Huntingtin Is Ubiquitinated and Interacts with a Specific Ubiquitin-conjugating Enzyme*

(Received for publication, March 22, 1996)

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Using the yeast two-hybrid system, we have identified a human ubiquitin-conjugating enzyme (hE2-25K) as a protein that interacts with the gene product for Huntington disease (HD) (Huntingtin). This protein has complete amino acid identity with the bovine E2-25K protein and has striking similarity to the UBC-1, -4 and -5 enzymes of Saccharomyces cerevisiae. This protein is highly expressed in brain and a slightly larger protein recognized by an anti-E2-25K polyclonal antibody to whom corresponded in brain regions affected in HD. The huntingtin-E2-25K interaction is not obviously modulated by CAG length. We also demonstrate that huntingtin is ubiquitinated. These findings have implications for the regulated catabolism of the gene product for HD.

Huntington disease (HD) is a member of a family of neurodegenerative disorders caused by CAG triplet expansion (1) which includes spinocerebellar ataxias types I and III, dentatorubropallidoluysian atrophy (DRPLA) and spinal bulbar muscular atrophy (SBMA) (2, 3).

Each of these diseases is characterized by selective neuronal loss in discrete regions of the brain or spinal cord (3). In HD the major site of neuropathology is the caudate nucleus and putamen (4). The cloning of the respective genes allowed testing of the hypothesis that the selective neuronal loss in these disorders was a reflection of restricted expression of the mRNA transcript or protein in those cells undergoing degeneration. However, the respective mRNA transcripts and the resulting proteins are widely expressed in the central nervous system and peripheral tissues (5–10), and are not obviously altered in their abundance in affected tissues (8–13).

Somatic mosaicism has also been invoked to account for the selective neuropathology. At both the DNA and protein levels, significant somatic mosaicism of the CAG (polyglutamine) repeat was demonstrated in tissues from affected patients with HD, with the predominant levels of mosaicism in the caudate nucleus and the cortex, those regions most severely affected in HD (14). Moreover, the cerebellum and brainstem show low levels of mosaicism and are infrequently involved in the neuropathology of HD (14). However, the similar patterns of mosaicism are evident in SCA-I and DRPLA with limited mosaicism in the cerebellum even though this is one of the regions of the brain that is predominantly affected. This clearly indicates that somatic mosaicism was not likely to be a major factor in the genetically determined selective neuronal loss in HD or these other disorders (15, 16).

It has recently been proposed that the selective neuropathology of these disorders could be related not only to expression of the gene containing CAG expansion, but to a protein with restricted expression with which the gene product normally associates (17, 18). To identify proteins interacting with the HD gene product, we exploited the yeast two-hybrid system (19, 20) and identified a human protein with an affinity for the amino terminus of the HD protein. This protein has complete amino acid identity with the previously described bovine ubiquitin-conjugating enzyme known as E2-25K (21). The E2-25K ubiquitin-conjugating enzyme belongs to a family of proteins that participate in the linking of COOH-terminal glycine residues of ubiquitin to specific lysine residues of target proteins (21). This ubiquitination leads to target protein degradation by the 26 S proteasome (22–25).

We demonstrate that he2-25k is highly expressed in brain and that cell-derived huntingtin interacts with purified he2-25k in vitro. In addition, we clearly show that huntingtin is ubiquitinated in peripheral cells, which may have implications for the regulated catabolism of this protein.

EXPERIMENTAL PROCEDURES

GAL4-HD cDNA Constructs—An HD cDNA construct (44EKpGBT9), with 44 glutamine repeats was generated containing amino acids 1–540 of the published HD cDNA (1, 26). This cDNA fragment was fused in-frame to the GAL4 DNA-binding domain (BD) of the yeast two-hybrid vector pGBT9 (Clontech). Another HD cDNA construct, 16EKpGBT9, was identical to 44EKpGBT9 but included only 16 glutamine repeats.

Another clone (DMK BamHIpGBT9) containing the first 544 amino acids of the myotonic dystrophy gene (a gift from R. Korneluk) was fused in-frame with the GAL4-DNA BD of pGBT9 and was used as a
negative control. Plasmids expressing the GAL4-4XBD 
and SIR3 (27) were used as positive controls for the β-galactosidase filter assay. Yeast Strains, Transformations, and β-Galactosidase Assays—The yeast strain Y190 (MATa leu2-3, 112, ura3-52, trp2-301, his3-120, ade2-101, gal4Δgal80A, URA3::GAL1-1ac2, Lys2::GAL-His3 cya) (28) was used for all transformations and assays. Yeast transformations were performed using a modified lithium acetate transformation protocol (29) and grown at 30°C using appropriate synthetic complete dropout media.

