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Expansion of polyglutamine repeat in huntingtin leads to abnormal protein interactions involving calmodulin

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ABSTRACT **Huntington's disease (HD) is an inherited neurodegenerative disorder associated with expansion of a CAG repeat in the *IT15* gene. The *IT15* gene is translated to a protein product termed huntingtin that contains a polyglutamine (polyGln) tract. Recent investigations indicate that the cause of HD is expansion of the polyGln tract. However, the function of huntingtin and how the expanded polyGln tract causes HD is not known. We investigate potential protein–protein interactions of huntingtin using affinity resins. Huntingtin from brain extracts is retained on calmodulin(CAM)-Sepharose in a calcium-dependent fashion. We purify rat huntingtin to apparent homogeneity using a combination of DEAE-cellulose column chromatography, ammonium sulfate precipitation, and preparative SDS/PAGE. Purified rat huntingtin does not interact with CAM directly as revealed by ^{125}I-CAM overlay. Huntingtin forms a large CAM-containing complex of over 1,000 kDa in the presence of calcium, which partially disassociates in the absence of calcium. Furthermore, an increased amount of mutant huntingtin from HD patient brains is retained on CAM-Sepharose compared to normal huntingtin from control patient brains, and the mutant allele is preferentially retained on CAM-Sepharose in the absence of calcium. These results suggest that huntingtin interacts with other proteins including CAM and that the expansion of polyGln alters this interaction.**

Huntington's Disease (HD) is an autosomal dominant progressive neurodegenerative disorder that is characterized by chorea, psychiatric disturbances, and dementia (1). Recently, the gene causing HD, designated *IT-15*, was identified by the Huntington's Disease Collaborative Research Group and was found to contain an unstable CAG repeat whose length correlates with disease severity (2). *IT-15* lacks homology to other genes except for the CAG repeat which is also present in four other neurodegenerative diseases that have expansion of CAG in the coding region of their genes (3–5). In addition, in the N terminus of huntingtin there is a proline repeat, and a proline-rich region which fits the consensus for an SH3-binding domain.

The mRNA and protein product of *IT-15* and its mutant allele are widely distributed in both the brain and peripheral tissues, with no enrichment in the basal ganglia (6–16). There are no changes in the level of expression of mRNA or protein in patients with HD, except for decreased levels that correlate with cell loss in the striatum (6–16). Thus, the pathophysiology of HD likely depends on the expansion of polyglutamine (polyGln) in the mutant allele. Recent evidence clearly shows that the CAG repeat in *IT-15* is translated into polyGln in both normal and expanded alleles (11–16). Gene targeted disruption indicates that HD is not due to a loss of function, because neither heterozygote nor homozygote animals for the disrupted gene have an HD-like phenotype (17, 18). A number of investigators have proposed that HD is due to a gain of function caused by abnormal protein–protein interactions related to the expanded polyGln tract (2–5, 11–21). Perutz and colleagues (19–21) have proposed that polyGln may function as polar zippers and that their expansion may cause disease by increasing the amount or affinity of potential protein–protein interactions. In this study we purify huntingtin to apparent homogeneity from rat brain. We show that huntingtin interacts indirectly with calmodulin (CAM) only in the presence of calcium. Huntingtin from HD patient brains also interacts indirectly with CAM and it appears to bind more avidly than huntingtin from control brains. Moreover, the mutated allele, unlike the normal allele, interacts indirectly with CAM in the absence of calcium.

MATERIALS AND METHODS

Immunoblot Analysis. Immunoblot analysis of huntingtin is performed as described (11, 16). Briefly, SDS/3–12% gradient PAGE is used to separate huntingtin. After electrophoresis, proteins are electroblotted onto a nitrocellulose membrane and incubated with anti-huntingtin antibody AP78 (diluted 1:5000) (11). Huntingtin is visualized by enhanced chemiluminescense (Kirkegaard & Perry Laboratories).

