Huntingtin Is Present in the Nucleus, Interacts with the Transcriptional Corepressor C-terminal Binding Protein, and Represses Transcription*

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Huntingtin is a protein of unknown function that contains a polyglutamine tract, which is expanded in patients with Huntington’s disease (HD). We investigated the localization and a potential function for huntingtin in the nucleus. In human fibroblasts from normal and HD patients, huntingtin localized diffusely in the nucleus and in subnuclear compartments identified as speckles, promyelocytic leukemia protein bodies, and nucleoli. Huntingtin-positive nuclear bodies redistributed after treatment with sodium butyrate. By Western blot, purified nuclei had low levels of full-length huntingtin compared with the cytoplasm but contained high levels of N- and C-terminal huntingtin fragments, which tightly bound the nuclear matrix. Full-length huntingtin co-immunoprecipitated with the transcriptional corepressor C-terminal binding protein, and polyglutamine expansion in huntingtin reduced this interaction. Full-length wild-type and mutant huntingtin repressed transcription when targeted to DNA. Truncated N-terminal mutant huntingtin repressed transcription, whereas the corresponding wild-type fragment did not repress transcription. We speculate that wild-type huntingtin may function in the nucleus in the assembly of nuclear matrix-bound protein complexes involved with transcriptional repression and RNA processing. Proteolysis of mutant huntingtin may alter nuclear functions by disrupting protein complexes and inappropriately repressing transcription in HD.

Huntingtin, a protein with an estimated molecular mass of 350 kDa, contains a polyglutamine tract near its N terminus that when expanded beyond 37 glutamines causes HD1 (1).

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‡ The abbreviations used are: HD, Huntington’s disease; aa, amino acid; CtBP, C-terminal binding protein; CtIP, C-terminal interacting corepressor protein; PML, promyelocytic leukemia protein; TBP, TATA-binding protein; snRNP, small nuclear ribonucleoprotein; PBS, phosphate-buffered saline; CSK, cytoskeletal buffer; IP, immunoprecipitation; PIPES, 1,4-piperazinediethanesulfonic acid.
ter promyelocytic leukemia protein (PML) (26). These results suggest that the N terminus of mutant huntingtin may disrupt neuronal function in HD by interfering with nuclear organization and transcriptional regulation. Recent studies have not addressed the ability of full-length normal huntingtin to repress transcription or the ability of larger N-terminal fragments of mutant huntingtin to repress transcription through a mechanism that directs huntingtin to DNA.

We investigated the localization of huntingtin in the nucleus using immunohistochemical and biochemical methods. We also tested the hypothesis that full-length wild-type huntingtin interacts with CtBP. Finally, we assessed normal and mutant huntingtin’s ability to repress transcription and the potential effects of huntingtin truncation on this function.

**EXPERIMENTAL PROCEDURES**

**Cell Culture—**Human fibroblasts were obtained from the Coriell Cell Repository (Camden, NJ) (normal, repository number GM08399A; homozygous for HD gene, repository number GM04857) and cultured according to the repository’s recommended conditions. Clonal striatal cells (COS-1 cells), and MFC-7 cells were cultured as previously described (14, 27). COS-1 cells derived from African green monkey kidney obtained from the American Type Culture Collection (Manassas, VA) were grown in 10% Dulbecco’s modified Eagle’s medium containing 4.5 g/liter glucose, 4 mM glutamine, and 1.5 g/liter sodium bicarbonate. All cells were maintained with 5% CO₂ at 37 °C.

**Antibodies—**Four polyclonal anti-huntingtin antisera have been characterized in previous publications (2, 28) and were used to the following regions of huntingtin: Ab1, aa 1–17; Ab585, aa 585–754; Ab2527, aa 2527–2547; and Ab2911, aa 2911–3140. Another polyclonal antibody, Ab1173, made to aa 1173–1196 of huntingtin, recognizes full-length huntingtin in protein extracts from brain (results not shown). Monoclonal antibodies mAb2170 and mAb2168 made to aa 1247–1645 and aa 2146–2541 of huntingtin, respectively, were obtained from Chemicon (Temecula, CA). The monoclonal antibody mAb1574, which specifically recognizes expanded polyglutamine stretches, was also obtained from Chemicon. Polyclonal anti-CtBP was made against a synthetic peptide containing the N-terminal 20 amino acids of CtBP. An N-terminal lysine residue was added for coupling to KHL. The antibody was purified against the synthetic peptide bound to bovine serum albumin. Antibodies against the following proteins were obtained from the indicated sources: splicing factor SC-35 (monoclonal; FLAG, FLAG-Affi-Gel (monoclonal, clones M5 and M2), and β-tubulin, Sigma; snRNP 70 kDa (monoclonal), American Research Products (Belmont, MA); nucleolin, also known as C23 (monoclonal, clone MS-3), and GallDBD (monoclonal, clone RK531), Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); calnexin, Stressgen (Victoria, BC); panhistone, Roche Molecular Biochemicals; monoclonal antibody to PML (clone 5E10), Dr. J. Don Chen (University of Massachusetts Medical Center, Worcester, MA); and body-labeled secondary antibodies, Molecular Probes, Inc. (Eugene, OR); and Cy3- and peroxidase-labeled secondary antibodies, Jackson Immunoresearch Laboratories (West Grove, PA).