The β-galactosidase chromogenic filter assays were performed by transferring the yeast colonies onto Whatman filters. The yeast cells were partially lysed by submerging the filters in liquid nitrogen for 5 min and placed onto filter paper presoaked in Z-buffer (100 mM sodium phosphate (pH 7.0) 10 mM KCl, 1 mM MgSO4) supplemented with 50 mM β-mercaptoethanol and 0.07 mg/ml 5-bromo-4-chloro-3-indolyl β-galactosidase. Filters were placed at 37°C for up to 8 h.

Liquid β-galactosidase assays were performed by inoculating a single yeast colony into appropriate synthetic complete dropout media and grown to OD600 0.6–1.5. Five milliliters of overnight culture was pelleted and washed once with 1 ml of Z-buffer, then resuspended in 100 μl of Z-buffer supplemented with 38 mM β-mercaptoethanol and 0.05% SDS. Acid-washed glass beads (−100 μl) were added to each sample and vortexed for 4 min, by repeatedly alternating a 30-s vortex, with 30 s on ice. Each sample was pelleted and 10 μl of lysate was added to 500 μl of lysis buffer. The samples were incubated in a 30°C water bath for 30 s and then 100 μl of 4 mg/ml o-nitrophenyl β-galactopyranoside solution was added to each tube. The reaction was allowed to continue for 30 min in the ice bath and stopped by the addition of 500 μl of 1 M Na2CO3 and placing the samples on ice. Subsequently, OD420 was taken in order to calculate the β-galactosidase activity with the equation 1000 × OD420(t × V × OD600 (30), where t is the elapsed time (minutes) and V is the amount of lysate used.

Screening for Huntingtin Interacting Protein (HIP) cDNAs—A human adult brain Matchmaker library (Clontech) was transformed into the yeast strain Y190 already harboring the 44KgPegt9 construct. The transformants were plated onto 100 150 × 15-mm circular culture dishes containing synthetic complete media deficient in Trp, Leu, and His. The herbicide 3-amino triazole (25 μM) was utilized to limit the number of false His+ positives (31). The yeast transformants were plated at 30°C for 5 days and β-galactosidase filter assays were performed on all colonies found after this time, as described above, to identify β-galactosidase+ clones. Primary His+ β-galactosidase+ colonies were then ordered patched onto a grid on synthetic complete -Trp/Leu/His (25 μM 3-amino triazole) plates and assayed again for clones were then orderly patched onto a grid on synthetic complete media overnight, lysing the cells with acid-washed glass beads and electrophorating the bacterial strain, KC8 (Leu uxauxotrophic) with the yeast lysate. The KC8 ampicillin-resistant colonies were replica plated onto M9. Fluorescent DNA from M9+ colonies was transformed into DH5α for further manipulation.

DNA Sequencing—Oligonucleotide primers were synthesized on an ABI PCR-mate oligo-synthesizer. DNA sequencing was performed using an ABI 373 fluorescent automated DNA sequencer. The HIP cDNAs were confirmed to be in-frame with the GAL4-4XBD by sequencing across the AD-4XBD junction using an AD oligonucleotide (5′-GAA GAT ACC CCA CCA AAC-3′). Subsequently, primer walking was used to determine the remaining sequences. In order to obtain the most 5′ of the sequence of the HE2-25K gene, direct sequencing of a gel purified RT-PCR product was performed. First strand cDNA was generated using Superscript II reverse transcriptase, according to the manufacturer’s recommendation (BRL) following annealing of the antisense oligonucleotide 5′-CCG TCG GGA GAC TGA TTG CGT CTG-3′ to total RNA. Subsequent PCR was performed using the same reverse primer used for the RT reaction and a forward primer (5′-GAC ATG GCC AAC ATC GGC GCG-3′) derived from the β-2-25K nucleotide sequence. GST Fusion Protein Expression—The HIP-2 cDNA was released from the yeast strain Y190 (MATa trp1-901, his3-112, ade2-101, galA gal80A, URA3::GAL1-1ac2, Lys2::GAL-His3 cya) (28) and the derived HIP cDNA from nucleotide 5345 in exon 39 to nucleotide 6257 in exon 44 was cloned in-frame into the pGEX-4T-2 vector (32). Approximately 100 μg of the fusion protein was injected subcutaneously into BALB/c mice in the presence of Freund’s adjuvant. Following three additional boosts, spleen cells from the immunized mice were fused with NS1 myeloma cells to generate antibody secreting hybridomas. Culture supernatants were screened for anti-HD antibodies by enzyme-linked immunosorbent assay using bacterial TrpE as a negative control. Approximately 600 hybrid clones were generated, of which 35 recognized the fusion protein. Seven of these did not react with the TrpE portion of the fusion protein and were used for further analysis. Western blot analysis revealed that one of the clones secreted antibodies (GHM1) that recognized the 350-kDa human HD protein but did not recognize the HD murine homolog.

