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ORIGINAL ARTICLE

Characterization of Huntingtin Pathologic Fragments in Human Huntington Disease, Transgenic Mice, and Cell Models

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Abstract

Huntington disease (HD) is caused by the expansion of a glutamine (Q) repeat near the N terminus of huntingtin (htt), resulting in altered conformation of the mutant protein to produce, most prominently in brain neurons, nuclear and cytoplasmic inclusion pathology. The inclusions and associated diffuse accumulation of mutant htt in nuclei are composed of N-terminal fragments of mutant protein. Here, we used a panel of peptide antibodies to characterize the htt protein pathologies in brain tissues from human HD, and a transgenic mouse model created by expressing the first 171 amino acids of human htt with 82Q (htt-N171-82Q). In tissues from both sources, htt pathologic features in nuclei were detected by antibodies to htt peptides 1–17 and 81–90 but not 115–129 (wild-type huntingtin numbering with 23 repeats). Human HEK 293 cells transfected with expression vectors that encode either the N-terminal 233 amino acids of human htt (htt-N233-82Q) or htt-N171-18Q accumulated smaller N-terminal fragments with antibody-binding characteristics identical to those of pathologic peptides. We conclude that the mutant htt peptides that accumulate in pathologic structures of human HD and httN171-82Q in mice are produced by similar, yet to be defined, proteolytic events in a region of the protein near or within amino acids 90–115.

Key Words: Huntingtin, Huntington disease, Inclusion bodies, Neurodegenerative disease, Proteolytic processing.

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INTRODUCTION

Huntington disease (HD) is an autosomal-dominant, progressive, and fatal neurodegenerative disease that usually starts in midlife (1–3). HD occurs when a CAG (glutamine codon) repeat near the N terminus of huntingtin (htt) expands to a length greater than 36 consecutive glutamines (1). The symptoms of HD include a movement disorder with cognitive impairment and psychiatric disturbances that progress to death over 15 to 25 years (for review, see Reference 2). Neuropathologic characteristics of the disease include inclusion bodies in neurons of the central nervous system, general brain atrophy, a dramatic loss of medium spiny neurons in the striatum, and loss of neurons in the deeper layers of the cortex (4, 5).

Neurons throughout the central nervous system harbor inclusion bodies, in both the nucleus and cytoplasm, that are immunoreactive with antibodies directed against N-terminal but not C-terminal regions (6–8). Transgenic or knockin mice that express full-length htt with expanded CAG repeats have also been shown to develop inclusions formed by N-terminal fragments of mutant htt (9–12). N-terminal fragments of mutant htt have been detected in immunoblots of homogenates from HD brains (6) and from transgenic mouse models created by expressing full-length versions of mutant htt (13, 14). Transgenic mice that express only N-terminal portions of mutant htt develop inclusion pathology that is very similar to human HD (15–17), including nuclear and cytoplasmic inclusions. In mice, another prominent pathologic manifestation is the accumulation of mutant htt diffusely in nuclei (17). With one recent exception that has yet to be explained (18), mice that express mutant htt fragments develop profound motor function abnormalities at early ages with premature death (17, 19).

The precise C termini of mutant htt fragments that comprise pathologic inclusions or the proteases responsible for generating such fragments have not been definitively identified. Lunkes et al (20) demonstrated that the pathologic htt fragments terminate between the polyglutamine domain (begins at amino acid 18) and amino acids 115–129 (all amino acid numbers are based on wild-type htt with 23Q). Sieradzan et al (8) demonstrated that an antibody to a htt peptide encompassing amino acids 80–113 recognized nuclear and cytoplasmic aggregates of mutant htt in human HD brain, localizing the C terminus to a 33-amino acid region of htt. The protease that produces this fragment has

been suggested to be a yet to be identified aspartyl protease (20). Wild-type and mutant htt are also substrates for caspases (21, 22), but these cleavage sites are located in the vicinity of amino acid 500. Mutant and wild-type htt can also be cleaved by calpain 1 (23), with one site located in a region near the putative C terminus of pathologic fragments (24). Lysosomal proteases have also been implicated in htt processing (25). Precise understanding of the proteolytic pathways and htt fragments generated remains to be completely resolved.

