Tissue-Specific Proteolysis of Huntingtin (htt) in Human Brain: Evidence of Enhanced Levels of N- and C-Terminal htt Fragments in Huntington's Disease Striatum

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Proteolysis of mutant huntingtin (htt) has been hypothesized to occur in Huntington's disease (HD) brains. Therefore, this *in vivo* study examined htt fragments in cortex and striatum of adult HD and control human brains by Western blots, using domain-specific anti-htt antibodies that recognize N- and C-terminal domains of htt (residues 181–810 and 2146–2541, respectively), as well as the 17 residues at the N terminus of htt. On the basis of the patterns of htt fragments observed, different "protease-susceptible domains" were identified for proteolysis of htt in cortex compared with striatum, suggesting that htt undergoes tissue-specific proteolysis. In cortex, htt proteolysis occurs within two different N-terminal domains, termed protease-susceptible domains "A" and "B." However, in striatum, a different pattern of fragments indicated that proteolysis of striatal htt occurred within a C-terminal domain termed "C," as well as within the N-terminal domain region designated "A". Importantly, striatum from HD brains showed elevated levels of 40–50 kDa N-terminal and 30–50 kDa C-terminal fragments compared with that of controls. Increased levels of these htt fragments may occur from a combination of enhanced production or retarded degradation of fragments. Results also demonstrated tissue-specific ubiquitination of certain htt N-terminal fragments in striatum compared with cortex. Moreover, expansions of the triplet-repeat domain of the IT15 gene encoding htt was confirmed for the HD tissue samples studied. Thus, regulated tissue-specific proteolysis and ubiquitination of htt occur in human HD brains. These results suggest that the role of huntingtin proteolysis should be explored in the pathogenic mechanisms of HD.

Key words: Huntington's disease; huntingtin; proteolytic fragments; brain; neurodegenerative disease; ubiquitin

Huntington's disease (HD) is an inherited neurodegenerative disorder characterized by psychological, motor, and cognitive impairments (Vonsattel and DiFiglia, 1998; Petersen et al., 1999). The onset of HD generally occurs in adults in midlife, with a long-term duration of 15–20 years. The genetic mutation in HD has been identified as a CAG expansion near the 5′ region of the IT15 gene that encodes the 350 kDa huntingtin (htt) protein, resulting in a greater number of polyglutamines near the N terminus of htt. Normal individuals contain <35 CAG repeats, whereas individuals with adult-onset HD possess an expansion of 38/39–55 CAG repeats (MacDonald et al., 1993; Rubinsztein et al., 1997); expansions of 70 or more repeats occur in juvenile-onset HD. HD brains display characteristic neuropathological alterations, graded from 0 to 4, with grade 4 representing severe brain atrophy. Advanced grades show a reduction in striatum, cerebral cortex, as well as hippocampus, amygdala, and thalamus brain tissues (de la Monte et al., 1988; Vonsattel and DiFiglia, 1998). Neuronal loss is especially severe in striatum.

Studies of the role of the polyglutamine expansion within mutant huntingtin in HD pathogenesis in transgenic mice demonstrated that expression of an N-terminal mutant htt fragment with 100–150 CAG repeats, corresponding to exon 1 of the human HD gene (IT15 gene), was sufficient for development of brain nuclear inclusions that reflect the characteristic neuropathology in HD brains (Bates et al., 1997; Davies et al., 1997). Moreover, these mice developed a neurological phenotype that resembles behavioral features of HD (Carter et al., 1999). The nuclear inclusions in transgenic mice contained huntingtin immunoreactivity, as well as ubiquitin, which occurred before development of the neurological phenotype in HD. In addition, YAC transgenic mice expressing htt with 72 triplet repeats showed nuclear inclusions and neurodegeneration with translocation of N-terminal fragments to the nucleus (Hodgson et al., 1999). Moreover, the resemblance of nuclear inclusions in transgenic mouse brains with that in human HD brains was remarkable (Bates et al., 1997; Davies et al., 1997; DiFiglia et al., 1997; Hodgson et al., 1999). Initial immunohistochemical examination of nuclear inclusions in brains of juvenile cases of HD suggested the presence of N-terminal htt fragments (DiFiglia et al., 1997), predicting that proteolysis of htt occurs.

However, proteolysis of htt *in vivo* in human HD brains (DiFiglia et al., 1997) has not been extensively characterized. Therefore, to understand the proteolytic processing of htt in HD, this

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study characterized htt fragments in cortex and striatum from HD and control human brains by Western blots with domain-specific antibodies that recognize different regions of htt. Results demonstrated that htt undergoes tissue-specific proteolysis and that elevated levels of certain N- and C-terminal htt fragments were observed in striatum of human HD brains. Moreover, results suggested ubiquitination of selected N-terminal htt fragments. These findings indicate that tissue-specific proteolysis, as well as selective ubiquitination, of huntingtin occurs in HD brains.

MATERIALS AND METHODS

Brain tissue samples and SDS-PAGE. Control and HD brain tissues were obtained from the Harvard Brain Tissue Resource Center. The brain regions examined in this study were the cortical regions corresponding to Brodmann areas 4 and 6, striatum (putamen), and cerebellum from control and HD brains. Tissue samples from control brains and HD grade 3 brains (five to six different samples for each group of control and HD tissue samples) were from adults of 55–75 years of age. Brain samples were collected by the Brain Bank ~9–16 hr postmortem from neurologically characterized cases. Tissues were stored frozen at −70°C.

Dissected tissue samples were homogenized in freshly prepared buffer consisting of 0.1 M Tris-HCl, pH 7.4, 50 mM NaCl, 1 mM EDTA, and a cocktail of protease inhibitors consisting of pepstatin A, leupeptin, and chymostatin at 10 μM each and E64c and PMSF at 1 μM each. Homogenates were sonicated three times for 5 sec on ice. The protein content of homogenates was determined using the Bradford protein assay (Bio-Rad, Hercules, CA), as described by the manufacturer. The same amount of protein (75 μg or as indicated in figure legends) from each tissue sample was subjected to fractionation on SDS-PAGE gels (Novex precast gels, San Diego, CA) and electrophoretically transferred to nitrocellulose membranes [enhanced chemiluminescent (ECL) Hybond; Amersham/Pharmacia Biotech, Piscataway, NJ] for Western blots as described previously (Hook et al., 1999a,b).