In order to generate an HD amino-terminal polyclonal antibody, a peptide corresponding to amino acids 3-16 (TLEKLMKAFESLKS) was synthesized and coupled to keyhole limpet hemocyanin using succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate. Two female New Zealand White rabbits were immunized with this peptide antigen with Freund’s adjuvant. Antibodies were purified on an affinity column of peptide bound to Sepharose. Purified antibodies (BK/P1) were used for Western blotting and detected a 350-kDa protein in human and mouse tissues and also in cell lines, including 293 cell and ES cell lines containing the HD gene, but not in ES cell lines lacking the HD gene.

Interaction of GST-HIP-2 and HD Protein—Five microliters of in vitro translated HD proteins (amino acids 1-1540 with either 44 or 16 glutamine repeats) were incubated with GST-HIP-2 and GST (10 μg each) in 500 μl of reaction buffer (20 μM Tris-HCl (pH 7.5), 120 mM NaCl) for 2 h at 4°C. Glutathione-Sepharose beads (10 μl) were then added and incubated for an additional 2 h. The beads were pelleted for 5 min, and washed 3 times with reaction buffer containing 3% Nonidet P-40. Samples were mixed with Laemmli sample buffer, applied to 7.5% SDS-PAGE gel, and transferred to PVDF membrane (Millipore). Immunodetection was performed using one of two HD NH2-terminal polyclonal antibodies (AP78 or BK/P1). For the experiments with 293 cell lysates, GST-HIP-2, GST-PTPase and GST were incubated with 300 μl of cell lysate (~500 μg of total protein) and 200 μl of reaction buffer.

Western Blot Analysis Using an Anti-Hip-e2-25K Antibody—In order to confirm that HIP-2 is in fact encoded for the E2-25K protein, an affinity-purified polyclonal anti-e2-25K antibody (33) was immunoreacted against the HIP-2 fusion protein after transfer on to a PVDF membrane from a 10% SDS-PAGE gel. Although this antibody is highly specific for detection of the E2-25K protein, it has been shown not to be used as an immunoprecipitating antibody.3 The membranes were blocked in 5% skim milk in Tris-buffered saline (TBS) (pH 7.4) and immunoreacted in blocking buffer with the anti-e2-25K polyclonal antibody (1:5000) for 1 h. After washing the membrane three times, the membranes were incubated with 1/5000 dilution of goat anti-rabbit IgG conjugated to horseradish peroxidase (Amersham) and visualized with ECL (Amersham).
times in TBS-T (Tris-buffered saline (pH 7.4), 0.05% Tween 20), a horseradish peroxidase-conjugated secondary antibody (1:10000) (Bio-Rad) was immunoreacted against the blots for 1 h followed by washing as described previously. The blots were subsequently incubated with ECL solution (Amersham) and exposed to ECL-Hyperfilm (Amersham). An aliquot of purified bE2-25K was used as a positive control. Fluorescent in Situ Hybridization Detection System and Image Analysis—The HE-25K was mapped to chromosome 4p14 by fluorescence in situ hybridization (FISH) (34) to normal human lymphocyte chromosomes counterstained with propidium iodide and DAPI. Biotinylated probe was detected with avidin-fluorescein isothiocyanate. Images of metaphase preparations were captured by a thermoelectrically cooled charge coupled camera (Photometrics). Separate images of DAPI banded chromosomes (35) and FITC targeted chromosomes were obtained. Hybridization signals were acquired and merged using image analysis software and pseudo colored blue (DAPI) and yellow (fluorescein isothiocyanate) as described (36) and overlaid electronically.