**Immunocytochemistry—**Immunofluorescence labeling of cultured cells was performed as previously described (29). Cells were washed in PBS, fixed for 10 min with 4% paraformaldehyde in PBS containing calcium and magnesium ions, washed three times, permeabilized with 0.1% Triton X-100 for 30 min, and then blocked with 4% normal goat serum for 1 h at room temperature. Alternatively, cells were fixed in ice-cold methanol for 1 min, followed by permeabilization and blocking steps. Fixed cells were then incubated overnight in primary antibodies at 4 °C, washed with PBS, incubated with secondary antibodies for 2 h at room temperature, washed again, and then dehydrated stepwise and mounted in Cytoseal (Stephens Scientific, Riverdale, NJ). Individual images for each excitation wavelength (488 or 568 nm) were obtained using a Bio-Rad 1024 laser confocal microscope through a ×100 objective with oil immersion and merged in Adobe Photoshop (Salinas, CA). Propidium iodide (Molecular Probes) was added with the secondary antibody at 1 μg/ml. Immunoperoxidase labeling of mouse brain sections was performed as previously described (17).

**Isolation of Nuclei—**Cells were washed three times with ice-cold PBS and then incubated on ice for 5 min in lysis buffer (0.1% Triton X-100, 20 mM N-2-hydroxy-1,1-bis(hydroxymethyl)ethylglycine-NaOH, 250 mM sucrose, 25 mM KCl, 5 mM MgCl₂, pH 7.8, plus complete, mini, EDTA-free protease inhibitor tablets (Roche Molecular Biochemicals). Plates were scraped, and crude homogenates (T) were collected and then centrifuged at 2000 × g at 4 °C for 10 min to obtain a low speed supernatant containing cytoplasmic constituents and a crude nuclear pellet. The crude nuclear pellet was washed twice with 1 ml of lysis buffer, and nuclei were reisolated by centrifugation. The pellet was resuspended in 25% iodixanol in lysis buffer (Optiprep; Accurate Chemicals, Westbury, NY) and layered on a discontinuous iodixanol gradient (30 and 35%) and centrifuged in a SW41 swing bucket rotor at 10,000 × g for 20 min at 4 °C. Nuclei were collected from the 30–35% interface. All fractions were stored on ice for the duration of the experiment before storing at −70 °C. Total protein was estimated using the Bradford method. Fractions were analyzed by SDS-PAGE and Western blot analysis performed as previously described (2). Antibody dilutions for Western blots were as follows: Ab1, 0.5 μg/ml, calnexin, 1:1000; anti-β tubulin, 1:1000; panhistone, 5 μg/ml.

**Nuclear Matrix Preparations—**Nuclear matrices were prepared as described by Nickerson et al. (30) and originally described by He et al. (31). For immunocytochemistry, cells were washed twice in PBS with Ca²⁺/Mg²⁺ and then incubated sequentially with cytoskeletal buffer (CSK: 0.5% Triton X-100, 10 mM PIPES, 100 mM NaCl, 300 mM sucrose, 3 mM MgCl₂, 1 mM EGTA, pH 6.8), extraction buffer (CSK buffer with 250 mM ammonium sulfate, no NaCl), digestion buffer (CSK buffer containing DNase I 400 units/ml, RNase-free; Roche Molecular Biochemicals), 50 mM NaCl, and high salt buffer (CSK buffer with 2 mM NaCl). Cells were fixed with paraformaldehyde after extractions were complete. All buffers contained RNasin inhibitor (Promega) with 1 mM dithiothreitol and protease inhibitors. For biochemical analysis, nuclei...
as template. The PCR fragment was gel-isolated, restricted with Bax/H11032 and ligated into pcDNA3-Gal4DBD restricted with BamHI and ApoI, and ligated into pcDNA3-Gal4DBD restricted with BamHI and ApoI. The construct was verified by sequencing. Plasmid DNA was prepared using Qiagen EndoFree Plasmid Maxi Kit for all applications (Valencia, CA).

Immunoprecipitations—BDS-1 cells grown in 60-mm plates and exposed to transfection reagents but no DNA (mock) or transfected with 5 µg of each plasmid alone (pcDNA3, pFH774-18 pFH774-100, pcDNA-Gal4DBD, and pcDNA-Gal4-CtBP) or 2.5 µg of two plasmids together. Transfections were performed using 30 µl of Superfect reagent (Qiagen). Twenty hours after transfection, cells were washed in PBS and lysed into 0.5 ml of IP buffer containing 50 µM Tris, 1% Nonidet P40, 250 mM NaCl, 5 mM EDTA, pH 7.4. Lysates were collected and centrifuged at 10,000 × g for 10 min at 4 °C to remove insoluble material. One-tenth volume of each supernatant was saved as “total lysate.” The remaining supernatant was incubated with 1.2 µg of Gal4DBD monoclonal antibody for 2 h on ice. 30 µl of protein A-Sepharose (Sigma) slurry in IP buffer (50 mg/ml) was added to each tube and incubated for another 1 h at 4 °C. The entire complex was collected by centrifugation at 10,000 × g, and the supernatant was discarded. The pellet was washed four times in IP buffer. Proteins were eluted by the addition of 50 µl of SDS sample buffer containing dithiothreitol and boiled for 5 min. Input lysates and precipitates were analyzed by SDS-PAGE and Western blot using standard methods, and detection was performed with ECL (Amersham Biosciences, Inc.).