Affinity Chromatography of Huntingtin. Rat brain is homogenized in 10 vol of 50 mM Tris·HCl, pH 7.3, containing 1 mM 2-mercaptoethanol and a cocktail of protease inhibitors [1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml pepstatin A, 10 μg/ml leupeptin, and 1 mM benzamidine]. The homogenate is centrifuged at 100,000 × *g* for 1 h at 4°C. The supernatant is incubated with various resins (1 ml/100 μl of 50% resin) (Table 1). The resin suspension is incubated for 2 h at 4°C with constant mixing. After a brief centrifugation at 10,000 × *g*, the supernatant is discarded and the pellet is extensively washed with the homogenization buffer. The proteins are disassociated from the resin by boiling in SDS/PAGE sample buffer for 5 min at 100°C. The proteins are then resolved by an SDS/3–12% gradient or 4% PAGE and huntingtin is monitored by immunoblot analysis as described above.

For CAM-Sepharose affinity chromatography, 0.1% Triton X-100 and various concentrations of $CaCl_2$ or EGTA are included in the homogenization buffer during the incubation and washing steps. In the experiments using human huntingtin, the human brain tissue is homogenized in 50 mM Hepes buffer (pH 7.3), containing 0.5% Triton X-100, 1 mM 2-mercaptoethanol, and the cocktail of protease inhibitors. The samples of human neocortex were obtained from Johns Hopkins University, Division of Neuropathology, HD Brain Resource

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Abbreviations: HD, Huntington's disease; polyGln, polyglutamine; CAM, calmodulin.

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Table 1. Binding properties of huntingtin

Resin	Binding
2′–5′ ADP-Sepharose	negative
Agarose beads	negative
CAM-Sepharose	positive
Cellulose	negative
CM-cellulose	negative
DEAE-cellulose	positive
Glutathione-agarose	positive
Heparin-agarose	positive
Wheatgerm agglutinin-agarose	negative
ConA-agarose	negative

The retention of huntingtin on various resins is monitored by immunoblot analysis as described. Retention of huntingtin on the various resins is indicated by a positive and the lack of retention is indicated by a negative.

Center. The postmortem delay is 6, 6, and 7 h for the control brains and 6, 6, 7, and 7.5 h for the HD brains. The CAG repeat length of the mutant allele is 42, 43, 60, and 63, respectively, for heterozygote HD cases used in this study.

Purification of Huntingtin. All procedures are performed at 4°C unless otherwise indicated. Immunoblot analysis for huntingtin is used to monitor the purification. Typically, 300 g of frozen rat brain (Pel-Freez Biologicals) is homogenized in 3000 ml of 50 mM Tris·HCl (pH 7.3), containing 1 mM 2-mercaptoethanol, 2 mM EGTA, and the cocktail of protease inhibitors. The homogenate is centrifuged at 100,000 × *g* for 1 h at 4°C. The supernatant obtained is applied to a DE-52 column (Whatman, 2.7 × 127 cm), pre-equilibrated with the homogenization buffer. The column is washed with the homogenization buffer, followed by pre-elution with the homogenization buffer containing 100 mM NaCl. Huntingtin is eluted from the column by the homogenization buffer containing 300 mM NaCl. Huntingtin is further enriched by ammonium sulfate precipitation (25% saturation). The precipitated pellets are redissolved in the homogenization buffer. The huntingtin sample is applied to a preparative SDS/3–12% gradient PAGE. After electrophoresis, the gel is horizontally cut into 0.25-cm wide slices and the gel slices are ground in the homogenization buffer. After a brief centrifugation at 14,000 × *g*, proteins are collected from the supernatant and an aliquot of each fraction is used for huntingtin immunoblot analysis. The fraction containing the highest amount of huntingtin is then applied to a second preparative SDS/PAGE. Proteins are reversibly visualized by copper negative-staining of the SDS/PAGE (Bio-Rad). Gel pieces containing individual protein bands are sliced out. The proteins are unfixed from the gel according to the procedure provided by the manufacturer (Bio-Rad) and are extracted by grinding the gel in the homogenization buffer, followed by collecting the supernatant after a brief centrifugation (14,000 × *g*). The authenticity of purified huntingtin is verified by SDS/PAGE and immunoblot analysis.