One goal of this study was to compare the mutant human huntingtin fragments that accumulate in inclusions and diffuse nuclear pathology that is found in human HD to the pathology present in our previously described N171-82Q mouse model of HD (17). As the name implies, the N171-82Q mouse model was generated by expressing the first 171 amino acids of htt (with 82Q), and, as such, if this entire fragment formed pathologic lesions, then epitopes C-terminal to 115–129 should be present. We also generated additional antibodies, targeting several sequences in the N terminus of huntingtin, to further characterize the C termini of htt fragments found in pathologic structures. We demonstrate that the huntingtin fragments that compose nuclear inclusions in the brains of N171-82Q mice and HD patients possess C termini that end between antibody epitopes defined by 2 htt peptides (81–90 and 115–129). Immunoblots of aggregated forms of mutant htt extracted from nuclear and cytoplasmic fractions from the brains of symptomatic N171-82Q mice confirmed the presence of the 81–90 sequence, but not the 115–129 sequence. We identified a human cell culture model in which htt proteins were cleaved to produce fragments similar to pathologic fragments. Human HEK 293 cells expressing a htt-N233-82Q construct (N-terminal 233 amino acids of human htt) and httN171-18Q constructs accumulated peptides of the expected length and shorter peptides, the latter of which have antibody-binding properties that are identical to pathologic inclusions in mouse and human brains. Collectively, our studies define the location of proteolytic site in the N terminus of htt as a domain between residues 81–90 and 115–129 and define 2 model systems (N171-82Q mice and HEK 293 cells) that appear to recapitulate the proteolytic processing involved in producing pathologic htt fragments.

MATERIALS AND METHODS

Antibodies

Synthetic peptide antigens were used in the generation of antibodies to specific sequence epitopes. Peptides to defined regions of htt were 10 to 15 amino acids in length. The synthesis of the peptide, coupling to carrier, injection into rabbits, and affinity purification were all done under contract with a commercial vendor (Cambridge Research Biochemicals, Billingham, Cleveland, UK). An antibody to amino acids 1–17 of human htt (MATLEKLMKA FESLKSFC) has been described previously (17). The N-del antibody, raised in rabbit, to portions of the N-terminal 90 amino acids of htt, excluding most of the poly Q domain and all of the first polyproline repeat, has been described previously (26). Two new peptide antibodies were generated for this study: the antibody designated htt55-65 was raised against amino acids 55–65 of human htt (QPPPQAQPLLPQC) and the antibody designated htt81-90 was raised against amino acids 81–90 of human htt (CAVAEEPLHRP); peptide sequences are numbered on the basis of wild-type human htt with 23Q (GenBank Accession Number NM_002111). Cysteines were added to the C terminus of the 55–65 peptide and to the N terminus of the 81–90 peptide to facilitate coupling to carrier protein. The huntingtin antibody 1H6 (raised against htt amino acids 115–129) was kindly provided by Dr. Trottier (Institut de Genetique et de Biologie Moleculaire et Cellulaire, CNRS, INSERM, Universite Louis Pasteur, Strasbourg, France) (20) initially; and later was obtained from Chemicon International (catalog number MAB5490; Chemicon International, Temecula, CA). The huntingtin antibody EM48 (Chemicon International) recognizes an epitope similar to N-Del.

Immunohistochemical Studies

All procedures involving animals were approved by The Johns Hopkins University Institutional Animal Care and Use Committee. Animals were anesthetized in ether and perfused with PLP (2% paraformaldehyde, 10 mmol/L sodium periodate, and 75 mmol/L lysine buffered in PBS). All chemicals, unless otherwise noted, were obtained from Sigma-Aldrich (St. Louis, MO). Brains were removed and postfixed overnight in PLP before transfer to PBS for storage at 4°C. Fixed brains were divided sagittally along the midline and processed for embedding in paraffin and immunocytochemistry as previously described (27).

Human tissues were obtained from the HD brain bank that is a component of The Johns Hopkins University Center for Huntington Disease Research, directed by Dr. Juan Troncoso. The 6 HD brains and 5 control brains were harvested no longer than 24 hours postmortem, and all tissues were fixed by immersion in 10% formalin. The HD cases selected for this study had been analyzed in a diagnostic manner for the presence of HD-related histopathology, including the presence of ubiquitin-immunoreactive neuronal nuclear inclusions and the quality of the tissue (short postmortem interval and evidence of good fixation). Control cases were selected on the basis of short postmortem interval, evidence of good fixation, and cause of death unrelated to neurologic disease.

Immunohistochemical staining of 8-μm paraffin sections was performed with mEM48 (MAB5374, Chemicon International) at a dilution of 1:50 and N-del at 1:100, htt1-17 at 1:100, htt55-65 at 1:500, htt81-91 at 1:500, 1H6 at 1:500, and anti-ubiquitin antibody at 1:1000. A subset of immunostained sections were lightly counterstained with hematoxylin and eosin to better visualize subcellular compartments. Images were generated at the original magnifications of 252×.

Analysis of Cytoplasmic Aggregates by Stereology

Adjacent sagittal sections of forebrain from 4 transgenic animals were immunostained with htt81-90 and 1H6

and then analyzed with the stereo investigator and software. The investigator performing the counting was blinded to the antibody used to immunostain tissue sections. In each section, the entire cortex area was outlined for analysis and the program was set to outline fields of 50 μm × 40 μm, with a minimal distance of 500 μm × 500 μm between the counting fields. Between 20 and 30 counting frames were analyzed for each section. Cytoplasmic inclusions were defined as focal accumulations of htt immunoreactivity that were not located within an obvious nuclear structure and that were of a size comparable to that of nuclear inclusions (visible in the same fields). The number of cytoplasmic aggregates in each field was averaged and standard deviations were calculated. Statistical significance of differences between groups was estimated by a two-tailed Student *t*-test.

