. An approximately 45-kDa amino-terminal fragment (arrow) was observed in the N854-68Q-transfected cells.

ing buffer or inclusion of caspase inhibitors (Z-VAD or Z-DEVD) in the lysis buffer did not alter the cleavage pattern observed by Western analysis (data not

shown). After centrifugation the supernatant was removed and protein concentration determined (BCA protein assay reagent; Pierce). Lysates were resolved by SDS-PAGE on 12% Tris-glycine acrylamide gels (Invitrogen), and the proteins were transferred onto PVDF membrane (Invitrogen). Blots were blocked for 1 h in TBST (20 mM Tris-HCl, pH 7.6, 137 mM NaCl, 0.1% Tween 20) containing 5% milk followed by incubation with primary antibodies for 2 h at room temperature. After three washes with TBST, blots were incubated with peroxidase-conjugated secondary antibodies (Amersham, Piscataway, NJ). Antibody binding was detected using enhanced chemiluminescence (Amersham).

Immunoprecipitation. Lysates (300 μg total protein in 600 μl volume) were preadsorbed with 50 μl protein G-agarose (Amersham) suspension (25-μl bed volume) overnight at 4°C on a rocking platform (all incubations were performed under this condition). Supernatants were transferred to fresh tubes and incubated with an appropriate amount of antibodies (2 μg anti-V5 or 5 μl polyclonal antiserum) for 1 h. Fifty microliters of protein G-agarose suspension was added to the mixture and incubated for 4 h. The pellets were washed two times with the lysis buffer, followed by two washes with washing buffer 2 (50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 0.1% Nonidet P-40, 0.05% sodium deoxycholate) and a final wash with washing buffer 3 (50 mM Tris-HCl, pH 7.5, 0.1% Nonidet P-40, 0.05% sodium deoxycholate). Pellets were resuspended in 50 μl SDS-loading buffer and heated in boiling water for 5 min. The mixtures were centrifuged and the supernatant were collected for SDS-PAGE analysis.

Metabolic labeling. For these studies 2×10^6 293T cells were plated on 10 cm tissue culture plates and transfected with pN854-23Q/68Q the following day. Sixteen hours later cells were serum-starved for 1 h in methionine-free DMEM. Following starvation, cells were labeled with 1 mCi ^{35}S-methionine (NEN, Boston, MA) for 1 h. The labeling medium was removed and cells were washed and chased for the indicated time. Following the chase, cells were washed with ice-cold PBS and lysed with STEN-lysis buffer and prepared for immunoprecipitation. The results were quantified using a Storm 860 phosphoimager (Molecular Dynamics, Sunnyvale, CA).

Calpain cleavage assay. Lysates of htt-transfected cells (100 μg of total protein) were prepared as described above and incubated with 0, 0.02, 0.1, 0.5, and 2.5 units of purified calpain II (Calbiochem, San Diego, CA) in a buffer (50 mM Tris, pH 7.5, 100 mM NaCl, 2

mM DTT, 1 mM EDTA, 5 mM CaCl$_2$) at 30°C for 15 min. Following incubation SDS-loading buffer was added and the mixture was boiled for 5 min and followed by SDS-PAGE analysis.

RESULTS

Identification of Polyglutamine Repeat-Dependent Proteolysis of Htt

To identify cleavage products of htt that correspond to those observed in HD patients we generated truncated carboxy-terminal V5-His-tagged htt expression constructs. These constructs contain the first 854 amino acids (N854) of htt and contain repeats of 23 or 68 glutamines. The amino-terminal fragments generated from these constructs were compared to those generated from full-length htt by analyzing extracts of transiently transfected 293T cells with the HD-1 antibody that recognizes the first 17 amino acids of htt (Fig. 1A). The predominant amino-terminal fragments generated from both full-length and truncated constructs include fragments of 70 kDa in 23-glutamine-(23Q) containing samples (lanes 2 and 4) and 90 kDa (68Q)-containing samples (lanes 3 and 5) (open arrows on right). These fragments are similar in size to those predicted to correspond to caspase cleavage of htt (Wellington *et al.*, 2000b). Larger amino-terminal fragments are also readily detected in the full-length htt extracts at approximately 110 and 130 kDa in the 23- and 68-glutamine-containing samples, respectively (open arrows on left). These data demonstrate the presence of a cleavage site carboxy-terminal to the described caspase sites. The upward shift in mobility of amino-terminal fragments, which is observed in the samples generated from expression of full-length and N854 constructs encoding 68 glutamines (lanes 2 and 4) when compared to samples with 23 glutamines (lanes 1 and 3), is due the presence of the expanded polyglutamine repeat.