Northern Blot Analysis—Northern blot analysis was performed as described previously (33). Filters were hybridized with a PAGE purified antisense riboprobe as described previously (37). The riboprobe was labeled to a specific activity of ~10^6 cpm/μl and approximately 2.0 × 10^6 cpm/ml of labeled probe was used to probe the filter.

Tissue Distribution of the HIP (E2-25K)—The human embryonic kidney cell line HEK293 was grown in Dulbecco’s modified Eagle’s medium F-12 media. Cultured cells, human, mouse, and rat tissues were sonicated in a lysis buffer containing protease inhibitors (0.25 mM sucrose, 20 mM Tris-HCl (pH 7.5), 10 mM EGTA, 2 mM EDTA, 1 mM NaVO₄, 20 μg/ml β-glycero-phosphate with 10 μg/ml each of leupeptin, aproustin, antipain, soybean trypsin inhibitor, pepstatin, and 100 μg/ml phenylmethylsulfonyl fluoride). Protein extracts (as specified in figure legends) were separated on 10% SDS-PAGE mini-gels and transferred to PVDF membrane. Filters were then probed with an affinity-purified antibody to E2-25K (33) with detection by ECL Co-immunoprecipitation of Ubiquitin and HD—An Epstein-Barr virus-transformed cell line was used to determine if the HD protein is a substrate for ubiquitin conjugation. Cells from lymphoblasts of a heterozygote for HD were lysed in buffer containing Nonidet P-40, and supplemented with N-ethylmaleimide to inactivate endogenous de-ubiquitinating enzymes (38). Fifty micrograms of cell lysate was mixed with dilution buffer (50 mM Tris-HCl, pH 7.6, 1 mM EDTA, 1% Triton X-100) to give a final volume of 50 μl. Five microliters of affinity-purified rabbit polyclonal antibodies against ubiquitin were added and the mixture rotated at 4 °C for 3 h. Protein A-Sepharose (Sigma), 50 μl of a 1:1 slurry in dilution buffer, was then added, and the suspension was rotated at room temperature for 30 min. The resin was pelleted, then washed four times with 500 μl of dilution buffer. The beads were suspended in 40 μl of 2 × SDS-PAGE sample buffer and boiled for 1 min. The protein in the resin pellet and 20-μl aliquots were electrophoresed on 5.5% and 10% mini-gels. Proteins in each gel were transferred to PVDF membrane in a buffer containing 10 mM CAPS (pH 10) and 10% methanol. The blots derived from the 10% gel were probed with anti-ubiquitin antibodies. The blots derived from the 5.5% gel was probed with the anti-HD monoclonal antibody GHM1. In both cases, detection was by ECL, peroxidase-conjugated goat anti-rabbit IgG (Amersham) and exposure to ECL-Hyperfilm. An aliquot of purified bE2-25K was used as a positive control.

Co-affinity purification experiments were also performed using a human embryonic kidney cell line (HEK293) cell lysates to assess the interaction between HIP-2 and endogenous full-length HD protein. Incubation of HEK293 lysate with GST-HIP-2 linked to glutathione-Sepharose beads resulted in specific affinity purification of the 350-kDa HD protein on the beads (Fig. 2B). The HD protein failed to copurify with the GST-PTPase or GST protein alone. The detection of the HD protein on Western blots could be blocked by preincubation with peptide antigen (data not shown).

HIP-2 Is the Human E2-25K Ubiquitin Conjugating Enzyme—Analysis of sequence data revealed that the HIP-2 protein had complete amino acid identity with a previously described bovine E2-25K (bE2-25K) ubiquitin-conjugating enzyme (21). Our original HIP-2 cDNA spanned all but the most 5' 99 nucleotides of the published bovine sequence (21). Thus the N-terminal 33 residues of the E2 protein are not necessary for the interaction of E2-25K with huntingtin. The DNA sequence spanning the coding region for the first 33 amino acids was generated by RT-PCR using a 5' primer based on the published E2-25K sequence ("Experimental Procedures"). There is 95% nucleotide identity and 100% amino acid identity between the bE2-25K and this human E2-25K (hE2-25K) protein, both of which comprise 200 amino acids (Fig. 3A). Residue 23 in the hE2-25K amino sequence is a serine while the published bE2-25K has a threonine at this codon (21). Residue 23 in the hE2-25K amino sequence is a serine while the published bE2-25K has a threonine at this codon.