CAM Overlay Analysis. CAM overlay experiments are performed as described (22, 23). Briefly, protein samples are resolved by SDS/PAGE and then transferred to a nitrocellulose membrane. Calcineurin, a known CAM-binding protein (22, 23), is used for the positive control. The nitrocellulose membrane is incubated in buffer A [4% BSA/0.05% sodium azide/0.1 mM $CaCl_2$ in Tris·HCl buffered saline (TBS) buffer (pH 7.4) for 3 h at 25°C]. ^{125}I-CAM is added into buffer A (0.1 μCi/ml) and the incubation is continued for an additional 3 h at 25°C. The membrane is then washed five times for 5 min each in buffer B (0.3% Tween-20/0.1 mM $CaCl_2$ in TBS buffer (pH 7.4) at 25°C). The membrane is air-dried and exposed to an autoradiography film or a PhosphorImager (Molecular Dynamics) cassette. A parallel experiment is performed in the presence of 1 mM EGTA instead of $CaCl_2$.

Gel-Filtration Column Chromatography of Huntingtin. Huntingtin is prepared as described above from rat brain. The homogenization buffer contains either 0.1 mM $CaCl_2$ or 4 mM EGTA. The sample is preconcentrated by ammonium sulfate precipitation (25% saturation). The concentrated sample (1 ml) is loaded to a Sephacryl S300 gel-filtration column (Pharmacia, 0.16 × 60 cm, V_t = 120 ml), pre-equilibrated with the homogenization buffer containing 0.1 mM $CaCl_2$ or 4 mM EGTA. Aliquots of the fractions are analyzed by immunoblot analysis for huntingtin. Molecular weight standards include thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), and aldolase (158 kDa) (Pharmacia). The column void volume (V_0) is determined by Blue Dextran 2000. The molecular weight of huntingtin is determined by a linear calibration curve according to the method provided by the manufacturer.

RESULTS

Huntingtin Interacts Indirectly with CAM. To characterize potential protein–protein interactions with huntingtin, we