In addition to these cleavage fragments, a number of other amino-terminal proteolytic fragments were observed, including a 45-kDa amino-terminal fragment observed in the full-length 68Q and N854-68Q sample (lanes 2 and 4, arrow). An amino-terminal fragment corresponding to the 45-kDa fragment was weakly observed at the predicted size 25 kDa in the N854-23Q and full-length 23Q extracts (lanes 1 and 3, arrow asterisk). Importantly, the cleavage pattern and amino-terminal fragments generated appeared the same between full-length and truncated htt with sim-

ilar polyglutamine repeats, indicating that full-length htt and htt truncated at amino acid 854 are processed similarly in 293T cells. None of the cleavage fragments were detected in nontransfected control extracts (data not shown).

To demonstrate that the 45-kDa amino-terminal fragment observed in 293T cells was of similar size to those reported in patients with HD, we used the HD-1 antibody to identify amino-terminal fragments in samples generated from HD (Fig. 1B, lanes 3–5) and control patients (Fig. 1B, lanes 1 and 2), and compared these results with those obtained with the N854-23/68Q extracts (Fig. 1B, lanes 6 and 7). Within the HD tissues extracts, but not present in the control patient extracts, amino-terminal fragments of approximately 40 kDa were observed (arrows asterisks). These fragments differ slightly in size and the size difference does not clearly reflect the polyglutamine repeat length, which is similar in all the HD patients. The amino-terminal fragments observed in HD patient samples are smaller than the amino-terminal fragment observed in the N854-68Q sample; likely reflecting differences in polyglutamine repeat length between the patient and the cell-based samples (40–43 glutamines versus 68 glutamines). The other fragments observed in the patient samples at 47 and 75 kDa are not specific to patients with HD since they are observed in both control and HD samples. A high-molecular-weight band corresponding to full-length htt was observed in all samples. Interestingly, no amino-terminal fragments of the size predicted by caspase cleavage were selectively observed in HD patient samples.

Next we sought to determine whether htt processing observed in 293T cells also occurred in cell lines of neural origin. In these experiments the cleavage patterns of truncated htt (N854-23/68Q) in cells of striatal (X57 and X58 cells) and neuroblastoma (NG108) origin were compared with cleavage observed in 293T cells. The amino-terminal fragments observed by HD-1 immunodetection were similar in all the cell lines tested (Fig. 2A), indicating that similar protease acts upon htt in these cell lines. The amino-terminal fragments observed included the predicted caspase cleavage products (open arrows) and a 45 kDa fragment that was generated in a polyglutamine repeat length-dependent manner in all cell lines (arrow). When the same extracts were analyzed using the carboxy-terminal epitope tag V5 antibody three prominent bands were observed in all cell lines (Fig. 2B). These bands include full-length recombinant form of N854 and a 45-kDa fragment (open arrow) that corresponds to the size

expected of the carboxy-terminal fragment generated from caspase-3 cleavage (Wellington *et al.*, 1998, 2000b). Interestingly, the other carboxy-terminal fragment observed, of approximately 110 kDa (arrow), was generated in a polyglutamine repeat length-dependent manner, similar to that observed for the 45-kDa amino-terminal fragment. In cells transiently expressing the expanded form of htt (N854-68Q) a greater proportion of this 110-kDa fragment relative to the full-length recombinant form was observed compared to cells expressing truncated htt with 23 glutamines. Similar to the 45-kDa amino-terminal fragment observed in N854-68Q samples, the polyglutamine repeat length-dependent cleavage that generated the 110-kb carboxy-terminal fragment was observed in all cell lines analyzed. We believe this 110-kDa carboxy-terminal fragment is the counterpart of the 45-kDa amino-terminal fragment. Importantly, we observed a 110-kDa carboxy-terminal fragment in the 23-glutamine-containing extracts, even though we were not able to readily detect the corresponding amino-terminal fragment generated from the N854-23Q sample. These data indicate that cleavage occurs in htt containing nonpathological glutamine stretches, albeit at greatly reduced levels in these cell-based systems. No polyglutamine repeat length-dependent cleavage was observed for the 45-kDa carboxy-terminal fragment. As expected, neither the 110-kDa polyglutamine repeat length-dependent carboxy-terminal fragment nor the 45-kDa carboxy-terminal fragment contain the polyglutamine stretch as is evident by the same size of the fragments in extracts from N854-23Q and 68Q.