[FIG. 2] a, confocal immunofluorescence of endogenous mouse huntingtin in clonal striatal cells using huntingtin antibodies Ab1 and Ab2527 fixed with paraformaldehyde (Para) and methanol (MeOH). b, immunohistochemistry of normal mouse brain using huntingtin antibody Ab2527 shows huntingtin in cell nuclei. Shown on the right is a neuron with diffuse nuclear staining and more intense huntingtin labeling in subnuclear structures (arrow). Scale bars in b, left, 25 µm; right, 10 µm. c, effects of sodium butyrate. Nuclear huntingtin changes from a mostly diffuse pattern to a punctate pattern after treatment with sodium butyrate. Cells were grown in normal growth medium (Controls) or in the presence of 20 mM sodium butyrate for 5 days and then fixed with paraformaldehyde and labeled with huntingtin antibody Ab2527.

were isolated as described above, and equal volumes of nuclei were suspended sequentially in 1 ml of PIPES buffer without Triton X-100, followed by CSK buffer, extraction buffer, digestion buffer, and high salt buffer. After each extraction, nuclei were collected by centrifugation at 2000 × g and then resuspended in 1 ml of the next buffer or in 100 µl of 2× SDS-PAGE sample buffer.

Plasmids—cDNAs for full-length FLAG-tagged huntingtin encoding 18 or 100 glutamines and designated FH774 were subcloned into the expression vector pcDNA3 and have been described previously (27). Plasmids pcDNA3-Gal4DBD (encoding the DNA binding domain of the yeast transcriptional activator Gal4), pcDNA3-Gal4-CtBP, and pcDNA-Gal4-CtBP, which contain PCR products encoding full-length CtBP1 or C-terminal interacting protein (CtIP) inserted in frame after Gal4, have been described (14). To create Gal4-huntingtin fusion constructs, pFH774-18, -46, and -100 (27) were restricted with BamHI and ApoI. The insert cDNAs encoding base pairs 314–3210 of the HD gene with 18, 46, or 100 CAG repeats were ligated into pcDNA3-Gal4DBD restricted with BamHI and ApoI, creating pGal4-H774-18, -46, and -100. To create pGal4-H774-18 and -46, an NsiI to NotI restriction fragment representing base pairs 3210–6343 of the HD cDNA was ligated into the pGal4-H774 vectors restricted with NsiI and NotI. Gal4-H774 constructs bearing larger glutamine expansions were unstable in bacteria. The Gal4-H774 full-length constructs were made by ligating a KpnI to NotI restriction fragment, representing base pairs 2078–9763 of the HD cDNA, into the pGal4-H774, -46, and -100 vectors restricted with KpnI and NotI. To create the pGal4-PLDL3 construct, a PCR product was generated using primers within the HD gene (5′-ATTGGATCCGCGCTAATTCTGCGA′-3′ and 5′-TAGCTGCCG-3′ primer sequences; Invitrogen) and full-length huntingtin cDNA as template. The PCR fragment was gel-isolated, restricted with BamHI and ApoI, and ligated into pcDNA3-Gal4DBD restricted with BamHI and ApoI. The construct was verified by sequencing. Plasmid DNA was prepared using Qiagen EndoFree Plasmid Maxi Kit for all applications (Valencia, CA).

RESULTS

Huntingtin Immunoreactivity in the Nucleus Is Diffuse and Localized in Subdomains with Other Nuclear Proteins—Huntingtin localization was examined in normal human fibroblasts with a panel of polyclonal antibodies (Ab1, Ab585, Ab1173, Ab2527, and Ab2911) and monoclonal antibodies (mAb2170 and mAb2168) that recognize different regions of huntingtin (Fig. 1a). Fixation of cells with paraformaldehyde or methanol revealed different compartments of huntingtin labeling in nuclei. In cells fixed with paraformaldehyde or methanol fixation reduced or eliminated the diffuse staining and revealed different compartments of huntingtin labeling in nuclei. Small dots and patches of intense labeling were seen with Ab585 and Ab2527 (Fig. 1b). Western blot analysis confirmed that similar levels of Gal4 fusion proteins were expressed among transfections. Cells cotransfected with Gal4 fusion constructs were expressed as a percentage of luciferase activity measured in cells cotransfected with pcDNA-Gal4DBD.

[FIG. 1] a,imunoreactivity staining pattern in normal human fibroblasts. Ab1, Ab585, Ab1173, Ab2527, and Ab2911. b, Methanol fixation reduced or eliminated the diffuse staining and revealed different compartments of huntingtin labeling in nuclei. Small dots and patches of intense labeling were seen with Ab585 and Ab2527 (Fig. 1b). Western blot analysis confirmed that similar levels of Gal4 fusion proteins were expressed among transfections. Cells cotransfected with Gal4 fusion constructs were expressed as a percentage of luciferase activity measured in cells cotransfected with pcDNA-Gal4DBD.

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staining observed with anti-huntingtin polyclonal antisera was blocked in the presence of the antigenic peptide but not unrelated peptide (data not shown).

Huntingtin immunoreactivity also was present in the nuclei of clonal striatal cells fixed with paraformaldehyde or methanol. When cells were grown under basal conditions, Ab1 and Ab2527 antisera detected diffuse nuclear labeling and small dots (Ab1) and large patches (Ab2527) (Fig. 2a). In normal mouse brain, cortical and striatal neurons also showed diffuse and patchy nuclear labeling with Ab2527 using the immunoperoxidase method (Fig. 2b).