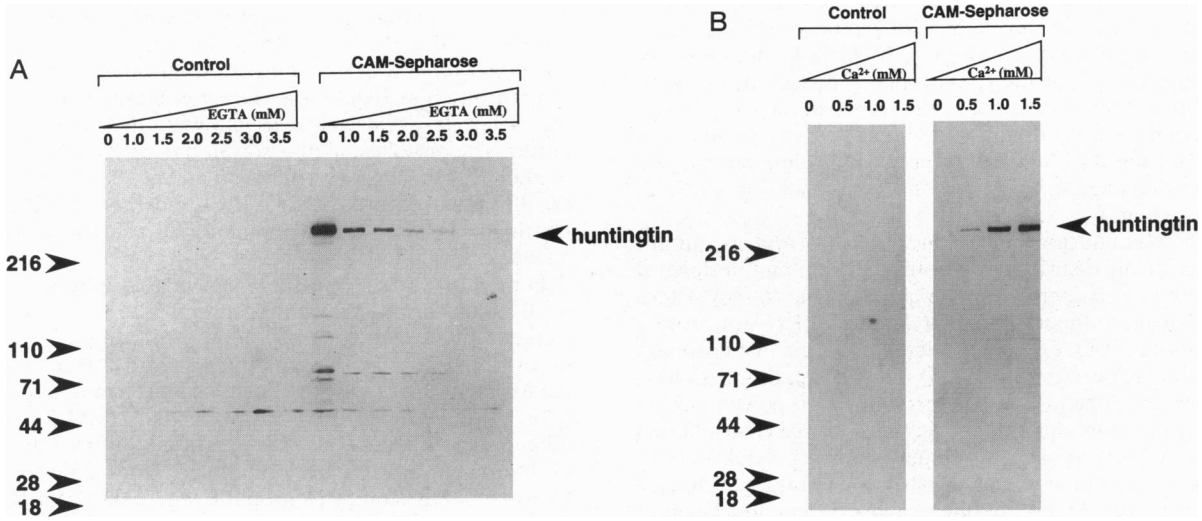

FIG. 1. Huntingtin binds to CAM-Sepharose in the presence of calcium. Huntingtin is prepared from rat brain in the absence (*A*) and presence (*B*) of 2 mM EGTA. Increasing amounts of EGTA or $CaCl_2$ are included in the incubation with CAM-Sepharose and the wash. G-25 Sephadex is used as the control resin. Proteins are released from the resins and resolved by a SDS/3–12% gradient PAGE. Huntingtin is monitored by immunoblot analysis. Molecular weight standards (in kDa) are indicated on the left. These results have been replicated at least five times and representative blots are shown.

analyzed the ability of huntingtin to bind to various affinity resins using an affinity-purified antibody directed against the N terminus of huntingtin (11) (Table 1). Huntingtin binds to DEAE-cellulose, but not CM-cellulose. It is eluted from DEAE-cellulose at 300 mM to 350 mM NaCl in a gradient from 100–600 mM NaCl (data not shown). DEAE-cellulose enriches huntingtin approximately 3- to 5-fold from a 100,000 $\times$ *g* supernatant. Thus, huntingtin has anionic binding properties. Huntingtin also binds to heparin-agarose; however, this interaction appears to be anionic, in that it is eluted from heparin-agarose with high salt concentrations (data not shown). Interestingly, huntingtin binds to glutathione-agarose and it is eluted by reduced glutathione (data not shown). Huntingtin does not bind to cellulose and agarose control resins. In addition, huntingtin does not bind to 2′-5′ ADP-Sepharose, wheatgerm agglutinin-agarose, and ConA-agarose.

Huntingtin binds to CAM-Sepharose in the presence of calcium (Table 1). To further evaluate the binding of huntingtin to CAM-Sepharose we examined the binding in the presence and absence of calcium (Fig. 1). When rat brain is homogenized in 50 mM Tris·HCl buffer, huntingtin avidly binds CAM-Sepharose (Fig. 1*A*). Increasing concentrations of EGTA diminish huntingtin interactions with CAM-Sepharose and 3.0 mM EGTA completely eliminates the binding. When huntingtin is homogenized in 50 mM Tris·HCl buffer containing 2 mM EGTA, huntingtin fails to bind CAM-Sepharose. The addition of calcium re-establishes the binding of huntingtin to CAM-Sepharose and binding is observed at calcium concentrations between 0.5–1.5 mM (Fig. 1*B*). At concentrations of calcium greater than 2 mM, huntingtin precipitates from the supernatant (data not shown).

To ascertain whether huntingtin binds directly to CAM we purified huntingtin to apparent homogeneity from rat brain (Fig. 2). Rat huntingtin is purified using a combination of DEAE-cellulose column chromatography, ammonium sulfate precipitation, and preparative SDS/PAGE (see *Materials and Methods* for details). Purified rat huntingtin migrates as a single band of 350 kDa on SDS/PAGE visualized by silver staining (Fig. 2*A*). Immunoblot analysis for huntingtin indicates that there is at least a 1000-fold purification of huntingtin (Fig. 2*B*). Utilizing rat purified huntingtin we performed ^{125}I-CAM overlay experiments. ^{125}I-CAM fails to bind to purified rat huntingtin, but avidly binds to the CAM binding protein, calcineurin (Fig. 3). Furthermore, huntingtin immunoprecipitated by anti-huntingtin antibodies fails to bind ^{125}I-CAM (data not shown).