To further confirm and quantify the polyglutamine repeat length-dependent cleavage 293T cells transiently transfected with pN854-23/68Q constructs were metabolically labeled with ^{35}S-methionine for 1 h and then chased for the indicated times (Fig. 2C). At the early time points the 110-kDa carboxy-terminal fragment generated from expanded polyglutamine-containing htt was present at about seven times the level of the comparable fragment generated from the 23-glutamine-encoding clone. Again, no difference in the 45-kDa cleavage product was observed between the 23- and 68-glutamine cleavage products (data not shown). These data support the premise that the polyglutamine repeat length modulates the proteolytic event that generates the 110-kDa carboxy-terminal fragment. Based on the aforementioned data, which identified an amino-terminal fragment of similar size to those observed in HD brain extracts that was generated in a manner consistent with polyglutamine re-
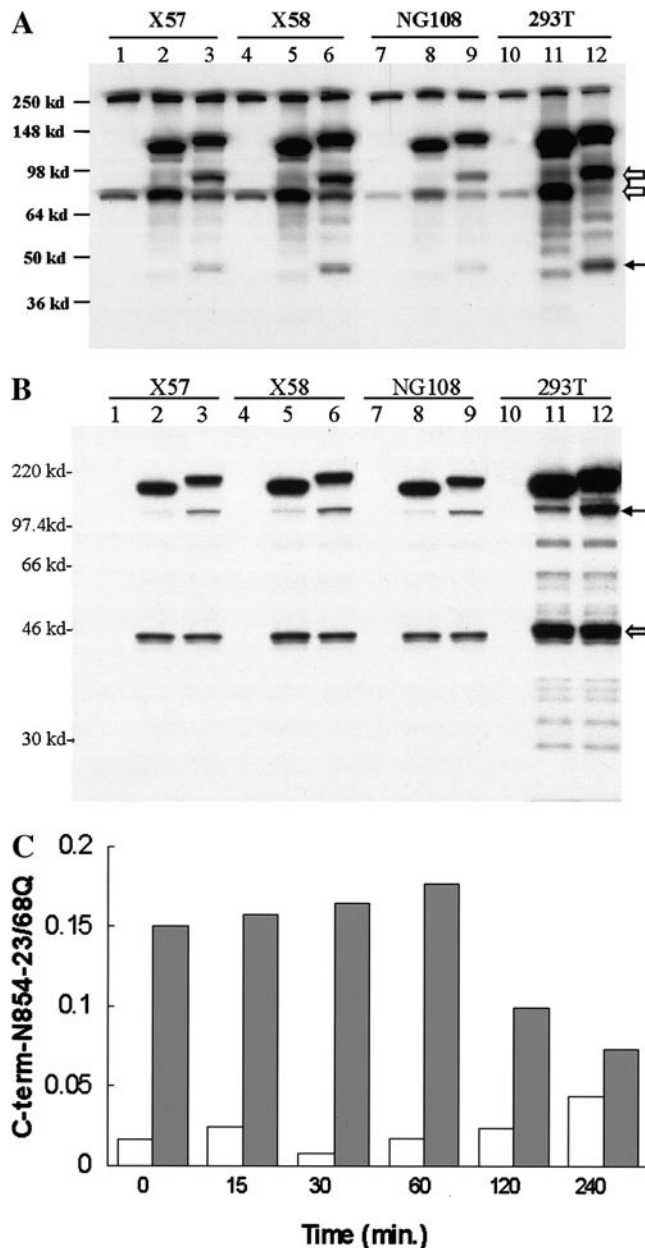
A

B

C

FIG. 2. Htt cleavage in various neuronal cell lines. (A) Western blot of extracts prepared from nontranfected (lanes 1, 4, 7, and 10), N854-23Q-transfected (lanes 2, 5, 8, and 11), and N854-68Q-transfected (lanes 3, 6, 9, and 12) X57 and X58 (striatal neuron) cell lines, NG108-15 (neuroblastoma/glioma fusion) cells and 293T (embryonic kidney) cells. Similar amino-terminal fragments are generated in all cell lines. The arrow indicates the amino-terminal fragment generated from N854-68Q in a polyglutamine repeat length-dependent manner. Open arrows denote the predicted caspase fragments. (B) The same extracts as in A analyzed with the carboxy-terminal V5 epitope antibody. The arrow indicates the 110-kDa carboxy-terminal fragment that is generated in a polyglutamine repeat length-dependent manner and is the counterpart of the 45-kDa amino-terminal fragment. The open arrow indicates the likely carboxy-terminal

peat dependence in cell-based models, we undertook the following experiments to further delimit the cleavage site responsible for generating this amino-terminal fragment.