Specifically, tissue homogenates (<50 μl) were each adjusted to contain final concentrations of sample buffer for SDS-PAGE (SB) consisting of 10 mM Tris-glycine, 6% β-mercaptoethanol, 20% glycerol, and 4% SDS, with bromophenol blue. These buffer conditions represent 2× SB conditions, in contrast to other studies that normally prepare tissue samples in 1× SB conditions. The 2× SB buffer condition provides optimum conditions for the reduction and denaturation of proteins, including htt fragments. Samples were then immediately mixed and heated at 95°C for 10 min and stored at −70°C. Immediately before SDS-PAGE, samples were thawed, reheated at 95°C for 5 min, and brought to room temperature before being loaded onto SDS-PAGE gels. SDS-PAGE used 4–20% polyacrylamide gradient gels or 12% polyacrylamide gels (Invitrogen, San Diego, CA), as indicated in figure legends. After electrophoresis at 125 V for ~2 hr, proteins from the SDS-PAGE gels were electrophoretically transferred (at ~25 V for 2 hr) to Hybond nitrocellulose membranes, as described previously (Hook et al., 1999a,b).

Antibodies and Western blots. Antisera that recognize different domains of huntingtin were used for Western blots. Monoclonal antibodies generated to an N-terminal domain (residues 181–810) and C-terminal domain (residues 2146–2541) of huntingtin were obtained commercially (Chemicon, Temecula, CA). Anti-ubiquitin serum (from rabbits; Chemicon) was also used in Western blots.

Antisera were generated against the peptide sequence corresponding to the first 17 residues of huntingtin. The peptide MATLEKLMKAFESLKSFC represents residues 1–17 of huntingtin, with addition of a Cys residue at the C terminus to allow conjugation to KLH protein for immunization of rabbits (custom antisera production was by Phoenix Pharmaceuticals, Inc., Mountain View, CA). ELISA assays [performed as described previously (Hook et al., 1985)] measured antisera binding to huntingtin peptide 1–17 and indicated production of high-titer antisera that showed effective binding to antigen (at an antisera dilution of 1:10,000).

For Western blots, membranes were blocked overnight at 4°C in 10% fetal calf serum in 20 mM Tris-HCl, pH 7.5, 0.5 M NaCl, and 0.05% Tween 20 (TBST). Membranes were incubated with anti-huntingtin sera (final dilutions of 1:1000) in TBST with 1% nonfat dry milk for 2 hr at room temperature. After washing, immunoreactive bands were detected with anti-rabbit IgGs coupled to horseradish peroxidase (final dilution of 1:4000) by use of the ECL detection system (Amersham/Pharmacia Biotech) as described previously (Saudou et al., 1998; Hook et al., 1999a). Immunoreactive bands were subjected to densitometric analyses with the Kodak Electrophoresis Documentation and Analysis System 120.

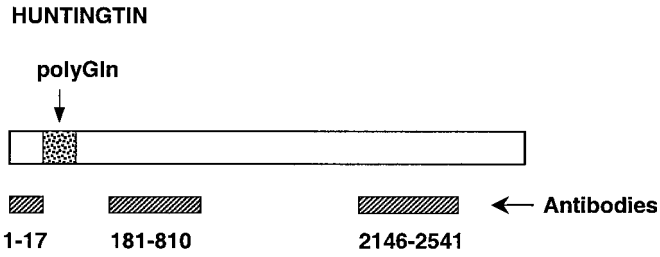

Figure 1. Domain-specific antibodies of htt. Htt in brain samples was analyzed by Western blots that used monoclonal antibodies recognizing an N-terminal domain (residues 181–810) and a C-terminal domain (residues 2146–2541). Antiserum (rabbit) was also generated against residues 1–17 of htt that recognize the N terminus.

Western blots were performed on at least three control and three HD tissue samples from each brain region. Also, each sample was analyzed two to three times by Western blots to confirm the reproducibility of results. Each group of triplicates from control or HD samples showed the same pattern of htt fragments; therefore, the figures illustrate Western blot profiles from a sample that is representative of the group of triplicates. It is noted that all of the control samples showed the same profile of htt fragments, although these samples contained a mixture of homozygous (15–17 repeats) and heterozygous (13–15 and 28–34 repeats) alleles of the IT15 gene that encodes huntingtin (see next section). Similarly, all HD samples showed the same profile of htt fragments, with HD tissues consisting of heterozygote alleles of the IT15 gene (13–18 and 36–46 repeats), as determined by PCR (explained in next section).

PCR of the triplet-repeat domain of the IT15 gene in control and HD tissue samples. PCR of genomic DNA with primers flanking the CAG-repeat domain of the IT15 gene encoding htt was performed, as described previously (MacDonald et al., 1993). PCR-generated DNAs were analyzed by DNA agarose gel electrophoresis, and DNAs were subcloned into the TA cloning vector (Invitrogen) for determination of the number of triplet repeats by DNA sequencing. Subcloning and DNA sequencing were performed as we have described previously (Hwang et al., 1994, 1999).

RESULTS

Domain-specific antibodies against htt

Antibodies that recognize different regions of htt (Fig. 1) allowed the evaluation of htt-derived fragments in brain by Western blots. Monoclonal antibodies generated against an N-terminal domain (residues 181–810 of huntingtin) and against a C-terminal domain (residues 2146–2541) of the 3136-residue htt protein were used to assess htt proteolytic fragments. Further analyses of N-terminal fragments used a high-titer polyclonal antibody that was generated against a synthetic peptide corresponding to the first 17 residues of huntingtin (Fig. 1).