Because huntingtin does not appear to interact with CAM directly, this indicates that huntingtin may be interacting with other proteins, some of which may bind to CAM. To examine for this possibility we conducted gel-filtration column chromatography in the presence and absence of calcium. Prior to size exclusion chromatography, huntingtin is concentrated by ammonium sulfate precipitation (25% saturation). Huntingtin is then redissolved with 50 mM Tris·HCl buffer containing protease inhibitors and 0.1 mM $CaCl_2$ or 4 mM EGTA. In the presence of calcium huntingtin migrates as a large protein complex ranging in size from 600 to more than 1000 kDa (Fig. 4*A*). Consistent with the notion that huntingtin interacts with CAM binding proteins is the observation that in the presence of EGTA huntingtin migrates as a much smaller protein complex ranging in size from 400 to 700 kDa (Fig. 4*B*). Native huntingtin migrates at approximately 500 kDa on native PAGE and sucrose gradients (data not shown).

Mutant Huntingtin Binds to CAM-Sepharose Abnormally. To ascertain how the expansion of polyGln in huntingtin may affect the interaction of huntingtin with CAM, we analyzed the ability of huntingtin from human autopsy tissues from control and HD patients to be retained on CAM-Sepharose in the presence and absence of calcium (Fig. 5). In control brains, moderate amounts of huntingtin bind to CAM-Sepharose in the presence of calcium and this binding is eliminated by 1.5 mM EGTA (Fig. 5*A*). As previously reported, mutant huntingtin from cortex is present at a significantly lower level (16) and it cannot be detected in the starting material (200 μg of total protein) on gradient SDS/PAGE (Fig. 5*B*). In striking contrast, marked amounts of huntingtin from HD tissue containing both the normal and mutant allele (63 Gln) bind to CAM-Sepharose. ^{125}I-CAM overlay experiments directed against immunoblots containing mutant huntingtin also suggest that mutant huntingtin does not directly interact with CAM similar to normal huntingtin (data not shown). The CAM-Sepharose appears to enrich for the mutant allele. In the presence of EGTA the amount of huntingtin interacting with CAM-Sepharose is reduced. Similar to the control brains, 1.5 mM EGTA eliminates interaction of normal huntingtin (lower allele) with CAM-Sepharose. However, the mutant huntingtin