Immunoblot Analyses of htt Aggregates in N171-82Q Mice

Fractionation procedures were performed at 4°C or on ice. Whole brains were Dounce homogenized in 10 volumes of buffer A (0.25 mol/L sucrose, 50 mmol/L triethanolamine, pH 7.5, 25 mmol/L potassium chloride, and 5 mmol/L magnesium chloride supplemented with 1 mmol/L dithiothreitol, 1 mmol/L phenylmethylsulfonyl fluoride, and 1x complete protease inhibitor cocktail (Roche Molecular Biochemicals/Boehringer Mannheim, Indianapolis, IN) and centrifuged at 800 × *g* for 15 minutes. Supernatants (S1 fraction) were centrifuged at 100,000 × *g* for 1 hour, and resultant supernatants (S2) were taken as cytoplasmic fractions. The pellet from the initial centrifugation (P1) was resuspended in 10 mL of buffer A and mixed with 18 mL of buffer B (1.8 mol/L sucrose, 50 mmol/L triethanolamine, pH 7.5, 25 mmol/L potassium chloride, and 5 mmol/L magnesium chloride supplemented with dithiothreitol, phenylmethylsulfonyl fluoride, and complete protease inhibitor cocktail). The resultant mixture was layered on top of 10 mL of buffer B, and nuclei were pelleted by centrifugation at 30,000 × *g* for 1 hour. The clear, viscous nuclear pellet (P2) was washed in buffer A by low speed centrifugation and resuspended to a final volume of 1 mL. Intact nuclei were observed in nuclear pellets using trypan blue staining.

Nuclear and cytoplasmic fractions were sonicated, and protein levels were quantified by a BCA protein assay (Pierce Biotechnology, Rockford, IL). For each fraction, 25 μg of protein was mixed with sodium dodecyl sulfate (SDS) loading buffer, sonicated, and loaded onto a 4% to 20% SDS-polyacrylamide gel electrophoresis (PAGE) gel (Bio-Rad Laboratories, Hercules, CA), run at 80 V for several hours and transferred onto a supported nitrocellulose membrane at 100 V for 1 hour at 4°C. The resultant blots were blocked for 1 hour at room temperature with 5% dry milk in Tris-buffered saline (TBS), except for the blot to be probed with htt81-90 antibody, which was blocked in 10% dry milk in TBS. Blots were incubated overnight at 4°C in 5% dry milk in TBS plus 0.07% Tween 20 (TBST) with htt81-90 antibody (1:1000) and 1H6 (1:1000, Chemicon MAB 5490). Parallel blots were probed with antibodies to histones (1:500, Chemicon MAB052) or actin (1:100, Chemicon MAB1501R) to confirm the relative purity of the fractions (data not shown). Immunoblots were washed 3 times for 15 minutes in TBST and incubated in 5% milk in TBS with the corresponding secondary antibody for 1 hour. Immunoblots were washed 3 times for 10 minutes in TBST, followed by a brief wash in TBS, and then developed by chemiluminescence for exposure to film as described previously (17, 27).

Cell Transfection and Immunoblotting

Two sets of recombinant htt constructs were used in transient transfection studies in human HEK293 cells. Initial transfections were performed with pRc/CMV vectors (Invitrogen, Carlsbad, CA) encoding the first 233 amino acids of human htt and 82 consecutive glutamines. Subsequent transfections were performed with the vector pcDNA3.1 (Invitrogen), which encoded a cDNA version of the first 171 amino acids of human htt with 18 consecutive glutamines. Each transfection and immunoblot experiment was repeated at least once.

HEK293 cells were transfected with plasmid DNA suspended in Lipofectamine 2000 (Invitrogen) at concentrations recommended by the manufacturer in serum-free Optimem (Invitrogen-Gibco). After 3 hours, complete medium was added (Dulbecco's modified Eagle's medium and 10% fetal bovine serum), and the cells were incubated at 37°C for 48 hours before harvesting. At harvest, the cells were washed 3 times in PBS, scraped into PBS, transferred to centrifuge tubes, pelleted by centrifugation, and then lysed by sonication (Microson XL2000; Misonix, Farmingdale, NY) in PBS for 10 seconds at 22.5 kHz on a power setting of 2 W. After dilution of the cell extract in 2× Laemmli sample buffer (28), equal amounts of protein from each cell lysate was loaded on 4% to 20% gradient gels (Bio-Rad Laboratories or Invitrogen) and electrophoresed until the dye front reached the bottom of the gel. Proteins within the gel were transferred overnight at 100 mA in 1× Tris-glycine-SDS buffer (Amresco, Solon, OH) onto BA85 nitrocellulose (Schleicher and Schuell; PerkinElmer Life and Analytical Sciences, Wellseley, MA). Immunoblots were then probed with corresponding first antibody and developed by chemiluminescence as described previously (17, 27).