Proteolysis of huntingtin in Brodmann areas 4 and 6 of cortex

Because cortical regions from HD brains are affected with the formation of nuclear inclusions that contain putative htt fragments, and loss of neurons (de la Monte et al., 1988; Vonsattel and DiFiglia, 1998; Gutekunst et al., 1999), proteolysis of htt was assessed in this region. Examination of cortex corresponding to Brodmann area 4 (Fig. 2*a*) and Brodmann area 6 (Fig. 2*b*) indicated the presence of full-length and several proteolytic fragments of htt that were identical in HD and control brains. Full-length htt in Brodmann areas 4 and 6 was detected by antibodies recognizing N- and C-terminal domains of htt. Full-length htt was observed as a slowly migrating band of ~350 kDa on SDS-PAGE that is consistent with the calculated molecular weight of htt of

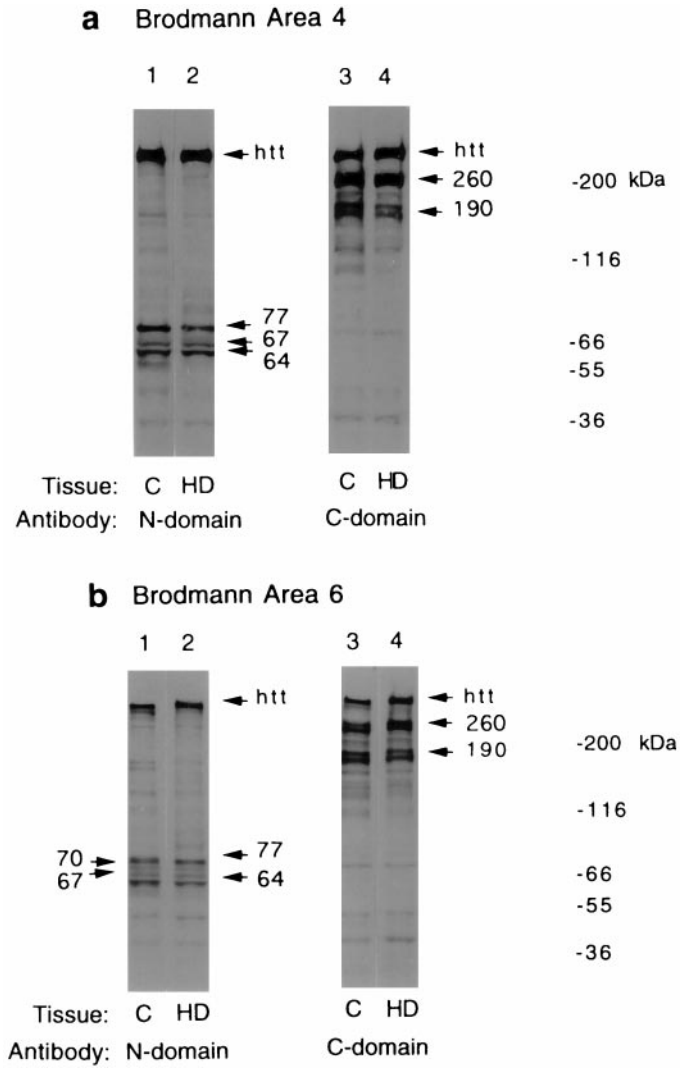

Figure 2. Proteolysis of huntingtin in cortex: htt fragments detected with N- and C-terminal domain antibodies. *a*, Brodmann area 4 of cortex from HD and control brains. Tissue homogenates from control (*lanes 1, 3*) and HD (*lanes 2, 4*) cortex corresponding to Brodmann area 4 were subjected to Western blots with N-terminal domain (*lanes 1, 2*) and C-terminal domain (*lanes 3, 4*) antibodies. Identical amounts of homogenate protein (70 μg) were applied to *each lane* of the SDS-PAGE gel (4–20% polyacrylamide gradient gel). *b*, Brodmann area 6 of cortex from HD and control brains. Tissue homogenates from control (*lanes 1, 3*) and HD (*lanes 2, 4*) cortex corresponding to Brodmann area 6 were subjected to Western blots with N-terminal domain (*lanes 1, 2*) and C-terminal domain (*lanes 3, 4*) antibodies. Identical amounts of homogenate protein (70 μg) were applied to *each lane* of the SDS-PAGE gel (4–20% polyacrylamide gradient gel). *C*, Control.

~345 kDa (MacDonald et al., 1993). Moreover, the relative amounts of full-length htt appeared similar in HD and control samples from Brodmann areas 4 and 6, suggesting that a similar degree of proteolysis of full-length htt occurred in cortex from HD and control brains.

The N-terminal domain antibody also detected low-molecular weight fragments of 60–80 kDa in Brodmann areas 4 and 6. These htt fragments consisted of 77, 67, and 64 kDa bands in area 4 and consisted of 77, 70, 67, and 64 kDa bands in area 6 from control and HD brains. The C-terminal domain antibody detected high-molecular weight htt fragments of ~260 and 190 kDa, with each band appearing as a doublet, in areas 4 and 6. Densitometry indicated similar levels of htt fragments in control and HD cortical regions. Moreover, highly reproducible detection of htt fragments by Western blots was observed with multiple brain samples (three to five samples for each tissue region).

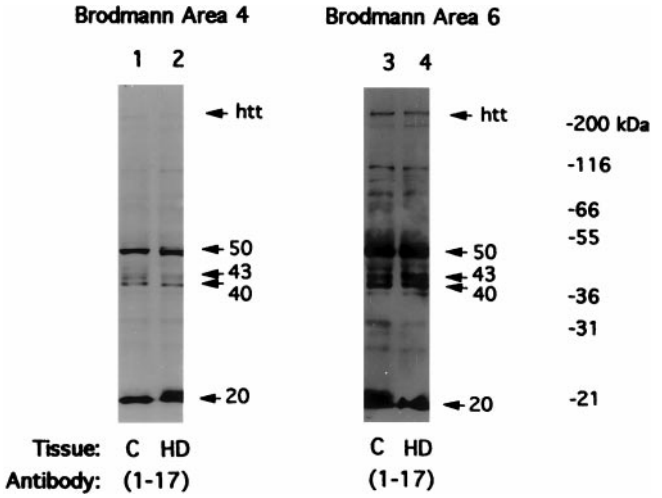

Figure 3. N-terminal fragments of huntingtin in cortex. Tissue homogenates from Brodmann area 4 (*lanes 1, 2*) and area 6 (*lanes 3, 4*) of cortex, from control (*lanes 1, 3*) and HD (*lanes 2, 4*) brains, were subjected to Western blots with anti-(1–17) serum that recognizes the N terminal of htt. *All lanes* of the gel (4–20% polyacrylamide gradient SDS-PAGE gel) contained equal amounts of homogenate protein (70 μg).