To identify the nuclear subdomains that contain huntingtin, we examined the co-distribution of huntingtin in fibroblasts with proteins found in the nucleolus, nuclear speckles, and PML bodies. Nucleolin was used to mark the nucleolus (32, 33); snRNP and SC-35 labeling was used to identify speckles (34–36); and PML localization was used to recognize PML bodies (37). The overlap between huntingtin and marker proteins was variable between cells within the same culture dish. Huntingtin labeling was clearly detected within the boundaries of the nucleolus (Fig. 3a). Huntingtin antiserum Ab2527 stained the nucleolus in 100% of the cells using methanol fixation. Huntingtin was frequently found in speckles and less frequently found in PML bodies (Fig. 3, b–d). Huntingtin co-distributed with 10–50% of the patches labeled for snRNP, 10–80% of the nuclear regions containing SC-35, and 0–30% of the PML bodies, depending on the cell. Using propidium iodide to visualize DNA, we found that huntingtin was located predominantly in the interchromatin space with small sites of interface with DNA (visualized with propidium iodide) throughout the nucleoplasm.

Fig. 3. Huntingtin localizes to multiple subnuclear domains. Primary human fibroblasts were double-stained with various huntingtin antisera (green, left column) and markers of different nuclear compartments (red, center column). Colocalization can be seen as yellow in merged images (right column). a, huntingtin is present in the nucleolus. Cells were fixed with paraformaldehyde (top panel) or methanol (bottom panel) and stained with Ab1 or Ab2527 and an antibody to nucleolin. b and c, Huntingtin localizes to nuclear speckles. b, cells were fixed with methanol and double-labeled with Ab585 or Ab2527 and the RNA splicing factor snRNP 70 kDa. c, cells were fixed with methanol and double-stained with Ab1173 or Ab2527 and SC-35. d, a small proportion of huntingtin can localize to PML bodies. Cells were fixed with methanol and double-stained with Ab1173 and a monoclonal antibody to PML. e, huntingtin detected with Ab2527 is located predominantly in the interchromatin space with small sites of interface with DNA (visualized with propidium iodide) throughout the nucleoplasm.
Two other drugs were tested for their effect on nuclear localization of huntingtin in COS-1 cells. Actinomycin D inhibits RNA synthesis and induces nuclear accumulation of p53 (39), a huntingtin-interacting protein (11). Leptomycin B blocks nuclear export of proteins that have CRM1 nuclear export receptor sequences (40). Treatment with actinomycin D (1–20 μg/ml) or leptomycin B (18–200 nM) had no effect on the nuclear localization of endogenous huntingtin. Treatment with these drugs also failed to affect the distribution of exogenously expressed N-terminal or full-length huntingtin, which localized in the cytoplasm as determined by immunofluorescence with antisera against N- and C-terminal epitopes (data not shown).

N-terminal and C-terminal Fragments of Huntingtin Are Present in Purified Nuclear Fractions—Nuclear fractions were prepared from normal human fibroblasts and clonal striatal cells and examined by Western blots with anti-huntingtin antisera. In normal fibroblasts, the signal intensity for full-length huntingtin was significantly lower in the nuclear fraction compared with the cytoplasm (Fig. 4a, compare lanes C and N). The nuclear fractions also contained N-terminal huntingtin fragments of about 160, 200, and 300 kDa, which were more abundant than full-length huntingtin and appeared specific to the nucleus. C-terminal huntingtin fragments of about 175 and 300 kDa were detected in the nuclear fraction with Ab2527 (Fig. 4a) and also with mAb2170, mA2168, and Ab2911 (data not shown). Based on signal intensity on the Western blot, the antisera had different efficiencies for the fragments. In mouse clonal striatal cells, full-length huntingtin could be detected in the nuclear fraction with Ab1 (Fig. 4b). Again the signal intensity for full-length huntingtin was low compared with the cytoplasm (Fig. 4b, compare lanes C and N). In addition, N-terminal fragments of ~140, 120, and 55 kDa were present in the nucleus. There was a marked enrichment of C-terminal huntingtin fragments of ~70, 120, and 200 kDa in the nuclear fraction compared with cytoplasm (Fig. 4b, lanes C and N). The two cell lines, which differ in both their species and cell type, had different arrays of fragments in the nucleus. Furthermore, whereas human fibroblasts showed an enrichment of N-terminal fragments, mouse clonal striatal cells showed an enrichment of C-terminal fragments. These results suggest that N-terminal and C-terminal huntingtin fragments may contribute to the huntingtin staining seen by immunofluorescence.