Epitope Mapping Reveals that the Polyglutamine Repeat Length-Dependent Proteolysis Occurs Amino-Terminal to Amino Acid 111

Antipeptide antibodies directed against htt were generated to epitope map the cleavage sites within htt (Fig. 3A) that were utilized in the 293T cell-based model. As demonstrated previously, the V5 antibody recognized a carboxy-terminal fragment that exhibited polyglutamine repeat length-dependent proteolysis (Fig. 3B, arrow C). All the antibodies depicted in Fig. 3A, with the exception of HD-1 recognized this same carboxy-terminal fragment (arrow C). Furthermore, the HD-1 antibody recognizes an approximately 45-kDa fragment (arrow B) that is generated in a polyglutamine-dependent manner. These data indicate that the polyglutamine repeat length proteolysis occurs between epitopes recognized by HD-1 and HD-111, thereby placing the cleavage site amino-terminal to amino acid 111. Antibodies HD-1, HD-111, HD-170 (data not shown), HD-331, and HD-494 recognize a nonpolyglutamine repeat length-dependent amino-terminal fragment that contains the polyglutamine repeat (arrows D and D′) as is evident by the upward shift in mobility of this fragment within N854-68Q compared to N854-23Q samples. HD-560 and anti-V5 do not recognize these fragments. Using this panel of antibodies we were also able to position the cleavage event that generates the 45-kDa carboxy-terminal fragment between the epitopes recognized by HD-494 and HD-560 (arrow E). These data correspond to caspase-3 cleavage at amino acids Asp513 or Asp552 (Wellington *et al.*, 1998, 2000b).

To better define and characterize the cleavage products recognized by the HD-111 antibody, extracts were immunoprecipitated with the HD-111 antibody and immunobloted and probed with HD-1, HD-111, anti-

fragments generated by caspase cleavage. (C) Quantification of the 110-kDa carboxy-terminal fragment generated in a polyglutamine repeat length-dependent manner over time. Transiently transfected 293T cells were metabolically labeled and immunoprecipitated with the anti-V5 antibody. The data indicate that the 110-kDa carboxy-terminal fragment generated from 854-68Q (closed bars) was more efficiently generated than the same carboxy-terminal fragment generated from N854-23Q (open bars).
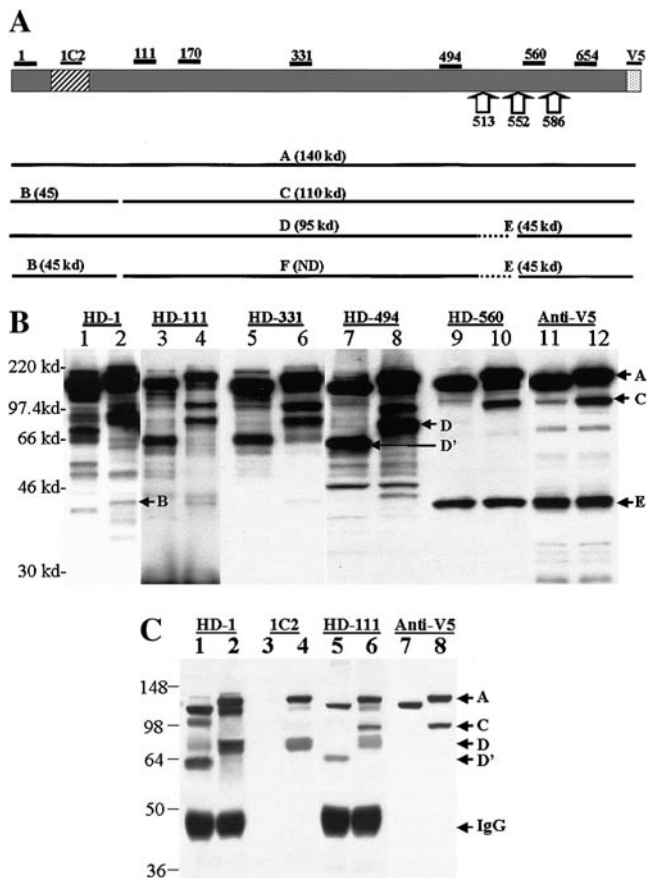
FIG. 3. Epitope mapping of the htt polyglutamine repeat length-dependent cleavage site. (A) Diagrammatic representation of the peptides used to generate anti-htt peptide antibodies used in these experiments and the observed htt cleavage products. The number above the bar indicates the amino-terminal amino acid of the peptide used to generate the antibodies. Below the antibody diagram is depicted full-length recombinant htt (A) and the 4 primary cleavage products described in this study (B–E). Fragment F was not conclusively identified. (B) Extracts prepared from N854-23Q-(lanes 1, 3, 5, 7, 9, and 11), N854-68Q- (lanes 2, 4, 6, 8, 10, and 12) expressing cells were analyzed by Western blot analysis using the antipeptide antibody indicated above the lanes. (C) Immunoprecipitation with HD-111. Extracts prepared from N854-23Q- (lanes 1, 3, 5, and 7) and N854-68Q- (lanes 2, 4, 6, 8) expressing cells were immunoprecipitated with HD-111, blotted, and probed with the indicated antibodies.