These results suggest that proteolysis of htt in cortex occurs near the N-terminal domain (near residues 181–810) to result in low-molecular weight N-terminal domain fragments of 64–77 kDa and high-molecular weight C-terminal domain fragments of 190–260 kDa. The sum of the molecular weights of these N- and C-terminal domain fragments is nearly equivalent to full-length htt. These findings demonstrate that similar proteolytic processing of huntingtin occurs in both Brodmann areas 4 and 6 of cortex, from control and HD brains.

Further characterization of N-terminal fragments of htt in cortex used antisera generated against residues 1–17 of htt. Western blots with anti-(1–17) serum demonstrated that Brodmann areas 4 and 6 contained similar N-terminal fragments of apparent molecular weights of 50, 43, 40, and 20 kDa (Fig. 3). In both Brodmann areas 4 and 6, 50 and 20 kDa N-terminal htt fragments were prominent; the 43 and 40 kDa bands were less abundant. The anti-(1–17) serum, however, did not detect bands of 64–77 kDa, which were recognized by the N-terminal domain antibody (generated to residues 181–810) (Fig. 2); these results indicated that these 64–77 kDa bands lack the first 17 residues at the N terminal of htt. Moreover, the 20–50 kDa bands detected by anti-(1–17) serum do not contain the N-terminal domain corresponding to residues 181–810. In addition, the nearly full-length htt band was minimally detected by the anti-(1–17) serum, suggesting that most of the nearly full-length htt lacks the N terminal. Thus, it is likely that the high-molecular weight htt band represents full-length and N-terminally truncated forms of htt, which would not be distinguished by small differences in relative electrophoretic mobilities on SDS-PAGE gels. Moreover, cortex from HD and control brains showed the same pattern and relative levels of htt N-terminal fragments.

Protease-susceptible domains of huntingtin in cortex

Analysis of htt fragments with domain-specific antibodies (Figs. 2, 3) demonstrated that two regions of huntingtin in cortex were

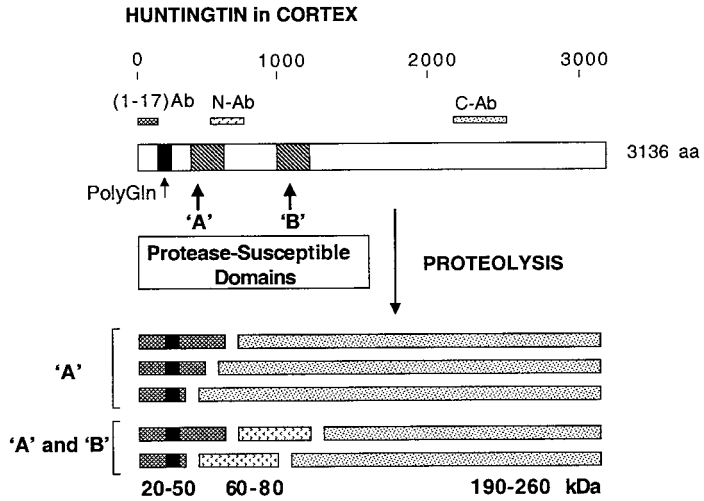

Figure 4. Protease-susceptible domains of huntingtin in cortex. Protease-susceptible domains indicated as *A* and *B* illustrate the predicted regions of htt that undergo proteolysis. Proteolysis within the *A* domain, or within both *A* and *B* domains, would generate low-M_r N-terminal fragments and high-M_r C-terminal fragments that are consistent with those detected in cortex by Western blots with N- and C-terminal domain antibodies (*N-Ab* and *C-Ab*, respectively), as well as by anti-(1–17) serum [*(1–17)Ab*]. Recognition of each predicted htt fragment (shown by *horizontal bars*) by the three different antibodies is indicated by antibody-specific *patterns* that fill the *horizontal bars*. *aa*, Amino acids.

particularly susceptible to proteolysis. One "protease-susceptible domain," the "A" domain, resides near the N terminus (Fig. 4). Cleavage within this region would generate low-molecular weight N-terminal fragments of 20–50 kDa [detected with anti-(1–17) serum] and nearly full-length htt [not detected by anti-(1–17) serum]. The second protease-susceptible domain, the "B" domain, is predicted to be situated further away from the N terminal and is located near the region corresponding to residues 181–810 that was detected by the N-terminal domain antibody. Cleavage within the B protease-susceptible domain, which occurs together with cleavage within the A domain, would generate fragments of 64–77 kDa that lack the N terminus. Proteolysis of htt within these two protease-susceptible domains would result in the high-molecular weight fragments of 190–260 kDa that are recognized by the C-terminal domain antibody (residues 2146–2541). Moreover, proteolysis within both A and B protease-susceptible domains occurs in HD and control tissue samples from Brodmann areas 4 and 6.

Distinct pattern of huntingtin proteolysis in striatum (putamen)

In striatum (putamen), proteolysis of huntingtin generated a pattern of htt fragments that differed from that found in cortex. Furthermore, striatum from HD brains showed elevated levels of N- and C-terminal fragments compared with those in controls.

Specifically, Western blots of striatum (putamen) with the antiserum recognizing the N terminal (residues 1–17) of htt detected increased levels of N-terminal fragments of 50, 45, and 43 kDa in HD compared with control brains (Fig. 5, *lanes 1, 2*). An N-terminal fragment of 20 kDa was also detected by the anti-(1–17) serum. The 50 and 45 kDa bands were also recognized by the antibody that detects the N-terminal domain (residues 181–810; Fig. 5, *lanes 3, 4*), suggesting that the 50 and 45 kDa bands represent extended N-terminal fragments. The 20 kDa N-terminal fragment was not detected by the antibody directed to residues 181–810, indicating that this smaller N-terminal fragment does not include the region corresponding to residues 181–810. Densitometry of the relative intensities of the htt fragments demonstrated that levels of the N-terminal 50 kDa fragment were at least fivefold greater in HD than in control striatum (putamen). Moreover, the 45 and 43 kDa N-terminal fragments were prominent in HD striatum and were nearly absent in controls.