Huntingtin Is Present in Soluble and Matrix Compartments of the Nucleus—Some proteins that localize to nuclear subdomains, such as SC-35 (41), are found within an insoluble compartment referred to as the matrix (31, 42). To determine whether endogenous huntingtin exists in the matrix compartment, purified nuclei from normal human fibroblasts were treated with a series of buffers to extract soluble proteins (see “Experimental Procedures”). Nuclei were first equilibrated in the PIPES-based buffer required for the procedure and then reisolated by centrifugation. This resulted in a significant loss of full-length huntingtin and many of the N-terminal huntingtin fragments, although some could still be detected on very long exposures of the Western blot (Fig. 5a). The 160-kDa N-terminal fragment detected with Ab1 (short arrow) was partly soluble, since its signal intensity was reduced after treatment with DNase I and 2 m NaCl, although histone was greatly reduced by this treatment. Further treatment with DNase I and 2 m NaCl reduced the level of the 160-kDa fragment somewhat, but a significant amount of signal remained, suggesting that it was tightly bound to the nuclear matrix (Fig. 5a, lanes 3–5). A portion of full-length huntingtin (detected with Ab2527; arrowhead) and the large C-terminal fragments of about 175 and 300 kDa (long arrow) were partly soluble, since their signal intensities were reduced after treatment with Triton X-100 (Fig. 5a, compare lane 2 with lane 1). To ensure detection of full-length huntingtin and the C-terminal bands after the extractions, we loaded much larger volumes in the Western blot probed with Ab2527. The subsequent addition of ammonium sulfate to the detergent buffer removed the small amount of full-length hun-
ammonium sulfate. Numerous small brightly fluorescent nuclear dots were resistant to extraction by ammonium sulfate (Fig. 5, b and c) and to treatment with DNase I or 2 mM NaCl (Fig. 5b). As expected, immunoreactivity for the nuclear matrix marker, SC-35, was present after all treatments (Fig. 5b), whereas histone labeling was largely removed by treatment with ammonium sulfate (Fig. 5b), and DNA staining with propidium iodide was absent after treatment with DNase I (data not shown). The results of these experiments suggested that while full-length huntingtin exists in a soluble state in fibroblast nuclei, N- and C-terminal fragments of huntingtin are anchored tightly to the nuclear matrix at numerous discrete foci. The partial sensitivity of huntingtin fragments to DNase I in Western blot assay suggests that huntingtin makes contacts with DNA. However, based on the observed segregation of huntingtin and propidium iodide labeling seen by fluorescence microscopy (Fig. 3c), a minority of the total nuclear huntingtin is present as these sites of interface.

**Full-length Huntingtin Interacts with CtBP**—Huntingtin contains a PXDLS motif (PLDLS at amino acids 1082–1086 for human). This motif is highly conserved and found in proteins that interact with the transcriptional corepressor CtBP (12–17, 43). To determine whether huntingtin interacts with CtBP, FLAG-tagged full-length huntingtin with 18 or 100 glutamines (FH9771-18 and -100) and full-length CtBP fused to the DNA binding domain of Gal4 (Gal4-CtBP) were co-expressed in COS-1 cells, and co-immunoprecipitates were isolated with a Gal4 antibody. Western blot analysis with anti-huntingtin antibody Ab1 showed that full-length normal and mutant huntingtin with 100 glutamines co-immunoprecipitated with Gal4-CtBP but not with the control, Gal4DBD (Fig. 6a, arrowhead). Another N-terminal huntingtin fragment of about 300 kDa was also recovered in the immunoprecipitates (Fig. 6a, arrow). Density of the immunoprecipitate signals (both bands) from four experiments showed that significantly more wild-type huntingtin was coprecipitated in the assay than mutant huntingtin per amount of Gal4-CtBP immunoprecipitated (one-sample t test, p < 0.01; see “Experimental Procedures”). These data suggest that polyglutamine expansion of huntingtin impaired the interaction with CtBP. In the inverse experiment, using anti-FLAG antiserum to immunoprecipitate full-length FLAG-tagged huntingtin, we detected Gal4-CtBP in the precipitate (Fig. 6b). Consistent with the previous experiment, less Gal4-CtBP coprecipitated with mutant huntingtin compared with wild-type huntingtin (Fig. 6b). To determine whether the region of the PLDLS site in huntingtin was sufficient for interactions with CtBP, we co-expressed full-length CtBP with a fusion protein containing Gal4DBD followed by amino acids 964–1136 of huntingtin (Gal4-PLDLS). This huntingtin construct encoding 172 aa included the PLDLS site. CtBP did not coprecipitate with Gal4-PLDLS isolated with the Gal4 antibody despite evidence for good protein expression from all plasmids and efficient precipitation of Gal4-PLDLS (data not shown). The polyclonal antiserum used to detect coprecipitated CtBP on Western blots was raised against the N terminus of CtBP1 (see “Experimental Procedures”) and recognized a protein of the predicted size of about 48 kDa in COS-1 cells over-expressing CtBP and in mouse brain (Fig. 6c). This result indicated that huntingtin sequences not contained in the Gal4-PLDLS construct must be required in addition to the PLDLS site for interaction with CtBP. In summary, results showed that full-length wild-type huntingtin is capable of interacting with CtBP, that polyglutamine expansion impedes this interaction, and that the huntingtin’s PLDLS site alone is insufficient for the interaction with CtBP and requires additional huntingtin sequence.
Localization of CtBP in Cultured Cells and in Neurons of Mouse Brain—In the mouse brain, CtBP1 immunoreactivity was seen primarily in neuronal nuclei (Fig. 7a), including neurons in the cortex and striatum that are affected in HD. Endogenous CtBP was detected primarily in the nuclei of human fibroblasts, with low level staining present in the cytoplasm; nuclear labeling was mainly diffuse in fibroblasts but with methanol fixation could be found in nuclear dots (Fig. 7, b and d). Similar to fibroblasts, endogenous CtBP in COS-1 cells was localized mainly in nuclei (Fig. 7c, long arrows). Exogenous expression of CtBP in COS-1 cells using full-length CtBP1 resulted in a predominately nuclear localization of CtBP, although staining was also observed in the cytoplasm (Fig. 7c, short arrow). These data are consistent with the results of others showing nuclear localization of CtBP (17). Confocal immunofluorescence of cells fixed with paraformaldehyde and then double-labeled with anti-CtBP1 and anti-huntingtin mAb2168 showed that CtBP1 and huntingtin show areas of overlap in fibroblast nuclei, although with this huntingtin antibody overlap was not extensive (Fig. 7d). A partial but not complete overlap in immunostaining was also described for the CtBP interactor human polycomb protein 2 (17). With methanol fixation, colocalization of huntingtin with CtBP was observed in nuclear dots in some cells (Fig. 7d, arrows).