V5, or 1C2 antibodies (Fig. 3C). The 1C2 antibody has been described as an antibody that recognizes expanded polyglutamine repeats (Trottier *et al.,* 1995). All the antibodies recognized full-length recombinant htt (Fig. 3C, arrow A). The amino-terminal 70- and 90-kDa fragments were detected with HD-1, and HD-111 antibodies (arrow D' and D). The 1C2 antibody only detected the 90-kDa fragment generated from

N854-68Q samples (arrow D) and did not detect the 23-glutamine-containing fragment (arrow D'). The 110-kDa fragment (arrow C) that was generated in a polyglutamine repeat-dependent manner was recognized by HD-111 and anti-V5, but not by HD-1 or 1C2 antibodies. These data suggest that the 110-kDa fragment does not contain the polyglutamine repeat, although we cannot formally exclude the possibility the cleavage occurred within the polyglutamine stretch, thereby altering the ability of 1C2 to recognize expanded polyglutamine.

Deletion Analysis Indicates That Htt Is Cleaved between Amino Acids 63 and 111

Htt deletion constructs were generated to further define and characterize the polyglutamine repeat length-dependent proteolysis of htt (Fig. 4A). Deletion constructs were transiently transfected into 293T cells and extracts were analyzed for both amino-terminal and carboxy-terminal fragments using HD-1 and V5 antibodies, respectively. The construct utilized in the epitope mapping experiments contained the first 854 (N854) amino acids of htt and was cleaved in a polyglutamine repeat length-dependent manner. To determine whether deletion of the caspase cleavage sites within htt would alter the polyglutamine repeat length-dependent cleavage observed in the N854 constructs, a construct encoding the first 508 amino acids (N508) of htt with either 23 or 68 glutamines was generated and like the N854 constructs, the N508 constructs were V5-epitope-tagged at the carboxy-terminus. Using the V5 antibody we were able to detect a 55-kDa carboxy-terminal fragment (Fig. 4B, arrow) that was generated from N508 in a polyglutamine repeat length-dependent manner similar to that observed for the 110-kDa fragment in N854 samples. Further epitope mapping of the 55-kDa carboxy-terminal fragment confirmed that the cleavage occurs amino-terminal to amino acid 111 (data not shown). The next deletion construct was truncated at amino acid 171 (N171) of htt and was similarly V5 tagged. We were not able to identify a carboxy-terminal fragment from the N171 constructs, potentially due to proteolysis of this fragment. However, an amino-terminal fragment generated in a polyglutamine repeat-dependent manner was identified from the N171-68Q construct (see below).