RESULTS

Immunocytochemical Studies of HD Postmortem Brains

A total of 6 HD (Grade 2 to 3) postmortem brains and 5 control brains were immunostained with a panel of htt antibodies (N-Del, htt1-17, htt55-65, htt81-90, and 1H6; epitopes defined in the Materials and Methods section) and antibodies to ubiquitin. All human brain tissues were obtained from parietal cortex. We also assessed another pathologic feature of HD, nuclei that stain diffusely with antibodies to htt, first seen in mouse models (15, 17). All of the HD cases studied had been previously screened in diagnostic assessments and were known to show a high frequency of ubiquitin-positive nuclear inclusions. All HD

cases were adult-onset cases. As a means to assess the frequency of inclusion pathology in various cases, we stained all tissues with anti-ubiquitin antibodies, which stain most nuclear inclusions in HD (6, 15, 16) in parallel to the various htt antibodies. Images from 2 HD cases and tissues from 2 control individuals, which were representative of all cases of each type, are shown in Figure 1. The N-Del and htt1-17 antibodies recognized small but well-defined nuclear structures in HD brains (Fig. 1). The htt55-65 antibody preferentially labeled diffusely accumulating protein with small inclusion bodies also being visible (these reproduce poorly in photomicrographs) in nuclei in HD brain sections (Fig. 1). The htt81-90 antibody preferentially recognized inclusion pathology in the HD brains. The frequency of inclusion recognition by these different htt antibodies was relatively equivalent to that of the anti-ubiquitin antibodies, whereas the frequency of nuclei diffusely labeled by htt55-65 was roughly equivalent to the frequency of nuclei containing ubiquitin-positive inclusions. None of the brain sections from the control cases showed evidence of nuclear labeling with these antibodies (Fig. 1). In all HD cases, the 1H6 antibody (epitope amino acids 115–129) failed to detect both the diffuse nuclear accumulation of mutant htt or nuclear inclusions, replicating the findings of Lunkes et al (20). 1H6 recognized discrete inclusion-like structures in the cytoplasm near the nucleus, but these structures were also found in control cases and their identity is therefore unclear.

Immunocytochemical Studies of the N171-82Q Line 81 and NLS-N171-82Q Line 8A Mouse Models

Using the same panel of antibodies, we next turned to immunocytochemical analysis of tissue from transgenic mice that express N171-82Q (line 81) and mice that express N171-82Q modified to encode an N-terminal nuclear localization sequence (NLS-N171-82Q [27]) (Fig. 2B). The animals from line 81 (n = 6) were all approximately 5 months of age at the time of perfusion; which is an age we have previously established as a point near the maximal life expectancy (17). The NLS-N171-82Q-8A animals (n = 4) were aged 7 to 8 months, which is also an age near the maximal life expectancy for his line of mice (27). At these ages, both lines of mice show abnormalities in motor performance, reduced weight (for the N171-82Q mice), and diminished spontaneous motor activity (17, 27, 29). Tissues from age-matched nontransgenic mice were immunostained in parallel with those from transgenic animals. We used EM48 and N-Del antibodies to establish the relative