Full-length Huntingtin Represses Constitutive Transcription, but N-terminal Fragments of Huntingtin Require Polyglutamine Expansion to Repress Transcription—Wild-type huntingtin interacts with two corepressors, CtBP (this study) and NCoR (10). We tested whether full-length huntingtin could repress transcription. Huntingtin proteolysis is thought to be important for HD pathogenesis, and others have reported that very short fragments of mutant huntingtin repress or interfere with transcription (11, 25). We asked whether larger fragments of mutant huntingtin, similar in size to those we found in nuclei, could repress transcription. The assay we used measured the ability of a protein that is brought to DNA to repress constitutive transcription of a reporter gene driven by the thymidine kinase promoter. Proteins or regions of interest are...
fused to the DNA binding domain of the transcriptional activator protein Gal4 (Gal4DBD), which targets the protein to Gal4 binding sites proximal to the promoter. We tested three sets of Gal4-huntingtin constructs (Fig. 8). For positive controls, we used Gal4-CtBP (14, 17) and CtIP, another PXDLS-containing protein that represses transcription in part by recruiting CtBP (14), fused to Gal4 (Gal4-CtIP). Repression was defined as a reduction in luciferase activity of the mean plus 1 S.D. value to 40% of the control, Gal4DBD (greater than 2.5-fold reduction in luciferase activity compared with control). This criterion was routinely met or exceeded by our positive controls, Gal4-CtBP and Gal4-CtIP (Fig. 9, G-CtIP and G-CtBP). We found that Gal4-full-length huntingtin with normal (18-glutamine) or mutant (100-glutamine) glutamine tracts repressed transcription to about 25 and 23% of control, respectively (Fig. 9a). Satisfactory expression levels could not be achieved with full-length mutant huntingtin with 46 glutamines. Significant repression to 20–29% of control was also observed with mutant Gal4-huntingtin fusion proteins containing the shortest huntingtin fragment (aa 1–969) with expanded polyglutamine tracts (Fig. 9c, G-H3221-46 and G-H3221-100). In contrast, the corresponding wild-type Gal4-huntingtin construct did not meet our criteria for repression (Fig. 9c, G-H3221-18). Constructs encoding the same region of huntingtin, with an N-terminal FLAG tag instead of Gal4DBD, did not repress transcription (data not shown). We found no repression with Gal4-H6343-18 and Gal4-H6343-46, although Western blots of the cell lysates confirmed that exogenous huntingtin had been expressed (Fig. 9b). We were unable to test Gal4-H6343-100 because it was unstable in bacteria during propagation.

These results show that full-length wild-type and mutant huntingtin can repress transcription. In addition, N-terminal fragments of mutant huntingtin (aa 1–969) containing 46 and 100 glutamines can repress transcription in a polyglutamine-dependent manner when brought to DNA, but the corresponding wild-type fragment does not repress transcription when targeted to DNA.

**DISCUSSION**

Our results show that full-length wild-type huntingtin is in the nucleus, interacts with CtBP, and can repress transcription. The presence of huntingtin in the nucleus has been difficult to demonstrate, despite the well documented interactions of huntingtin with proteins that function in the nucleus. We have shown that normal and mutant huntingtin are present diffusely throughout the nucleus, in subcompartments of the
nucleus, and in nuclear bodies that store proteins needed for pre-mRNA splicing (SC-35, snRNP) and transcription (PML). The subnuclear distribution of huntingtin, first recognized in mouse embryonic fibroblasts (8), occurred in nuclei of human fibroblasts and in mouse brain neurons. These results suggest that huntingtin’s heterogeneous localization in the nucleus may be a feature shared by many cell types. Huntingtin’s presence in the nucleolus was unexpected and suggests that huntingtin may function in RNA processing or ribosome biogenesis. The previous lack of success in detecting wild-type huntingtin in the nucleus with many huntingtin antisera may be due to fixation conditions. Some compartmentalized nuclear antigens such as Sm proteins (44) and PML (37) are optimally detected using methanol/acetone fixation rather than paraformaldehyde. Antibodies that easily detect huntingtin in the nucleus after paraformaldehyde fixation such as Ab2527 (this study) and antiseraum 1356 (8) recognize epitopes closer to the C terminus, suggesting that these epitopes are more accessible when this fixation method is used.

Nuclear bodies such as speckles, where huntingtin was found, are dynamic structures that may serve as storage areas for proteins but can also be sites of active transcription activity and RNA processing (45). Some proteins that concentrate in nuclear bodies rapidly move throughout the nucleoplasm, residing in nuclear bodies for relatively short periods of time (46, 47). Proteins in nuclear subdomains may also bind to the nuclear matrix to form stable multiprotein complexes, such as the human SWI-SNF complex, which influences chromatin remodeling and transcription (48, 49), and SC-35, a matrix bound splicing factor found in speckles (40). In clonal striatal cells, treatment with sodium butyrate, an agent that alters transcription through inhibition of histone deacetylases, caused changes in the immunostaining of huntingtin-positive nuclear bodies. This suggests that the movement of huntingtin in the nucleus may occur in response to changes in transcriptional activity. The abundant levels of N-terminal and C-terminal huntingtin fragments bound to the nuclear matrix imply that huntingtin proteolysis may be important in regulating its subnuclear location and function.