The HD-1 antibody was used to better define the polyglutamine repeat length-dependent cleavage site within the amino-terminus of htt (Fig. 4C). Amino-terminal cleavage products (Fig. 4C arrows) generated
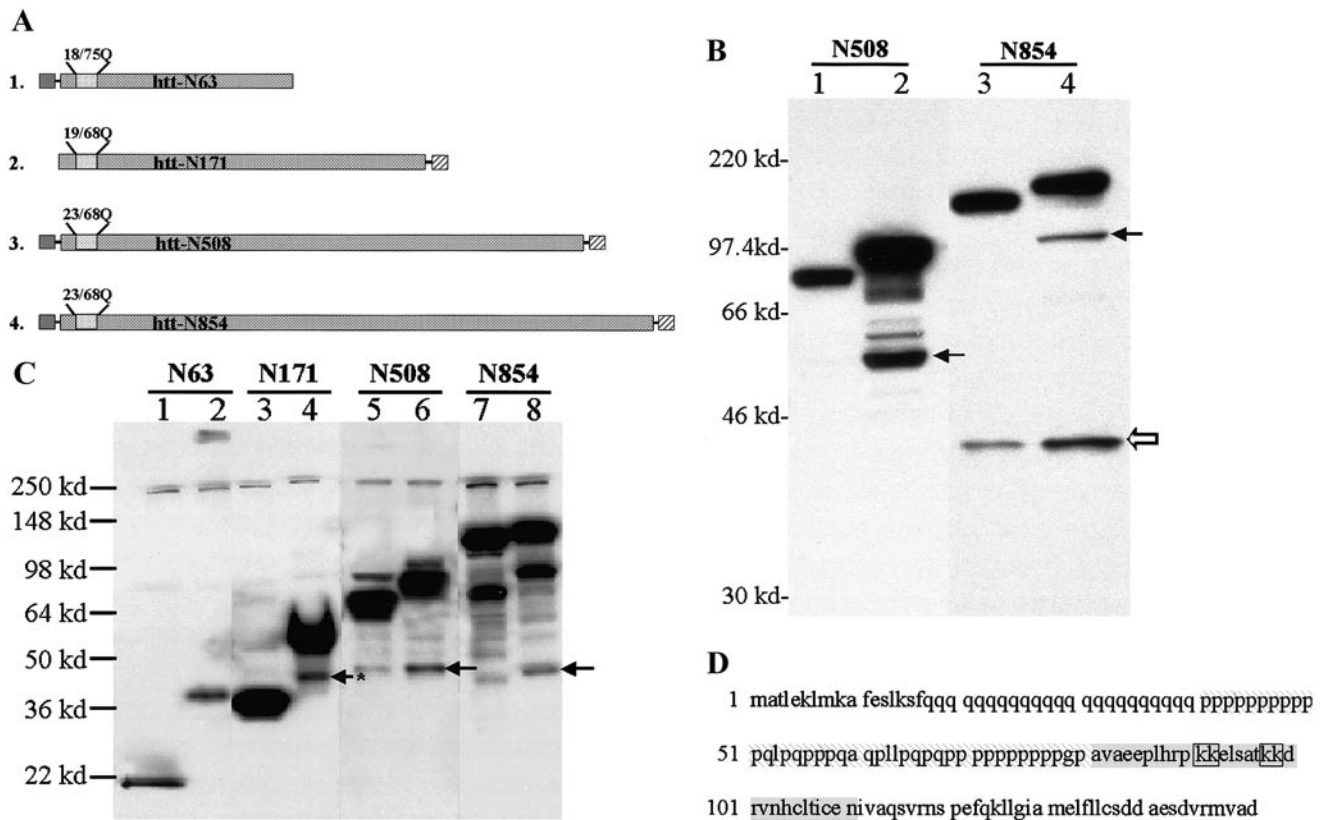
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101 rvnhcltice nivaqsvrns pefqkllgia melfllcsdd aesdvrmvad
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FIG. 4. Carboxy-terminal deletion analysis of htt cleavage. (A) Diagrammatic representation of the carboxy-terminal deletion constructs used in these analysis. The lengths of the polyglutamine tract in each construct are indicated and the presence or absence of a myc-epitope tag (shaded amino-terminal box) or V5-epitope tag (hatched carboxy-terminal box) is also indicated. The name of the construct corresponds to the number of amino-terminal amino acids present in the construct. (B) Analysis of polyglutamine repeat length-dependent cleavage in N508 (lanes 1 and 2) and N854 (lanes 3 and 4) using anti-V5 antibodies to detect carboxy-terminal fragments. Extracts prepared from 293T cells transiently transfected with 23Q (lanes 1 and 3) or 68Q (lanes 2 and 4) htt were analyzed. The arrow indicates the carboxy-terminal fragment generated in a polyglutamine repeat length-dependent manner from both of these constructs. The open arrow indicates the predicted caspase carboxy-terminal fragment product generated from N854. (C) Amino-terminal fragments generated from the various deletion constructs coding either nonpathogenic (lanes 1, 3, 5, and 7) or pathogenic (lanes 2, 4, 6, and 8) polyglutamine stretches. The arrow indicates the polyglutamine repeat length-dependent cleavage amino-terminal fragments. No amino-terminal cleavage fragments were observed in N63 extracts thereby placing the cleavage site carboxy-terminal to amino acid 63. (D) The sequence of htt surrounding the region in which the polyglutamine repeat length-dependent cleavage site is predicted to reside. Amino-terminal to this region is the polyglutamine stretch. The predicted region (shaded) contains the polyproline-rich region of htt (hatched) and dibasic residues (boxed), as well as arginines and hydrophobic residues which are the preferred amino acids at the P2 position in a calpain cleavage site.

in a polyglutamine-dependent fashion were observed in all extracts except that transfected with the N63 constructs. The sizes of the amino-terminal fragments generated from all the constructs with expanded polyglutamine repeats are similar, but do vary slightly depending upon polyglutamine repeat length and absence of an amino-terminal myc tag in N171 (arrow asterisk). This suggests that cleavage is likely occurring at the same site in all the constructs, except N63 where cleavage was not observed, which would place the cleavage site carboxy-terminal to amino acid 63.