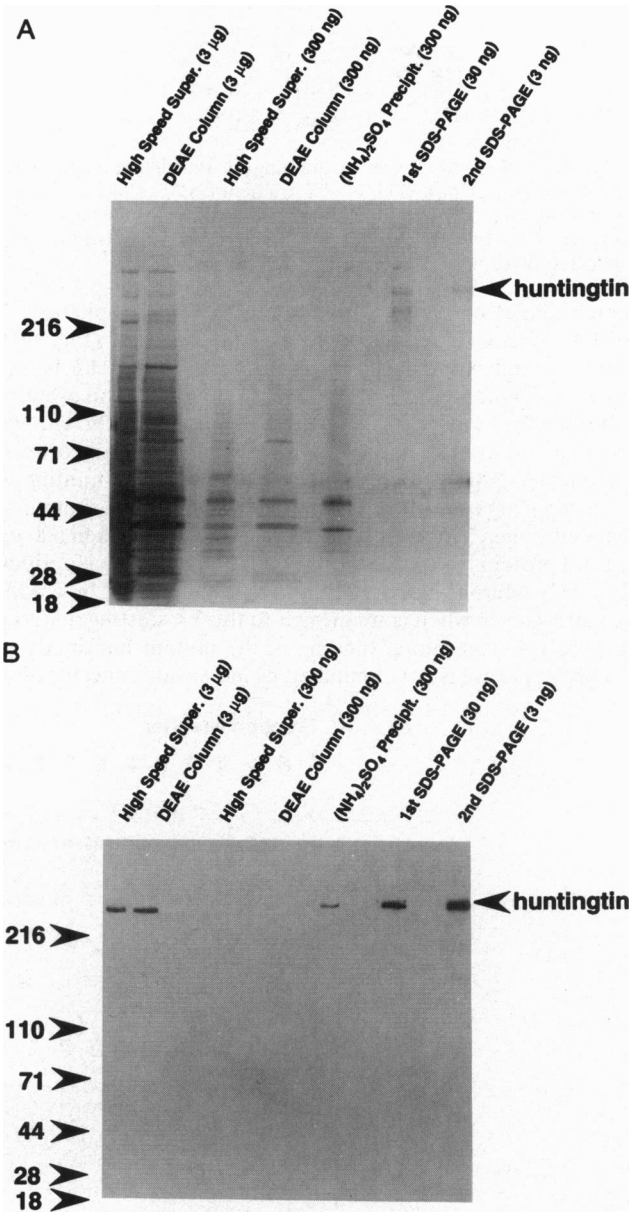

FIG. 2. Purification of huntingtin from rat brain. Huntingtin samples from various purification steps are loaded to a SDS/3–12% gradient PAGE. The proteins are visualized by silver-staining (*A*). Huntingtin is monitored by immunoblot analysis (*B*). Molecular weight standards (in kDa) are indicated on the left. To detect huntingtin in the crude brain extract, at least 3 μg of total protein is needed for immunoblot analysis. Super., supernatant; precipit., precipitation.

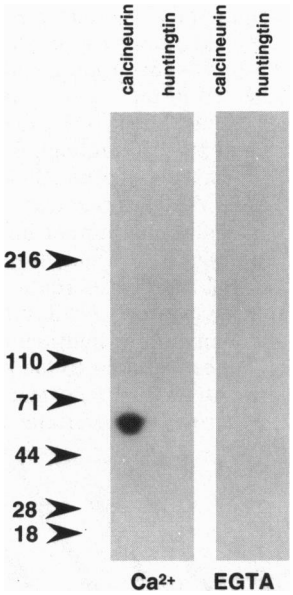

FIG. 3. ^{125}I-CAM overlay of huntingtin. Purified rat huntingtin (150 ng) is resolved by a SDS/3–12% gradient PAGE. Calcineurin (0.5 μg) is included for the positive control. The overlay is performed in the presence of 0.1 mM $CaCl_2$ or 1 mM EGTA. These results have been replicated twice and representative blots are shown.

(upper allele) continues to interact with CAM-Sepharose at EGTA concentrations as high as 3 mM EGTA (Fig. 5*B*). Previous studies indicate that mutant huntingtin can be revealed by loading 3× starting material (600 μg of total protein) and resolving by SDS/4% PAGE (Fig. 5*C*). Consistent with the previous report (16), mutant huntingtin from human cortex is present at a significantly lower level than the normal huntingtin and it appears as a smear of more slowly migrating immunoreactivity when 200 μg of total protein is loaded. When 600 μg of total protein is loaded the mutant allele is readily identified (Fig. 5*C*). Mutant huntingtin is markedly enriched by CAM-Sepharose even when comparing it to the 3× starting material (Fig. 5*C*). Furthermore, binding of the mutant huntingtin to CAM-Sepharose is not eliminated by increasing concentration of EGTA, whereas the binding of the normal huntingtin to CAM-Sepharose is markedly reduced with 3.0 mM EGTA. Similar results are obtained from three additional cases of HD with polyGln expansions of 42, 43, and 60 and with two additional age-matched control cases (data not shown).