We identified CtBP as a binding partner for full-length human huntingtin, which contains the amino acid sequence PLDLS. The PLDLS sequence is present in human, mouse, pufferfish, and pig huntingtin. The PXDLS motif is also found in other proteins that interact with CtBP (12–17, 43). The PLDLS site in huntingtin is significantly distal to the polyglutamine tract in a region where no other interactions have yet been identified. Protein interactions with CtBP can require specific modification (acetylation) of the target protein (50) and can be affected by the amino acid sequences flanking the PXDLS motif as well as by overall tertiary protein structure (51). This may explain why we did not see an interaction with the 172-aa internal region of huntingtin containing the PLDLS site or with the large N-terminal construct (aa 1–2009 of huntingtin), which also included the PLDLS site. CtBP was first iden-

**Fig. 9. Repression of constitutive transcription.** a, wild-type and mutant Gal4-full-length huntingtin (G-H9763-18 and -100) repress transcription. Western blots were probed with anti-Gal4DBD to verify the expression of Gal4-full-length wild-type huntingtin (top blot, wt) and Gal4-CtBP (bottom blots). Gal4-full-length mutant huntingtin was not recognized by the anti-Gal4 monoclonal but was detected using mAb1573, which specifically recognizes highly expanded glutamine repeats (middle blot, mt). A representative sample from each triplicate of the experiment is shown. Equal volumes were loaded per lane. b, G-H9763-18 and -46 with aa 1–2009 of huntingtin do not repress transcription. Western blots were probed with mAb2166 (top) or anti-Gal4DBD (bottom) to verify expression of Gal4-truncated huntingtin and Gal4-CtBP. Endogenous huntingtin is also indicated (arrowhead). c, mutant Gal4-huntingtin constructs with aa 1–969 of huntingtin repress transcription in a polyglutamine length-dependent manner. The graph shows results found with Gal4-H3221-18, -46, or -100. Western blots were also performed to confirm expression of fusion proteins (not shown). Repression assays were performed as described under “Experimental Procedures.” Results are expressed as a percentage of luciferase activity obtained from cells cotransfected with Gal4DBD (G), Gal4-CtBP (G-CtBP), and Gal4-CtBP (G-CtBP) were used as positive controls. Shown are the results of a typical experiment performed in triplicate; error bars indicate S.D. *, fusion protein met or exceeded our criteria for repression (mean + 1 S.D. ≤ 40% compared with the control protein Gal4DBD).
tified as a target of the viral oncoprotein E1A, an interaction that relieves repression of genes necessary for progression through the cell cycle (12). CtBP can repress transcription through histone deacetylase-dependent mechanisms (24) but also can repress promoters unresponsive to the drug trichostatin, which alleviates histone deacetylase-dependent repression (14, 15). Some CtBP binding partners that contain the PXDLS motif, such as CtIP, require interactions with CtBP to repress transcription (14), while others, such as human polycomb 2, can repress transcription even if interactions with CtBP are disrupted (17).

The polyglutamine expansion in huntingtin that causes HD may affect cellular function by altering the strength of interactions between huntingtin and its binding partners. Polyglutamine expansion strengthens interactions between huntingtin and the nuclear proteins HYPA/FBP-11 (9), NCoR (10), CREB-binding protein, or mSin3a (11), whereas polyglutamine expansion weakens interactions between huntingtin and CA150, a transcriptional coactivator (52), and CtBP, a transcriptional corepressor. In the case of CtBP, a weakened interaction with mutant huntingtin compared with wild-type may disrupt the stability of protein complexes through which CtBP regulates transcription. A reduced interaction between CtBP and huntingtin might also involve a function unrelated to gene transcription. Another CtBP family member, brefeldin A-ADP-ribosylated substrate, has 97% identity at the amino acid level to CtBP, differing only at the extreme N terminus (53). Brefeldin A-ADP-ribosylated substrate functions in the cytoplasm in vesicle scission at the Golgi by modifying phospholipids (54). Huntingtin is also present on Golgi structures and can promote membrane tubulation (28, 29), suggesting that an interaction between huntingtin and CtBP or brefeldin A-ADP-ribosylated substrate could affect Golgi function. The abnormal interaction between mutant huntingtin and CtBP is relevant to HD, since both huntingtin and CtBP are present in neurons known to be affected in HD.

We found that full-length wild-type huntingtin repressed transcription. The premise that wild-type huntingtin might repress transcription was based on evidence that huntingtin interacts with two transcriptional co-repressors, NCoR (10) and CtBP (this study). The repression we observed with wild-type huntingtin may be due to its ability to recruit CtBP, perhaps in coordination with other proteins such as NCoR or perhaps through another pathway. Further studies will be necessary to determine how huntingtin exerts repression. Our results do not rule out the possibility that wild-type huntingtin can have other effects on transcription. For example, CtBP can activate gene transcription, depending on the cellular context (55, 56); therefore, huntingtin through an interaction with CtBP might activate rather than repress transcription. Transcriptional activation of brain-derived nerve growth factor has therefore been shown to occur upon induction of wild-type huntingtin in stable cell lines (57).

Our results showing that N-terminal huntingtin fragments with an expanded polyglutamine tract repress transcription are in agreement with the results of Steffan et al. (11), who demonstrated repression with exon 1 of mutant huntingtin (aa 1–88 of wild-type huntingtin with an additional 70 glutamines) and a p53-dependent promoter. The mutant N-terminal fragments that repressed transcription in our assay were larger in size than the one studied by Steffan et al. (11) and were similar in size to the N-terminal huntingtin fragments that bound tightly to the nuclear matrix. Both normal and mutant huntingtin are subject to proteolytic processing in the brain (58, 59), although it is unclear where this processing occurs within neurons. Proteolysis of huntingtin in the nucleus may normally occur in cells to terminate or modulate huntingtin function. Limited proteolysis of mutant huntingtin in the nucleus might be sufficient to unveil a gain of function, abnormal transcriptional repression by N-terminal mutant huntingtin fragments.