Western blots with the C-terminal domain antibody [anti-(2146–2541)] showed that striatum (putamen) possesses numerous high- and low-molecular weight C-terminal domain htt fragments (Fig. 5, *lanes 5, 6*) that differed from those in cortex. Low-molecular weight C-terminal domain fragments of 35–48 kDa (35, 38, 42, and 48 kDa) were observed in striatum; such low-molecular weight htt fragments were not observed in cortex. Importantly, HD striatum (compared with that in controls) contained higher levels of the 42 and 48 kDa C-terminal domain fragments that were ~20- and 5-fold greater, respectively, than those in controls (estimated by densitometry). In addition, striatum contained a group of high-molecular weight C-terminal domain htt fragments, indicated by bands of 100, 120, 150, 210, and 250 kDa that were present in both HD and control samples.

Overall, these findings demonstrate tissue-specific proteolysis of huntingtin in striatum compared with cortex. Importantly, elevated levels of certain N- and C-terminal domain htt fragments are found in HD striatum compared with those in controls. The increased levels of these htt fragments may occur by a combination of increased production or retarded degradation of such fragments.

Unique protease-susceptible domains of huntingtin in striatum

Differences in the pattern of htt fragments were observed in striatum compared with cortex. The presence of N-terminal fragments of 20–50 kDa in striatum indicated proteolysis within a region near the N terminal of htt (Fig. 6), which resembles the A protease-susceptible domain observed for htt proteolysis in cortex. Apparently, regulated proteolysis within this region in the striatum from HD compared with control brains results in higher levels of 20–50 kDa N-terminal htt fragments and lower relative levels of full-length huntingtin. Moreover, some differences in proteolysis within the A domain generate a 45 kDa N-terminal htt fragment in striatum that was not detected in cortex.

Importantly, htt in striatum possesses a unique protease-susceptible domain that differs from that in cortex. In striatum, a second protease-susceptible domain, indicated as the "C" domain (Fig. 6), is predicted to reside within a C-terminal region of huntingtin. Proteolysis within this C domain would account for the increased production of low-molecular weight (35–48 kDa) C-terminal domain htt fragments. High-molecular weight (100–250 kDa) C-terminal htt fragments also result from proteolysis within the C domain. It is notable that proteolysis within the C domain does not occur in cortex.

As a control, the examination of huntingtin fragments in cerebellum, which is minimally affected in HD with respect to nuclear inclusions and neuronal loss, indicated a completely different profile of huntingtin-derived proteolytic fragments detected with the N- and C-terminal domain antibodies (Fig. 7). The N-terminal domain antibody detected numerous htt fragments (12–14 fragments) of 20–90 kDa; this contrasts with fewer N-terminal domain fragments of different molecular weights found in cortex (64–77 kDa) and in striatum (40–50

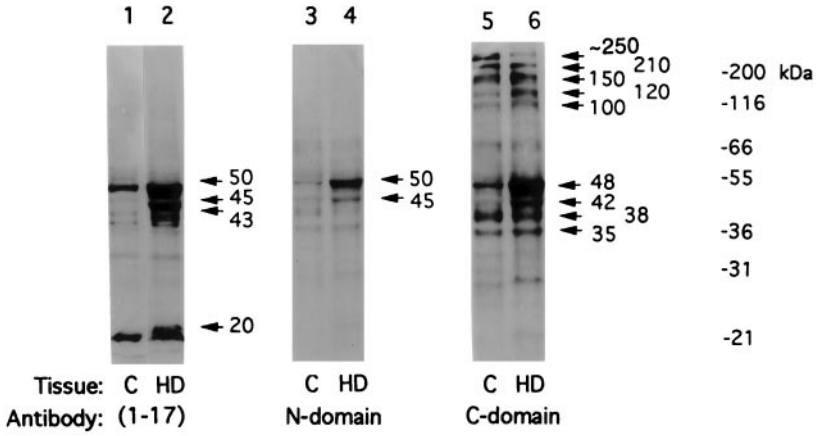

Figure 5. Striatum (putamen): huntingtin fragments detected with antibodies recognizing the N terminus, N-terminal domain, and C-terminal domain of htt. Striatum (putamen) tissue homogenates from control (*lanes 1, 3, 5*) and HD (*lanes 2, 4, 6*) brains were analyzed by Western blots with anti-(1–17) serum (*lanes 1, 2*), N-terminal domain antibody (*lanes 3, 4*), and C-terminal domain antibody (*lanes 5, 6*). *Each lane* of the gel (12% SDS-PAGE) contained identical amounts of homogenate protein (18 μg).

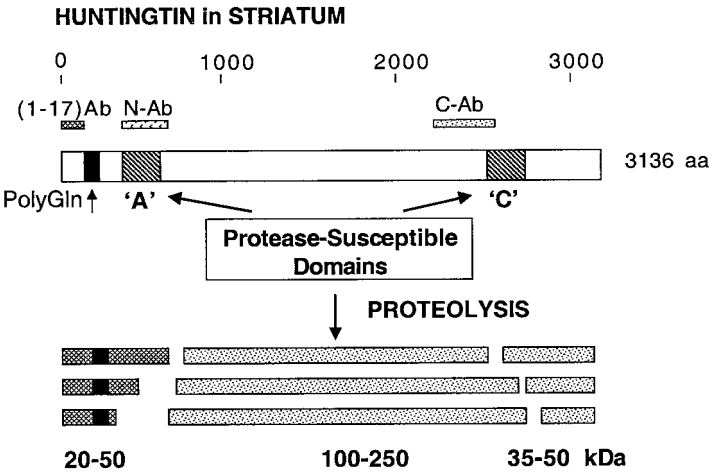

Figure 6. Protease-susceptible domains of huntingtin in striatum. Protease-susceptible domains indicated as *A* and *C* illustrate the predicted domains of htt proteolysis in striatum (putamen). Proteolysis within both "*A*" and "*C*" domains would generate low-M_r N-terminal fragments of 20–50 kDa, concomitantly with 35–50 kDa low-M_r and 100–250 high-M_r C-terminal fragments. These htt fragments are consistent with those detected in striatum (putamen) by Western blots with *N-Ab* and *C-Ab*, as well as by *(1–17)Ab*. Recognition of the predicted htt fragments (indicated by *horizontal bars*) by each of the three anti-htt antibodies is illustrated by antibody-specific *patterns* that fill the *horizontal bars*.