There are a total of 19 conservative nucleotide changes in the coding region and nine nucleotide changes in the known 3'-untranslated region sequence between human and bovine E2-25K cDNA (Fig. 3A). The HIP-2 cDNA isolated from the HD yeast two-hybrid screen contains an additional 3'-untranslated

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4 L. Mastrandrea and C. Pickart, unpublished experiments.
The complete identity between the bovine and human E2-25K enzymes places hE2-25K in the same class of conjugating enzymes as the E2- encoded by the UBC1, UBC4, and UBC5 genes of *S. cerevisiae* (21). The latter three E2 proteins have essential, and partially overlapping, functions in ubiquitin-mediated protein turnover (39–42). Cys-92 is the active site Cys of hE2-25K, based both on extensive homology of the surrounding sequence to the active site sequences of other E2s.

Fig. 1. Specific interaction of HIP-2 with the 5' region of the HD gene. A, the specificity of the HD-HIP-2 interaction is shown by the activation of the lacZ reporter gene only when the GAL4BD fusion protein containing amino acids 1–540 of HD (16 and 44 CAG) are co-transformed with HIP-2. The RAD7-SIR3 interaction was used as a positive control for the filter assays. Co-transformation of HIP-2 with other control constructs (pGBT9 and DMK∆BmH1pGBT9) failed to activate the yeast lacZ reporter gene. B, measurement of the HD-HIP-2 interaction using a liquid β-galactosidase assay. Only constructs containing amino acids 1–540 were able to produce β-galactosidase activity when co-transformed with HIP-2. The hatched box represents the GAL4 DNABD of the vector pGBT9. Both negative controls failed to produce β-galactosidase activity using the liquid assay. These assays were performed on three different (co-)transformants in triplicate, except for HD44EKpGBT9, where two transformants were evaluated in triplicate. BD, DNA-binding domain; AD, activating domain.
with ubiquitin. As expected based on its identity to the corre-
sonding portions of hE2-25K, the purified GST-HIP-2 fusion 
protein reacted strongly with affinity-purified antibodies 
 raised against bE2-25K (33), while GST exhibited no reaction 
protein reacted strongly with affinity-purified antibodies 
representing the amino terminus between amino acids 1 and 540, our data 
do not show any obvious influence of CAG length on the 
interaction. Furthermore, examination of patterns of expression of 
the hE2-25K in the frontal cortex from affected and unaffected 
individuals reveals no obvious differences (Fig. 4D). The HD Gene Product Is Ubiquitinated—Members of the 
family of ubiquitin conjugating enzymes participate in the con-
jugation of ubiquitin to cellular proteins. The interaction of 
E2-25K with HD thus suggested that HD might be a substrate 
for ubiquitination within the cell. To address this possibility, 
ubiquitin conjugates were immunoprecipitated from lysates of 
transformed lymphoblasts derived from an individual het-
ereozygous for HD. As seen in Fig. 5A, the immunoprecipitate 
obtained with the affinity-purified anti-ubiquitin antibodies 
faithfully reproduced the spectrum of conjugates present in the 
starting lysate (compare fourth and fifth lanes to first lane). As 
expected, no conjugates were observed in precipitates from 
control incubations lacking either antibodies or lysate (Fig. 5A, 
second and third lanes, respectively).

When the same immunoprecipitates were then probed with 
an HD-specific antibody (GHM1), precipitates from complete 
incubations (Fig. 5B, fourth and fifth lanes), but not precipi-
tates from control incubations (second and third lanes), were 
seen to contain an HD immunoreactive band running slightly 
above the major band seen in the starting lysate (first lane). An 
even higher molecular weight immunoreactive “smear” was 
also evident in the immunoprecipitates (fourth and fifth lanes, 
Fig. 5B). Similar results were obtained with a different anti-
HD antibody (BKP1 data not shown). The enhanced molec-
ular weight of these immunoreactive proteins, and their detec-
tion with anti-HD antibodies suggest that these proteins are 
ubiquitinated forms of HD. Immunoprecipitates derived from 
complete incubations also contained anti-HD-immunoreactive 
proteins that were smaller than intact HD (Fig. 5B, fourth and
FIG. 3. A, DNA sequence of the HIP-2 (hE2-25K) cDNA. The bovine E2-25K sequence is aligned and shows 95% nucleotide and 100% amino acid identity. The arrow indicates the first amino acid that was part of the cDNA isolated from the yeast two-hybrid screen. The putative active site cysteine is at residue 92 enclosed with a box. The sequence 5' to the arrow was generated from RT-PCR from human frontal cortex RNA. B, fluorescent in situ hybridization localized the hE2-25K protein to cytogenetic band 4p14.
fifth lanes). It remains to be determined whether these could represent ubiquitinated forms of processed or partially degraded forms of the HD protein.