The repression caused by N-terminal mutant huntingtin fragments could be mediated by polyglutamines, perhaps through steric hindrance, or may involve increased binding to the corepressor NCoR, which interacts more with the amino terminus of mutant huntingtin than wild-type huntingtin (10). The N-terminal fragments of mutant huntingtin repressed transcription only when targeted to DNA by the Gal4 DNA binding domain, suggesting that mutant huntingtin can repress transcription by directly affecting components of the basal transcription machinery rather than indirectly by sequestering essential transcription factors into aggregates. Studies by Steffan et al. (11) also suggest that transcriptional repression may occur independent of aggregate formation. Recently, p231HB/PYHB, a protein that interacts with the polyproline region in the N terminus of huntingtin, was shown to be a DNA-binding factor (60), further suggesting that huntingtin is in close proximity with DNA in situ.

In summary, our findings suggest several roles for huntingtin in the nucleus that could be affected by polyglutamine expansion. Huntingtin may act as a scaffold to support multi-protein complexes that regulate transcription or other nuclear events and link them to the nuclear matrix. Because of its altered interactions with CtBP and other nuclear proteins, mutant huntingtin may disrupt the formation of protein complexes that regulate transcription and RNA processing. Proteolysis of mutant huntingtin in the nucleus may produce N-terminal fragments of huntingtin that repress transcription aberrantly in HD and cause neuronal dysfunction.

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REFERENCES

1. Huntington’s Disease Collaborative Research Group (1993) Cell 72, 971–983
2. DiFiglia, M., Sapp, E., Chase, K., Schwarz, C., Meloni, A., Young, C., Martin, E., Vonsattel, J. P., Carraway, E., Boyce, F. M., and Aronin, N. (1995) Neuro 14, 1057–1081
3. Gutekunst, C. A., Levey, A. I., Heilman, C. J., Whaley, W. L., Yi, H., Nash, N. R., Rees, H. D., Maddox, J. J., and Hersch, S. M. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 8710–8714
4. Sharp, A. H., Loev, S. J., Schilling, G., Li, S.-H., Li, X.-J., Bao, J., Wagster, M. Y., Ketzuk, J. A., Stein, J. P. L., Hedgren, J., Sisodia, S., Snyder, S. H., Dawson, T. M., Ryugo, D. K., and Ross, C. A. (1995) Neuron 14, 1065–1074
5. Blakes, P. J., Day, M., Sapp, E., Schwarz, C., Sheth, A., Kim, J., Young, A. B., Penney, J., Golden, J., Aronin, N., and DiFiglia, M. (1996) J. Neurosci. 16, 5523–5535
6. Sapp, E., Schwarz, C., Chase, K., Blakes, P. G., Young, A. B., Penney, J., Vonsattel, J. P., Aronin, N., and DiFiglia, M. (1997) Ann. Neurol. 42, 604–612
7. Hoogeveen, A. T., Willemsen, R., Meyer, N. D., DeRooij, D. E., Roos, R. A. C., Van Ommeren, G. J. B., and Galjaard, H. (1993) Hum. Mol. Genet. 2, 2069–2073
8. DeRooij, D. E., Dorsman, J. D., Snoor, M. A., Den Dunnen, J. T., and Van Ommeren, G. J. (1996) Hum. Mol. Genet. 5, 1093–1099
9. Faber, P. W., Barnes, G. T., Srinidhi, J., Chen, J., Gussella, J. F., and MacDonald, E. M. (1998) Hum. Mol. Genet. 7, 1463–1474
10. Boutell, J. M., Thomas, P., Neal, J. W., Weston, V. J., Duce J., Harper, P. S., and Jones, A. L. (1999) Hum. Mol. Genet. 16, 3392–3403
11. Steffen, J. S., Razantes, A., Spasic-Boskovic, O., Greenwald, M., Zhu, Y.-Z., Gohler, H. Wanker, E. D., Bates, G. P., Regevan, D. E., and Thompson, L. M. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 6763–6768
12. Schaepfer, U., Boyd, J. M., Verma, S., Uhlmann, E., Subramanian, T., and Chinnadurai, G. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 10467–10471
13. Crippa-Chipil-Filipe, F., Ducrot, C., Maira, S.-M., and Wasylyk, B. (1999) EMBO J. 18, 3339–3403
14. Meloni, A. R., Smith, E. J., and Nevins, J. R. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 9764–9769
15. Koipally, J., and Georgopolous, K. (2000) J. Biol. Chem. 275, 19594–19602
16. Postigo, A. A., and Dean, D. C. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 6683–6688
17. Sewalt, R. G. A. B., Gunster, M. J., van der Blag, J., Satij, D. P. E., and Otte, A. P. (1999) Mol. Cell. Biol. 19, 777–787
18. Soudou, F., Finkbeiner, S., Deyva, D., and Greenberg, M. E. (1998) Cell 95, 55–66
19. Peters, M. F., Nucifora, P. F., Jr, Kushi, J., Seaman, H. C., Cooper, J. K., Herring, W. J., Dawson, V. L., Dawson, T. M., and Ross, C. A. (1999) Mol. Cell Neurosci. 14, 121–128
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