These data indicate that the polyglutamine repeat-dependent cleavage site resides between amino acids 63 and 111 in huntingtin (Fig. 4D).

Calpain Is Capable of Cleaving Huntingtin at or near the Polyglutamine Repeat-Dependent Cleavage Site

An examination of the amino acid sequence of htt between amino acids 63 and 111 revealed a number of hydrophobic amino acids and arginines that tend to be
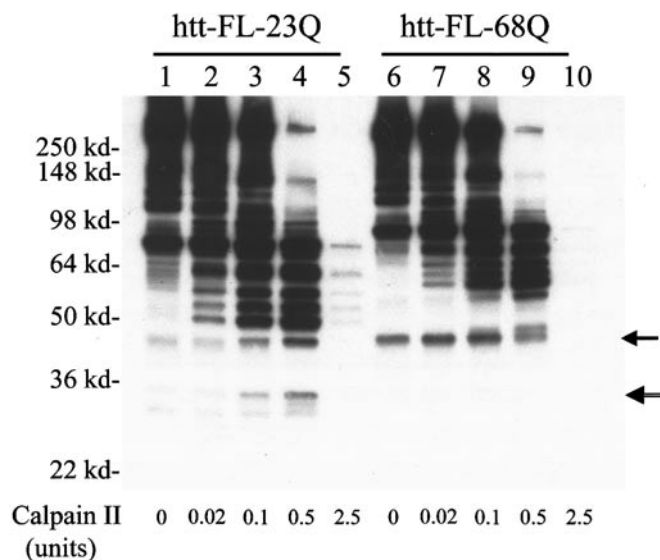
FIG. 5. Calpain cleavage of htt. Cellular lysates prepared from 293T cells transfected with 23-glutamine (lanes 1–5) or 68-glutamine-containing htt (lanes 6–10) were treated with the indicated concentrations of calpain II (below) and then analyzed by Western blot using the HD-1 antibody. The lower arrow indicates the position of the polyglutamine repeat length-dependent proteolytic fragment generated from 23Q-htt, whereas the upper arrow indicates the position of the similar fragment generated from 68Q-htt. Low levels of the polyglutamine repeat length-dependent amino-terminal fragment can be detected in the non-calpain-treated 23Q-containing lysate (lower arrow, lane 1). This fragment is also detected in the non-calpain-treated 68Q-containing lysate, albeit at increased levels (upper arrow, lane 6). A calpain concentration-dependent increase in this fragment is observed in all the calpain-treated 23Q-htt lysates with the exception of the highest concentration where htt is degraded (lane 5). A similar concentration-dependent increase is observed in 68Q-htt lysates except at the two highest concentrations of calpain (lanes 9 and 10). In addition, many other amino-terminal fragments of htt are generated by calpain cleavage.

that preferred amino acids at the P2 position for calpain-mediated cleavage (Sorimachi & Suzuki, 1998). Furthermore, htt has been recently shown to be a substrate for calpain (Kim *et al.,* 2001). To determine whether calpain was capable of generating a fragment *in vitro* of the same size as the fragment observed from polyglutamine repeat length cleavage of htt, we incubated lysates prepared from htt-transfected 293T cells with various concentrations of calpain II and then analyzed the lysates by Western analysis with HD-1 (Fig. 5). Incubation of extracts derived from either full-length 23- or 68Q-containing htt (Fig. 5, lanes 1–5 and 6–10, respectively) with various concentrations of calpain II led to a dosage-dependent increase in an amino-terminal fragment of the same size as generated by polyglutamine repeat length-dependent cleavage. This dose-dependent increase is most readily observed in samples containing a nonpathological polyglutamine repeat (Fig. 5, lanes 1–5), where only a small amount of the amino-terminal fragment is observed in the absence of calpain II (lane 1), although a dose-dependent increase in the fragment is also observed in 68Q-containing extracts. No evidence of polyglutamine repeat length-dependent cleavage was observed in these *in vitro* assays. These data also indicate that calpain II is capable of cleaving full-length htt at a number of sites *in vitro* and that a number of these sites appear to be at least as well utilized, if not more effectively utilized, than the polyglutamine length-dependent repeat cleavage site. At the highest concentration of calpain II utilized htt is degraded (lanes 5 and 10). This experiment indicates that calpain II is capable of cleaving htt at or near the polyglutamine repeat length-dependent cleavage site.