DISCUSSION

Using the yeast two-hybrid system we have identified a protein that interacts with the gene product for HD (huntingtin interacting protein or HIP). This protein (HIP-2) was identified from a human brain Matchmaker™ cDNA library and has complete amino acid identity with a previously described bovine ubiquitin-conjugating enzyme. Human E2-25K also shares significant homology to other members of the large family of E2 proteins with especially striking similarity to the UBC-1, -4, and -5 enzymes of *S. cerevisiae*. These three E2 enzymes in yeast play an essential role in the catabolism of abnormal proteins and have partially overlapping functions (41, 42).

Recent discoveries have indicated that protein ubiquitination plays a major role in cellular regulation (24). Substrates of this pathway include the tumor suppressor protein p53 (44), other oncoproteins, transcription factors (45), and cell cycle regulatory proteins (46). In addition, more recently it has been demonstrated that the gene product for cystic fibrosis is also ubiquitinated and degraded by the ubiquitin dependent pathway (47).

The ubiquitin pathway functions in many processes that occur broadly in many cell types, for example, cell cycle progression (47). However, the pathway can also function in processes that are tissue- or cell type-specific, for example, terminal erythroid differentiation (25) and programmed cell death (48).

In the latter two examples, the role of the pathway is mediated in part through the induction of enzymes responsible for the conjugation of ubiquitin to target proteins, including several ubiquitin conjugating enzymes (25, 48).

It has also recently been suggested that altered patterns of cellular ubiquitination of target patterns could play a role in neurodegenerative disorders. Elevated levels of free ubiquitin pools are seen in Alzheimer's disease (49), Parkinson's disease (50), and amyotrophic lateral sclerosis (51), although the precise relationship of these findings to the disease phenotype is not yet clear. An increase in the number of ubiquitin reactive neurites has also been reported in HD brains compared to controls (52). Here we have demonstrated that the gene product for HD interacts with a specific ubiquitin-conjugating enzyme and is itself ubiquitinated. These results suggest that the HD protein may also be degraded via the ubiquitin mediated proteolytic pathway.

The 167 amino acids encoded by the original HIP-2 cDNA were completely identical to residues 33 through 200 of the previously described bovine ubiquitin conjugating enzyme bE2-25K, and we have shown that hE2-25K and bE2-25K are identical over their entire respective 200 amino acid sequences (Fig. 3A). The two-hybrid results indicate that the interaction be-
between E2-25K and HD protein minimally requires residues 33 through 200 of E2-25K and the first 540 residues of the HD protein (Fig. 2). Although this region of the HD protein contains the polyglutamine tract that is amplified in Huntington disease, the interaction between huntingtin and hE2-25K was not sensitive to the length of this polyglutamine tract, as inferred from the results of quantitative two-hybrid assay (Fig. 2) and qualitative in vitro interaction assays (Fig. 3A).

As detected by binding of the HD protein to a GST-HIP-2 fusion protein, E2-25K forms a complex with a heterologously expressed HD protein derivative in rabbit reticulocyte lysate (Fig. 3A) and with the endogenous HD protein in human embryonic kidney cells (Fig. 3B). A similar complex can be formed in yeast cells, as shown by the two-hybrid results (Fig. 2). Within cells, both E2-25K (33) and the HD protein (5, 6, 8) are localized in the same (cytosolic) compartment, supporting the potential in vivo relevance of the complex revealed by our data. While in the simplest case this is a binary complex, our results do not exclude a model in which complex formation requires an additional, unidentified protein to be present in all three types of cells. A requirement for such a component, specifically a ubiquitin-protein ligase (E3), might be expected based on an emerging model for specificity in ubiquitination. In this model, the E3 interacts directly with the target protein, while E2 specificity arises at the level of the E2-E3 interaction (53). So far, this model based on the analysis of the interactions between specific ubiquitinating enzymes and substrate-based ubiquitination signals in only a few substrates, notably p53 (54), (synthetic) N-end rule substrates (55), and mitotic cyclins (reviewed in Ref. 46). Thus, it is not excluded that an E2 protein can make a substantial direct contribution to substrate recognition in selected cases. However, even if a third component is required for the formation of a complex between E2-25K and the HD protein, the involvement of E2-25K appears to be specific.