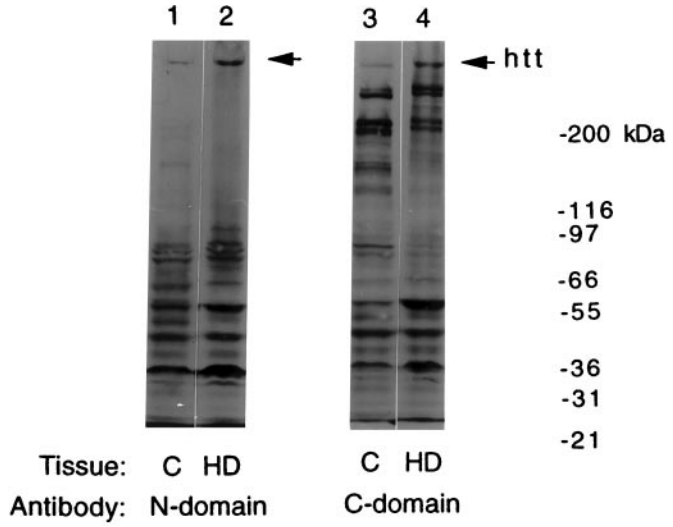

Figure 7. Huntingtin fragments in cerebellum. Cerebellum tissue homogenates from control (*lanes 1, 3*) and HD (*lanes 2, 4*) brains were analyzed by Western blots with the N-terminal domain antibody (*lanes 1, 2*) and the C-terminal domain antibody (*lanes 3, 4*). *Each lane* of the gel (12% SDS-PAGE) contained identical amounts of homogenate protein (18 μg).

kDa). Moreover, cerebellum contained a larger number of C-terminal domain htt fragments of ~20–60 kDa that differed in overall pattern from those in cortex or striatum. It was also noted that HD cerebellum showed larger amounts of full-length htt compared with that in control. These results further demonstrate tissue-specific proteolysis of huntingtin in different brain regions.

Ubiquitination of N-terminal huntingtin fragments in striatum and cortex

Immunohistochemical studies have suggested colocalization of htt and ubiquitin (Ub) in nuclear inclusions in human HD brains and in brains of transgenic mice expressing htt N-terminal fragments with an expanded polyglutamine region (Davies et al., 1997; DiFiglia et al., 1997). In addition, an htt fragment in lymphoblasts was found to be ubiquitinated (Kalchman et al., 1996). The proposed hypothesis of ubiquitination of htt fragments in human brain (DiFiglia et al., 1997) suggests recognition of htt-positive bands by anti-Ub sera. Therefore, this study examined ubiquitination of N-terminal fragments in striatum and cortex by parallel anti-Ub and anti-(1–17) huntingtin Western blots.

Differences in ubiquitination of htt N-terminal fragments in these regions were observed (Fig. 8*a*). The bands representing 50 and 45 kDa N-terminal fragments from striatum (putamen) were apparently recognized by anti-Ub serum. Moreover, increased ubiquitination of the 50 kDa band was observed in HD compared with control. Ubiquitination was observed for only some N-terminal htt fragments, because the 20 kDa N-terminal band in striatum was not readily detected by anti-Ub sera.

In contrast to striatum, the majority of N-terminal fragments in cortex did not appear to be ubiquitinated, with the exception of 45 and 100 kDa fragments in Brodmann area 6 (Fig. 8*b,c*). In Brodmann area 4, none of the N-terminal htt fragments appeared to be ubiquitinated at the level of sensitivity of these Western blots. However, in Brodmann area 6, 45 and 100 kDa N-terminal fragments appeared to be ubiquitinated; other N-terminal fragments of 40, 43, and 50 kDa did not appear to be ubiquitinated. In addition, full-length htt was not ubiquitinated. These results suggest tissue-specific ubiquitination of selected N-terminal htt fragments.