DISCUSSION

Using several cell-based models, including those of neural origin, we have defined a region within the amino-terminus of htt, which contains a proteolytic cleavage site that when cleaved generates an amino-terminal fragment with similar size to those observed in HD patients and animal models (DiFiglia *et al.,* 1997; Li *et al.,* 2000; Mende-Mueller *et al.,* 2001), to amino acids 63 and 111 of htt (Fig. 4D). The cleavage site that is utilized to generate the 45-kDa amino-terminal fragment appears to be more effectively utilized when the polyglutamine repeat is in the pathogenic range. The observation that htt cleavage at this site occurs in 293T cells, as well as in cells of neuronal origin, may suggest that this cleavage event in itself is unlikely to result in the regional pathology observed in HD. In addition to containing the polyglutamine repeat length-dependent cleavage site, the region of htt between amino acids 63 and 111 contains the polyproline stretch of htt. Repeated attempts to sequence the amino-terminus of the carboxy-terminal fragment have failed; therefore, we have been unable to define the exact cleavage site. Within this defined region, dibasic amino acids are present that can serve as protease recognition sites for many proteases. Additionally, as noted above, calpain has been reported to prefer hydrophobic residues and arginine at the P2 position, both of which occur in this region. And htt has been shown to be a substrate of calpain (Kim *et al.,* 2001). We were able to demonstrate that calpain II

cleavage of htt resulted in an amino-terminal fragment of htt of similar size to the one described in this paper. This result suggests that polyglutamine repeat length-dependent cleavage of htt may result from a calpain-like activity *in vivo*. In addition, it has been reported that substrate recognition by calpain is dependent upon the structural properties of the protein (Sorimachi & Suzuki, 1998), which could account for the differences in cleavage observed between 23- and 68-glutamine-containing htt. However, the identification of the exact cleavage site and the actual protease responsible for this cleavage *in vivo* remains to be conclusively determined, as does whether this cleavage could account for the regional specificity of neurodegeneration observed in HD.

In addition to the identification of a polyglutamine repeat length-dependent cleavage site, we mapped a cleavage site between amino acids 494 and 560 that was consistent with the caspase-3 cleavage sites in htt (Wellington *et al.,* 1998, 2000b). The length of the polyglutamine stretch did not consistently regulate the cleavage event occurring in this region. Blocking caspase cleavage of htt has been shown in cell-based models to diminish the toxicity associated with expanded polyglutamine-containing htt (Wellington *et al.,* 2000b). Whether caspase cleavage of htt contributes to the pathology of HD *in vivo* remains to be determined. However, the sizes of the amino-terminal fragments generated by caspase cleavage would likely prevent passive entry of these fragments into the nucleus (Goerlich & Mattaj, 1996; Allen *et al.,* 2000), and a functional nuclear localization signal has not been described in this region of htt (Hackam *et al.,* 1999). This does not preclude the ability of the caspase-generated fragments to enter the nucleus; however, further truncation of these caspase generated htt amino-terminal products or full-length htt to approximately 40 kDa would conceivably allow passive diffusion of polyglutamine-containing htt fragments into the nucleus where they would further augment the cellular toxicity elicited by expanded polyglutamine. Furthermore, we cannot exclude the possibility that caspase cleavage of htt influences cleavage between amino acids 63 and 111.

The amino-terminus of htt has been used as bait in two-hybrid experiments that have identified htt interacting factors, some of which show a decreased interaction with expanded polyglutamine repeat-containing htt (Gusella & MacDonald, 1998). One of these, HIP1, has been shown to be associated with the cytoskeleton (Kalchman *et al.,* 1997; Wanker *et al.,* 1997; Hackam *et al.,* 2000). These data support the idea that decreased interaction with htt-associated proteins reveals the polyglutamine repeat length-dependent cleavage site allowing it to become susceptible to proteolytic cleavage or conversely that polyglutamine repeat length-dependent cleavage of htt may be responsible for the decreased interaction of HIP1 and other htt-associated proteins with htt. Regardless, once the htt amino-terminal fragment is released from these interactions it could become mislocalized within the cell, leading to cellular dysfunction, which may occur via the ability of expanded polyglutamine to interact with and sequester factors required for cellular function (Preisinger *et al.,* 1999; Cha, 2000; McCampbell *et al.,* 2000; Shimohata *et al.,* 2000; Steffan *et al.,* 2000; Nucifora *et al.,* 2001).

The identification of the region involved in polyglutamine repeat length-dependent cleavage of htt is significant in that it may provide a link between the pathology of HD, and its increasing severity with increasing polyglutamine length, and polyglutamine repeat length-dependent generation of the toxic htt amino-terminal fragment. Future efforts aimed at conclusive identification of the polyglutamine repeat length-dependent protease may lead to the identification of a therapeutic target for HD that directly addresses the mechanism of the disease.

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