A high degree of specificity in the interactions of E2-25K with either substrates or enzymatic cofactors (if any) had been apparent from the results of prior biochemical analyses of this E2 protein (see, for example, studies outlined in Ref. 56). Prior to the present work, the only substrates known to be recognized by E2-25K was ubiquitin itself. This latter reaction is E3-independent in vitro, and results in the formation of long mult ubiquitin chains such as are known to efficiently target proteins for degradation by the 26S proteasome (57). It remains to be determined whether E2-25K can generate such ubiquitin chains on the HD protein.

E2-25K is highly related to yeast UBC4 and -5. These E2s function, probably with an unidentified E3, in the turnover of a large body of short-lived proteins in yeast (21). There are numerous homologs of yeast UBC4 in mammals, many of which appear to be broadly expressed (58). None of these homologs was detected in the two-hybrid screen, providing an indication of specificity in the HD-E2-25K interaction. Moreover, in a converse two-hybrid screen of a Matchmaker library from murine brain using bE2-25K as the bait, we identified only a single non-HD positive. Our failure to detect HD is not surprising, since E2-25K interacts with the amino-terminal region of the 350-kDa HD protein (Fig. 2), and the relevant region of the cDNA is likely to be under-represented in the library. However, the results of this converse screen provide a strong indication that E2-25K does not engage in a broad, nonspecific set of protein-protein interactions.

Our finding of an interaction between the HD protein and E2-25K immediately suggested that the HD protein might be a substrate for ubiquitination within the cell. Immunoprecipitated ubiquitin conjugates indeed contained protein species that reacted with monoclonal and polyclonal anti-HD antibodies (Fig. 5 and data not shown). These results contrast with those obtained in a prior study by Aronin et al. (12). It is likely that the failure to detect ubiquitinated HD in this earlier work reflected the use of postmortem material. The steady-state level of conjugated forms of a given substrate depends on the...
relative rates of substrate ubiquitination and de-ubiquitination (38). Since ubiquitination but not de-ubiquitination is ATP-dependent, ubiquitin conjugates will rapidly decay postmortem (59). Consistent with this expectation, in simple Western analysis of extracts prepared from postmortem human brain (with anti-ubiquitin antibodies), we did not observe ubiquitin conjugates at a detectable level (data not shown). On the other hand, inclusion of a thiol alkylating agent during lymphoblast lysis quantitatively inhibited endogenous de-ubiquitinating enzymes and enabled us to detect ubiquitinated HD in these cells (Fig. 5).

While our results on ubiquitination (Fig. 5) were necessarily obtained in peripheral cells rather than in brain tissue (above), it is expected that these events occur in brain as well, since E2-25K and HD protein are co-expressed in those cells (e.g. Fig. 4). On the other hand, these results do not resolve an unanswered enigma for HD and other disorders associated with CAG expansion, which is that degeneration is specifically observed in neurons, even though the genes harboring these mutations are widely expressed. Similar questions must be raised for the presenilin 1 and 2 genes which contain mutations associated with some forms of familial Alzheimer’s disease (44, 60, 61). While E2-25K is broadly expressed (Ref. 25 and Fig. 4), it is intriguing that a slightly larger protein recognized by the polyclonal anti-E2 antibody is predominately expressed in the central nervous system (Fig. 4C), with a pattern of expression that appears to parallel the neuropathology of HD. This expression pattern might be expected for an interactive protein with potential to explain the selective neuronal loss in this disease. Additional studies will be needed to determine the molecular identity of this protein.

While our results indicate that the interaction between the HD protein and E2-25K is not modulated by CAG expansion in the HD gene, it is possible that the mutated HD protein interacts differentially with other proteins, and that such interactions in turn influence either the interaction observed here or the activity of E2-25K.

Recently using the yeast two-hybrid system, Li et al. (62) have identified another protein (HAP-1) interacting with the HD protein and E2-25K is not modulated by CAG expansion in the HD gene, it is possible that the mutated HD protein interacts differentially with other proteins, and that such interactions in turn influence either the interaction observed here or the activity of E2-25K.

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