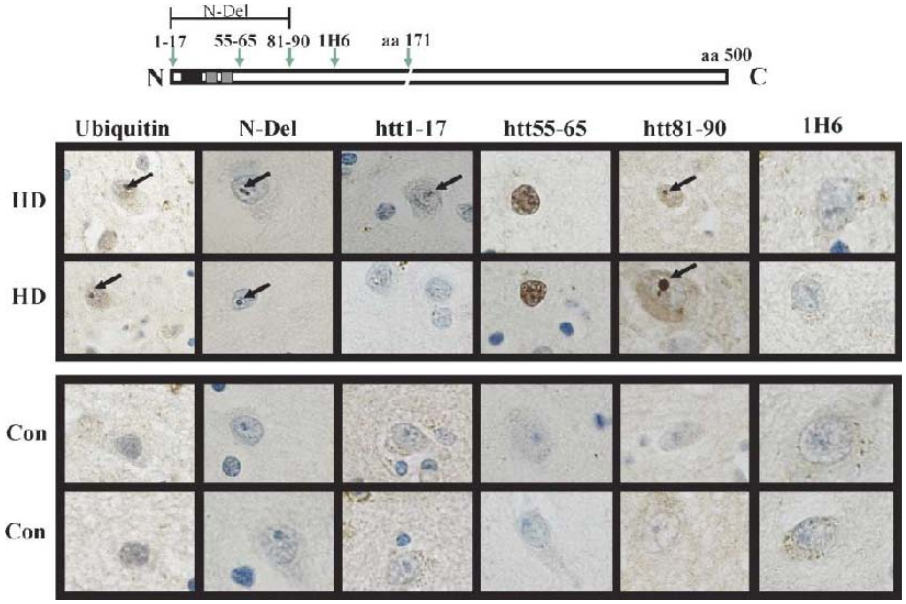

FIGURE 1. Epitopes present in huntingtin (htt) proteins that accumulate in neuronal nuclei of Huntington disease (HD) postmortem brains. We used the panel of antibodies described in the Materials and Methods section to define htt epitopes present in nuclear and cytoplasmic inclusions in postmortem brains of HD patients. The images shown in this figure were from 2 different HD cases (designated HD 239 and HD 231, postmortem interval [PMI] 7 and 3 hours, respectively) and 2 different control (Con) cases (designated Control 783 and 710, PMI 8 and 14 hours, respectively), which were representative of all cases of each type. The schematic diagram above the figure shows the first 500 amino acids (aa) of htt. The black box marks the position of the polyglutamine repeats. The gray boxes mark polyproline repeats. The relative position of different antibody epitopes is also noted in the diagram. Anti-ubiquitin antibodies were used as positive controls because they reliably detect neuronal nuclear inclusions in HD brains (15,16). Ubiquitin antibodies detect large discrete aggregates in HD postmortem brain but not in control brains. The N-Del polyspecific antibody detected small discrete nuclear aggregates in HD cases. Immunostaining with htt55-65 antibody revealed diffuse nuclear accumulations of reactivity, whereas htt81-90 antibody stained well-defined nuclear inclusions. Immunostains with 1H6 antibody revealed similar patterns of reactivity in both HD and control brains with an absence of both diffuse nuclear reactivity and reactivity to inclusions in the HD brain sections. Original magnification: 252×.

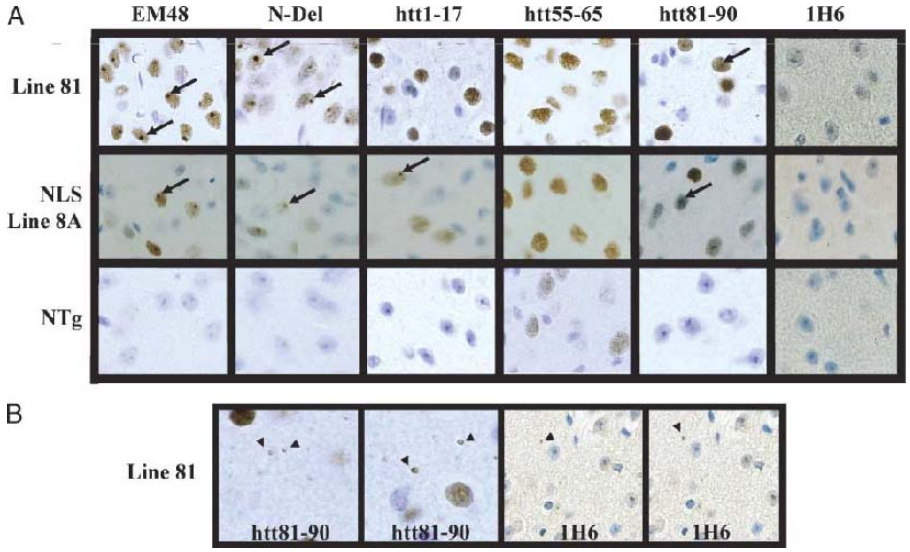

FIGURE 2. Pathologic features in neuronal nuclei of HD-N171-82Q and NLS-HD-171-82Q mice show epitope antibody binding characteristics similar to that of human Huntington disease (HD). **(A)** Tissue sections from the cortex of 5-month-old HD-N171-82Q (line 81) and 7- to 8-month-old NLS-HD-171-82Q (line 8A) mice were immunostained along with age-matched nontransgenic (NTg) littermates for each. To establish the relative frequency of nuclear staining in a given animal we stained sections with monoclonal antibody EM48 (7) and our N-Del antibody, which have been used in previous studies of these mice. Both antibodies show the same pattern of diffuse nuclear and punctate labeling of nuclear inclusion structures in both lines of transgenic mice. In both the HD-N171-82Q and NLS-N171-82Q mice htt1-17, htt55-65, and htt81-90 antibodies preferentially stained diffuse material in nuclei, which was absent in nontransgenic animals. Diffusely stained nuclei and nuclear inclusions in both lines of mice were not detected by the 1H6 antibody (htt115-129). Original magnification: 160×. **(B)** Images of cortical neuropil fields from symptomatic N171-82Q mice (line 81) stained with htt81-90 and 1H6. Cytoplasmic inclusion structures are noted by arrowheads.