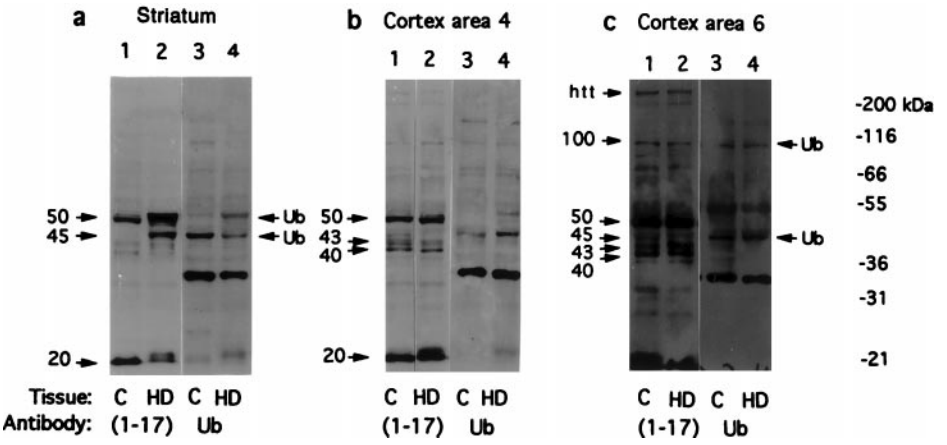

Figure 8. Analysis of ubiquitination of N-terminal fragments of huntingtin by Western blots. *a*, Striatum. Homogenate samples of striatum (putamen) from control (*lanes 1, 3*) and HD (*lanes 2, 4*) brains were subjected to parallel Western blots with anti-(1–17) serum (*lanes 1, 2*) and anti-Ub serum (*lanes 3, 4*). *Each lane* of the gel (12% SDS-PAGE) contained identical amounts of homogenate protein (18 μg). *b*, Cortex area 4. Homogenate samples of cortex, from Brodmann area 4, from control (*lanes 1, 3*) and HD (*lanes 2, 4*) brains were subjected to parallel Western blots with anti-(1–17) serum (*lanes 1, 2*) and anti-Ub serum (*lanes 3, 4*). *Each lane* of the gel (12% SDS-PAGE) contained identical amounts of homogenate protein (70 μg). *c*, Cortex area 6. Homogenate samples of cortex, from Brodmann area 6, from control (*lanes 1, 3*) and HD (*lanes 2, 4*) brains were subjected to parallel Western blots with anti-(1–17) serum (*lanes 1, 2*) and anti-Ub serum (*lanes 3, 4*). *Each lane* of the gel (12% SDS-PAGE) contained identical amounts of homogenate protein (70 μg).

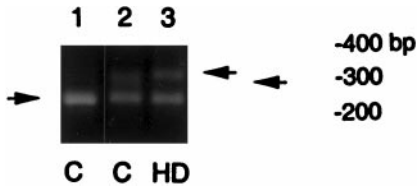

Figure 9. PCR amplification of the triplet-repeat domain of the IT15 gene from control and Huntington's disease brain samples. PCR amplification of genomic DNA used primers flanking the triplet-repeat domain of the IT15 gene encoding huntingtin. PCR used DNA isolated from four control and four Huntington's disease brains. Two control samples showed a single band of ~250 bp generated by PCR (*lane 1*); two other controls showed lower and upper bands of ~250 and 300 bp, respectively (*lane 2*). All four Huntington's samples showed lower and upper DNA bands of ~250 and 320–330 bp, respectively (*lane 3*). The lower and upper bands from each PCR reaction were subjected to DNA sequencing to determine the number of CAG repeats (see Table 1).

Table 1. Triplet-repeat length in control and Huntington's disease samples

	Repeat length (CAG)	
Tissues	Lower PCR band	Upper PCR band
Controls		
Sample 1	15	—
Sample 2	17	—
Sample 3	13	34
Sample 4	15	28
Huntington's disease		
Sample 1	~13–18 (ND)	46
Sample 2	18	43
Sample 3	15	36
Sample 4	~13–18 (ND)	46

PCR amplification of DNA fragments that contain the CAG triplet region of the IT15 gene generated lower and upper bands of ~250 and 300 bp, respectively, from controls, and HD samples showed lower and upper bands of 250 and 320–330 bp, respectively (Fig. 9). PCR-generated fragments were subjected to DNA sequencing to determine the number of triplet repeats. ND (not directly determined by DNA sequencing) indicates that the size (in base pairs) of the lower band predicts a length of 13–18 repeats.

Analysis of triplet repeats in control and Huntington's tissue samples

To confirm that the neurologically characterized tissues contain normal and mutant IT15 genes encoding htt, the number of CAG triplet repeats in the IT15 gene in control and Huntington's brain tissues illustrated in Western blot analyses (Figs. 2, 3, 5, 7, 8) was determined by PCR of genomic DNA with primers flanking the triplet-repeat domain of the gene. In four control samples, two samples showed a single PCR-generated band of ~250 bp (Fig. 9, *lane 1*), suggesting the presence of similar alleles. In two other control samples, PCR generated two bands consisting of a lower band of ~250 bp and an upper band of ~300 bp (Fig. 9, *lane 2*), indicating the presence of two different alleles. In four Huntington's disease samples, PCR generated two distinct bands (Fig. 9, *lane 3*). The lower band of ~250 bp was similar in size to that found in controls; however, the upper band of 320–330 bp was slightly larger than the upper band from controls.

To determine whether the upper and lower DNA bands generated by PCR may represent different alleles of the IT15 gene with different numbers of CAG repeats, DNA sequencing was performed to determine the length of the triplet repeats (Table 1). In controls, the lower 250 bp band contained 13–17 repeats, and the upper 300 bp band contained 28–34 repeats; these results show that the neurologically diagnosed controls contain CAG repeats within the normal range of 11–34 repeats that may appear as homozygous or heterozygous alleles of the IT15 gene (MacDonald et al., 1993).

The HD tissue samples showed two alleles that contained normal and expanded repeat lengths (Table 1). One allele (lower PCR band of 250 bp) contained a normal repeat length of 13–18 repeats, but the other allele (upper PCR band of 320–330 bp) contained an expanded repeat domain of 36–46 triplet repeats. These results demonstrate that the clinically diagnosed HD tissue samples shown in Western blots contain expanded triplet repeats, consistent with previous observations that HD cases possess 40 or more repeats and with some HD cases having 36–39 repeats (MacDonald et al., 1993; Vonsattel and DiFiglia, 1998).

Determination of the number of CAG triplet repeats and the size of the polyglutamine expansion within normal and mutant htt protein indicates that mutant full-length htt would possess a predicted apparent molecular weight of ~351–353 kDa, whereas normal full-length htt would have a calculated apparent molecular weight of ~350 kDa. The difference of 1–3 kDa between normal and mutant htt is small compared with the large size of the htt protein. Because it is known that differences of 1–3 kDa are not usually resolved for proteins of 100–400 kDa by SDS-

PAGE, it is predicted that these small relative differences in molecular weight of mutant and wild-type htt would not be resolved by SDS-PAGE gels, as shown in the Western blots of normal and mutant htt in this study (Figs. 2, 3, 5).

Overall, analyses of htt fragments with domain-specific antibodies in HD and control human brain cortex and striatum, combined with determination of the CAG triplet-repeat expansion by PCR in individual samples, provide a strong association of tissue-specific proteolysis of huntingtin in HD with expanded triplet repeats in the IT15 gene encoding huntingtin.