DISCUSSION

The major findings of this study are the observations that huntingtin binds indirectly to CAM in a calcium-dependent fashion and forms a large calcium/CAM-dependent protein complex and that the expansion of the polyGln tract in huntingtin leads to an altered interaction with CAM. Several lines of evidence indicate that huntingtin interacts indirectly with CAM. ^{125}I-CAM overlays have been used to identify several CAM-binding proteins (22, 24). The failure of purified and immunoprecipitated huntingtin to bind ^{125}I-CAM indicates that it does not directly bind CAM. Furthermore, there are no CAM consensus binding domains within huntingtin. However, huntingtin from brain extract is retained on CAM-Sepharose only in the presence of calcium, similar to most other CAM-binding proteins (22–25). Thus, huntingtin probably interacts with other proteins that bind to CAM in a calcium-dependent fashion. Consistent with our notion that huntingtin binds in a calcium/CAM-dependent manner to a protein complex is our observation that huntingtin migrates as a complex of 600 to over 1000 kDa on a gel-filtration column in the presence of calcium, but only migrates as a complex of 400–700 kDa in the absence of calcium. Because CAM is an acidic protein, it is unlikely that huntingtin is being retained on a CAM-Sepharose column because of its anionic binding properties. Thus, the ability of huntingtin to be retained on a CAM-Sepharose column is not due to huntingtin directly interacting with CAM, but it is due to its ability to bind to another protein that has calcium/CAM-binding properties. Alternatively, calcium may promote huntingtin forming complexes with itself that has calcium/CAM-binding properties.

Our demonstration that mutant huntingtin with expanded polyGln from HD patients appears to bind more avidly to CAM-Sepharose and that mutant huntingtin continues to bind to CAM-Sepharose in the absence of calcium may have important implications for the pathogenesis of HD. Recent studies suggest that huntingtin is enriched in vesicle prepara-

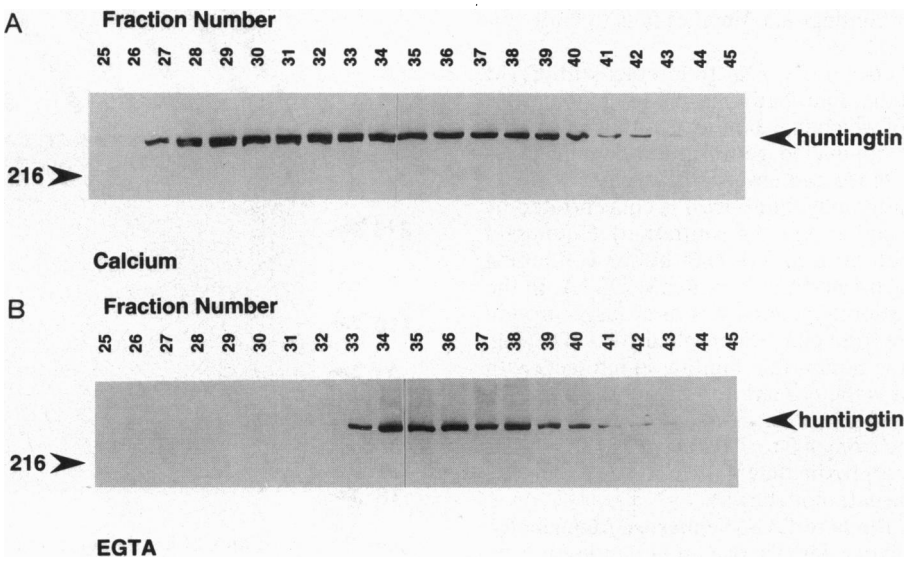

FIG. 4. Gel-filtration chromatography of huntingtin. Huntingtin sample prepared in the presence of 0.1 mM $CaCl_2$ (*A*) and 4 mM EGTA (*B*) is separated by Sephacryl S-300 column (V_t = 120 ml). The column flow rate is 0.5 ml/min and 1.5 ml is collected for each fraction. Aliquots of individual fractions are used for immunoblot analysis for huntingtin. A mixture of standard proteins is applied to the column to determine the molecular weight of huntingtin. The peak of Blue Dextran 2000, thyroglobulin (670 kDa), ferritin (440 kDa), and catalase (232 kDa) is fractions 26, 35, 39, and 53, respectively. These results have been replicated twice and representative blots are shown.