frequency of nuclear structures that accumulate htt immunoreactivity in a given tissue section. These antibodies reliably detect diffuse nuclear accumulation, nuclear inclusions, and cytoplasmic aggregates in cortical sections (29). The htt1-17 antibody showed the best reactivity to diffusely accumulated htt in the nucleus with reactivity to nuclear aggregates also being visible (Fig. 2). Antibodies to htt55-65 epitopes labeled diffuse nuclear protein strongly, with some reactivity to small discrete inclusions in the nucleus (Fig. 2B). As was the case in humans, the htt55-65 antiserum preferentially recognized the diffuse nuclear accumulation of mutant htt. However, there was some cross-reactivity with nuclei in cortical sections of the nontransgenic mice. The htt81-90 antibody showed great affinity for diffusely distributed nuclear htt and nuclear inclusions were usually visible but somewhat obscured by the diffuse antibody labeling. The patterns of immunoreactivity in the N171-82Q and NLS-N171-82Q mice with these antibodies were largely similar. Moreover, as is readily seen in these images, the frequencies of nuclei in any given field of view that contained htt immunoreactivity (with each antibody) were similar.

As was the case for the human tissues, the 1H6 antibody (amino acids 115–129) did not recognize either diffusely accumulated htt or nuclear inclusions in either mouse mode. Together, these data suggest that nuclear inclusions are formed by fragments of htt with C termini ending between the 81–90 and 115–129 antibody epitopes.

In our analysis of the HD-N171-82Q mouse tissues, we noted that cytoplasmic inclusions, which are more diffusely distributed in the neuropil, were visible in tissues stained with 1H6 (Fig. 2C). However, by simple inspection, the frequency of structures staining with 1H6 seemed to be far less frequent than those stained with htt81-90. To determine whether there was a difference in frequency, we used stereologic software to randomly define fields in the cortex and then counted cytoplasmic inclusions in sections stained with htt81-90 and 1H6 as described in the Materials and Methods section. From this study we found that fewer cytoplasmic structures with characteristics of htt inclusions stain with the 1H6 antibody. An analysis of sections taken from 4 animals stained with each antibody indicated that the number of cytoplasmic aggregates staining with htt81-90 was at least 3-fold greater than the number stained by 1H6 ($p < 0.00004$).

Characterization of htt Aggregates in HD-N171-82Q Mice

The lack of 1H6 staining in the immunohistochemical stains could be due to masking of the 115–129 epitope by the local conformation of aggregated htt fragments or by binding of other molecules to this region of the protein. To determine whether aggregating species of mutant htt contain the 115–129 epitope, we prepared nuclear and cytoplasmic fractions from brain tissue of symptomatic N171-82Q line 81 mice and analyzed these fractions by denaturing/reducing

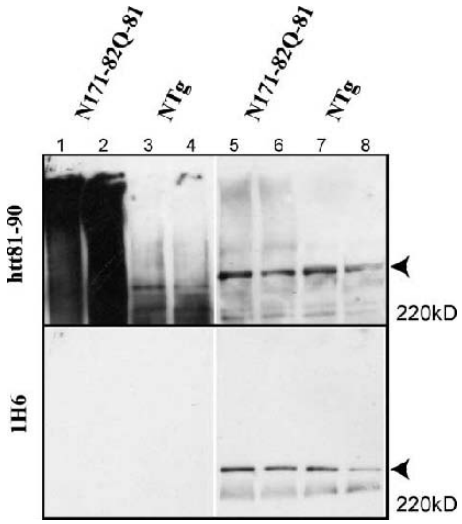

FIGURE 3. Antibody-binding characteristics of high molecular weight complexes of huntingtin (htt) in nuclear and cytoplasmic fractions from N171-82Q line 81 transgenic mouse brain. High molecular weight insoluble mutant htt is recognized by anti-htt81-90 antibody (top panel), but not by antibody 1H6 (htt115-129) (bottom panel). Nuclear fractions from HD-N171-82Q transgenic (lanes 1 and 2) and nontransgenic (NTg, lanes 3 and 4) mice and cytoplasmic fractions from HD transgenic (lanes 5 and 6) and NTg (lanes 7 and 8) mice are shown. Arrowheads indicate endogenous full-length htt.

SDS-PAGE (Fig. 3). Nuclei from symptomatic N171-82Q mice contained relatively large amounts of high molecular weight htt81-90 immunoreactivity, which predominantly appeared as a smear near the top of the gel (Fig. 3, top, lanes 1 and 2). This material was present in very low amounts in cytoplasmic fractions (Fig. 3, top, lanes 5 and 6). In contrast, the 1H6 antibody readily recognized a protein of the expected size for endogenous mouse htt but failed to recognize the high molecular weight smear of mutant N171-82Q protein (Fig. 3, bottom, lanes 1, 2, 5, and 6). Nuclei from nontransgenic animals lacked these high molecular weight immunoreactive structures and the majority of endogenous mouse htt was found in cytoplasmic fractions (lanes 5–8) in both transgenic and nontransgenic animals.