DISCUSSION

Results from this study demonstrate that htt undergoes tissue-specific proteolysis in cortex compared with striatum in HD and control brains. Htt fragments in these tissues were characterized with domain-specific antibodies against htt (Fig. 1). Different protease-susceptible domains were identified for proteolysis of htt in cortex compared with striatum. In cortex, htt proteolysis occurs within two different N-terminal domains, termed A and B (Fig. 4). However, in striatum, a distinct pattern of different and similar htt fragments compared with those in cortex indicated that proteolysis of striatal htt occurred within a unique C-terminal domain termed C (Fig. 6), as well as within the N-terminal domain A. Selective proteolysis within these protease-susceptible domains results in the observed differences in the pattern of htt fragments in cortex and striatum. Importantly, striatum from HD brains showed increased levels of 40–50 kDa N-terminal and 30–50 kDa C-terminal fragments compared with those in controls. Elevated levels of these htt fragments may occur by a combination of increased production or retarded degradation of such fragments. In cortex, no differences in htt fragments were observed in HD compared with control brains. Results also implicated tissue-specific ubiquitination of certain htt N-terminal fragments in striatum compared with cortex. Moreover, expansions of the triplet-repeat domain of the IT15 gene encoding htt were confirmed in the clinically diagnosed HD tissue samples used in this study. These findings suggest that regulated tissue-specific proteolysis and ubiquitination of htt occur in HD brains.

In cortex corresponding to Brodmann areas 4 and 6, the N-terminal domain antibody detected fragments of ~60–80 kDa (64, 67, and 77 kDa) that lacked the N terminus of htt, because they were not recognized by the anti-(1–17) htt serum. Moreover, the N-terminal fragments of 20–50 kDa (20, 40, 43, and 50 kDa) detected by anti-(1–17) were not recognized by the N-terminal domain antibody. Together, the pattern of these fragments indicates the presence of two protease-susceptible domains, A and B (Fig. 4), within htt in cortex. Proteolysis within the A domain would generate the 20–50 kDa N-terminal fragments. Proteolysis within both A and B domains would generate fragments of ~60–80 kDa that lack the N terminal. Moreover, proteolysis of htt was identical in cortex from HD and control brains.

In striatum, a different pattern of htt fragments was observed compared with that in cortex. In striatum, N-terminal fragments (20, 43, 45, and 50 kDa) were detected by the anti-(1–17) serum, whereas cortex contained N-terminal fragments of slightly differing molecular weights (20, 40, 43, and 50 kDa). These findings indicate the presence of a similar protease-susceptible domain in both striatum and cortex, designated the A domain, which undergoes differential processing in these two tissue regions to generate N-terminal fragments that vary in apparent molecular weights. In addition, striatum does not possess 60–80 kDa bands detected by the N-terminal domain antibody, which are present in cortex. These results indicate that proteolysis of htt in striatum does not occur within the B domain, but proteolysis in this region occurs in cortex (Fig. 4).

Importantly, htt in striatum possesses a unique protease-susceptible domain, the C domain (Fig. 6), located near the C-terminal region (residues 2146–2541) of htt. Proteolysis within the C domain was not detected in cortex. In striatum, proteolysis within the C domain generates low (35–50 kDa) and high (100–250 kDa) molecular weight htt fragments. These results suggest that striatum, compared with cortex, contains unique proteases that cleave within the C domain of htt.

Significantly, proteolysis of htt in striatum occurs within both A and C domains and results in elevated levels of N-terminal (43, 45, and 50 kDa) and C-terminal (42 and 48 kDa) domain fragments. The parallel increases in proteolysis within A and C domains suggest simultaneous involvement of proteases that recognize N- and C-terminal domains of htt, resulting in production of unique htt fragments in HD. In addition, elevated levels of these htt fragments may also occur by retarded degradation of these fragments. It is of interest that N-terminal fragments, but not C-terminal fragments, are detected in nuclear inclusions (de la Monte et al., 1988; DiFiglia et al., 1997; Petersen et al., 1999). Presumably, N-terminal fragments of htt are translocated to nuclei from the cytoplasm where htt and its fragments are normally located. Further investigations of the kinetics and cell biology in the formation of htt fragments can define the metabolism of htt, as well as the cellular location for htt proteolysis.

Assessment of ubiquitin immunoreactivity in Western blots provided evidence of ubiquitination of certain N-terminal htt fragments in a tissue-specific manner. In striatum, the bands representing 45 and 50 kDa N-terminal fragments were ubiquitinated to a greater extent in HD brains compared with controls. However, N-terminal fragments in Brodmann area 4 of cortex did not appear to contain ubiquitin. In Brodmann area 6 of cortex, an N-terminal fragment of 45 kDa showed increased ubiquitination in HD compared with control brains. These findings suggest that selected N-terminal fragments may be targeted for ubiquitin-dependent proteolysis in a tissue-specific manner.

The N-terminal fragments (20–50 kDa) present in adult-onset HD striatum and cortex are consistent with the N-terminal htt fragments (30–40 kDa) found in cortex of juvenile HD brains (DiFiglia et al., 1997). The findings from this study with grade 3 HD brains indicate altered htt proteolysis in striatum at a late stage of the disease. Examination of tissues from early grades of the disease will be necessary to determine whether these differences in htt proteolysis precede neuropathology or motor abnormalities of HD.

In summary, tissue-specific proteolysis of htt has been demonstrated in striatum and cortex of HD and control brains. The observed increase in htt N-terminal fragments in HD striatum, compared with cortex, suggests a possible association of such htt fragments with the more severe neuronal loss in striatum of HD brains. Moreover, the observation that affected neurons in HD are not altered in htt transcription or translation (Li et al., 1993; Bhide et al., 1996) suggests that regulated proteolysis of htt may contribute to tissue-specific alterations in HD. Htt contains caspase cleavage sites and may be cleaved by caspases (Goldberg et al., 1996; Wellington et al., 1998, 2000); in addition, several studies suggest that formation of nuclear inclusions that contain htt N-terminal fragments may be dissociated from events that regulate cell death (Saudou et al., 1998; Kim et al., 1999). Clearly, it will be important to identify and inhibit the proteases that

convert the mutant htt to N-terminal fragments to define the role of htt proteolysis in the pathogenic mechanisms involved in the development of HD.

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