Proteolytic Processing of htt in Cultured Cells

To further analyze the endoproteolysis of htt, we examined cultured cells transfected with expression plasmids encoding an htt fragment that encompassed the first 233 amino acids of human htt with 82Q consecutive glutamines. In lysates of HEK293 cells transfected with httN233-82Q, the N-Del, htt55-65, and htt81-90 antibodies detected 2 predominant proteins; one of the expected size of ~45 kDa (adjusting for the effects of polyglutamine on SDS-PAGE migration) and a second product ~10 kDa smaller (Fig. 4A). Of note, the htt81-90 antibody appeared to preferentially recognize the smaller htt protein and also was strongly reactive to high molecular weight aggregated forms of N233-82Q migrating near the top of the gel. By comparison, 1H6 only recognized the band running at the expected size of 45 kDa, suggesting that the epitope of 115–129 amino acids is C-terminal to the cleavage site that generates this truncation product (Fig. 4A). Processing of htt constructs with a normal number of polyglutamine repeats was also detected in the 293 cells. Transfection with htt-N171-18Q constructs produced htt fragments that were ~10 kDa smaller and were recognized by the htt81-90 antibody (Fig. 4B).

DISCUSSION

We present several lines of evidence to indicate that the htt fragments that accumulate in pathologic inclusions of human HD and the N171-82Q mouse model of HD are generated by a proteolytic event between 2 antibody epitopes defined by amino acids 81–90 and 115–129. Immunocytochemical staining of tissues from human HD patients and from N171-82Q mice demonstrate that nuclear inclusions and diffusely accumulating mutant htt in the nucleus are recognized by antibodies to an epitope within amino acids 81–90 but not by antibodies to an epitope within

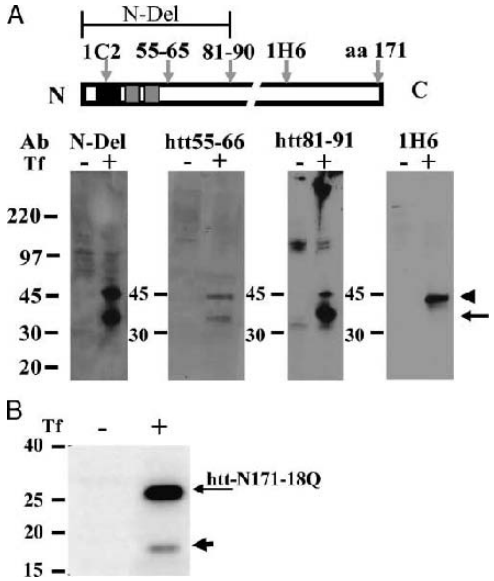

FIGURE 4. Western blot analysis of htt-N233-78Q and N171-18Q cleavage in human HEK293 cells. **(A)** Cells were transfected with pCMV-htt-N233-82Q vectors as described in the Materials and Methods section. After 48 hours, cells were lysed and analyzed by immunoblot with the following antibodies: N-Del, htt55-66, htt81-90, and 1H6 (antibody dilution noted below each panel). The full-length htt-N233-82Q protein migrates at ~45 kDa (arrowhead). The antibodies N-Del, htt55-66, and htt81-90 recognize a truncation product that appears to be ~10 kDa smaller and lacking epitopes recognized by 1H6 (arrow). **(B)** Cells were transfected with pcDNA3.1 encoding htt-N171-18Q vectors as described in the Materials and Methods section. After 48 hours, the cells were analyzed by immunoblot with htt80-91 antibody. In both **(A)** and **(B)**, the antibody dilutions used were as follows: N-del (1:1000), htt55-65 (1:100), htt81-90 (1:400), and 1H6 (1:2000).

amino acids 115–129. Four other antibodies to N-terminal epitopes (N-Del, EM48, htt1-17, and htt55-65) all similarly recognized mutant htt accumulating within the nucleus (either diffusely or in inclusions). Cytoplasmic inclusions may contain some protein fragments that extend to the 1H6 (115–129) epitope or beyond (see below). In studies of human HEK 293 cells transfected with recombinant htt constructs, we also demonstrate the existence of proteolysis between the 81–90 and 115–129 epitopes. Thus, the HEK293 cells appear to replicate the cleavage pattern of htt seen in HD brain and HD mouse models. This cell model may have utility in future work to identify proteases involved in the production of pathologic htt fragments. Based on the patterns of immunoreactivity seen in our cell transfection studies and immunohistochemistry, we conclude that htt encodes a site of specific endoproteolysis in the region between antibody epitopes within amino acids 81–90 and 115–129. These findings are in agreement with previous work by Lunkes et al (20), who had reported that nuclear inclusions and the majority of cytoplasmic inclusions are generated by fragments that terminate after the polyglutamine domain and before the 115–129 epitope for the 1H6 antibody.

The protease responsible for the cleavage that creates these pathologic htt fragments remains to be identified. The amino acid sequence of the region that spans these epitopes is 80-PAVAEEPLHRPKKELSATKKDRVNHCLTICENI VAQ-115. Analysis of this amino acid sequence reveals interesting double lysine motifs at residues 91–92 and 98–99 that are similar to sites of cleavage by dibasic endoproteases of the endoplasmic reticulum and Golgi, noting that these enzymes prefer Arg/Arg and Lys/Arg over Lys/Lys (30). In a cell culture model of htt proteolytic processing, a site of htt cleavage by aspartyl proteases was mapped to a region between amino acids 104 and 114 (20). Lunkes et al (20) also detected a second site of cleavage in cell models, between amino acids 146 and 214. The sequence encompassing residues 86-E**PL**HR**P**KKELS-96 contains 3 amino acids (in bold) that are found at high frequency at these positions in known calpain 1 recognition sites where cleavage would occur between H89 and R90 (31). However, calpain possesses no definitive consensus cleavage site and is highly influenced by local conformation. Two other studies have previously investigated the role of calpain in cleavage of htt with one study describing a cleavage site in proximity to the region compassed by amino acids 80–130 (24). However, another study of htt cleavage by calpain did not identify cleavage sites in this region, but rather calpain was found to cleave more C-terminal near amino acid 500 (23).

Although we are certain that the vast majority of htt fragments that comprise nuclear inclusions do not contain the 1H6 epitope (115–129), it is possible that some of the fragments that comprise cytoplasmic inclusions are longer. We noted that the 1H6 antibody recognized cytoplasmic inclusions in symptomatic N171-82Q mice, albeit at a lower frequency. Fractionation of tissues from these mice failed to detect 1H6-reactive htt aggregates in nuclear fractions as expected, and reactivity to htt aggregates in cytoplasmic fractions was weak. Notably, the level of high molecular weight, aggregated, htt immunoreactivity seen in cytoplasmic fractions with the htt81-90 antibody was much less than that found in nuclear fractions. It is possible that cytoplasmic aggregates are lost in the centrifugations required to obtain pure cytoplasmic protein preparations, or that the more infrequent reactivity of cytoplasmic inclusions to 1H6 antibody reflects a difference in processing for a small amount of htt that aggregates in cytoplasm. It is also possible that in the cytoplasm some full-length mouse htt becomes loosely associated with cytoplasmic inclusions at a low frequency and accounts for their 1H6 reactivity (1H6 recognizes both mouse and human htt).

We do not know at present whether cleavage at the site bordered by the htt81-90 and 115–129 epitopes occurs independently of cleavages at other sites within the protein. Gafni et al (23) demonstrated that in vitro translated htt is cleaved by calpain 1 at amino acids 469 and 586. Sites of cleavage by caspase 3 and 6 have also been mapped between amino acids 450 and 550 (21, 22). Thus, it is possible that the final fragment that appears in inclusions is derived from sequential cleavage of full-length htt. However, if sequential cleavage is involved, then the intermediate fragments are efficiently processed to the shorter fragments that produce pathologic structures. It is notable that we do not typically see an accumulation of endogenous N-terminal fragments of wild-type htt in normal mouse brain. In contrast, the brains of mice that express httN171-18Q were found to contain both the 171 htt peptide and a shorter peptide (17). This finding is consistent with the notion that the direct precursor of htt pathologic fragments may be htt molecules already cleaved by other proteases.

Our study demonstrates that pathologic inclusions in the brains of HD patients and the N171-82Q mouse model of HD are composed of N-terminal fragments of htt that terminate after an epitope contained within amino acids 81–90 and before an epitope contained within amino acids 115–129. Thus, the N171-82Q mice appear to accumulate htt fragments that are generated by events similar to the processing of htt in human HD. The C termini of the pathologic htt fragments in humans are also likely to be similar to the htt fragment expressed in the R6/2 mouse model of HD (the transgene encompasses only exon 1, which ends at amino acid 90), which displays profound behavioral phenotypes and premature death similar to the N171-82Q mice (19). In contrast the htt fragment expressed in the recently described shortstop HD mouse (18), which was surprisingly free of behavioral abnormalities and long-lived, appears to terminate at residue 117. The shortstop HD mice were found to develop abundant nuclear inclusion pathology. This latter study could suggest that specific proteolytic events are crucial in the genesis of toxic of htt fragments. A better understanding of the proteases involved in the generation of htt pathologic fragments could provide a new therapeutic approach to HD.

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