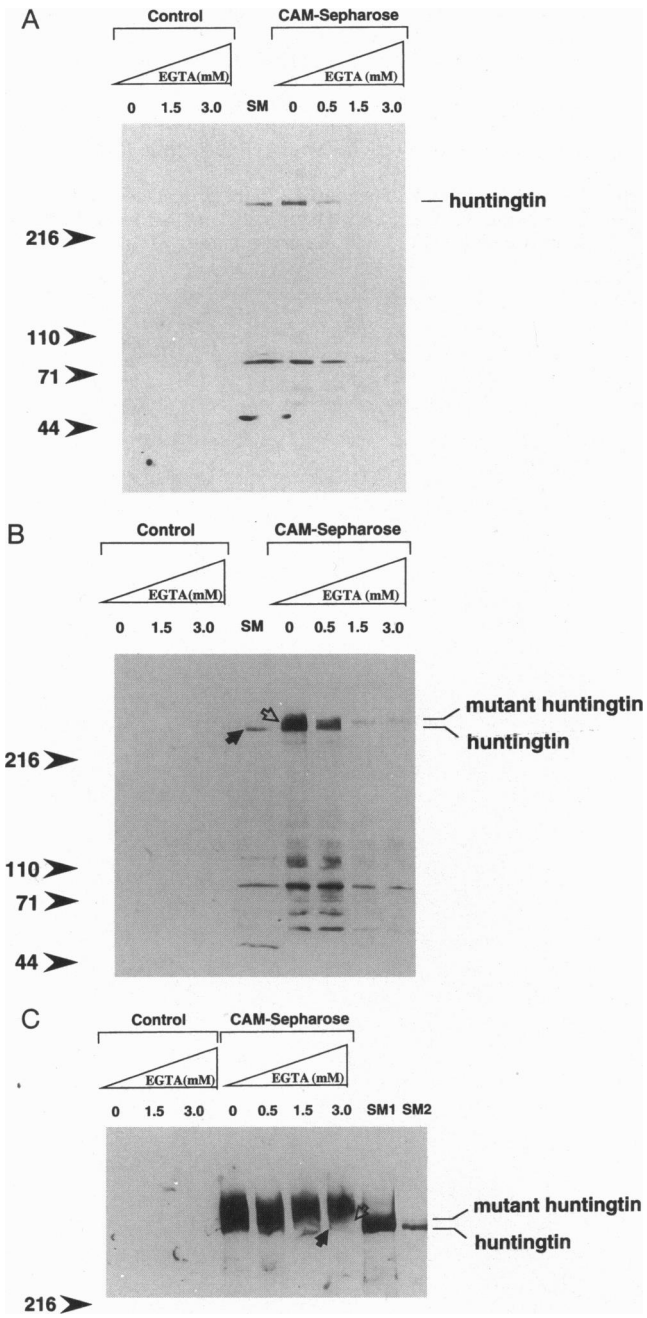

FIG. 5. Increased binding of huntingtin with expanded polyGln to CAM-Sepharose. Huntingtin sample is prepared from control brain BRC915 and HD brain HD144. G-25 Sephadex is used for the control resin. Proteins are released from resins and resolved by a SDS/3–12% gradient PAGE (*A* and *B*) or SDS/4% PAGE (*C*). To separate normal huntingtin from mutant huntingtin, electrophoresis is performed for 20 h compared to the usual 14 h. Huntingtin is monitored by immunoblot analysis. Molecular weight standards (in kDa) are indicated on the left. Results are representative of several experiments. (*A*) Control brain BRC915. Equivalent amounts of protein (≅200 μg) are loaded in the starting material (SM) and 0 mM EGTA lanes. (*B*) HD brain HD144. Equivalent amounts of protein (≅200 μg) are loaded in the SM and 0 mM EGTA lanes. Solid arrow indicates normal huntingtin and open arrow indicates mutant huntingtin. (*C*) HD brain HD144. Equivalent amounts of protein (≅200 μg) were loaded in the SM2 and 0 mM EGTA lanes. Protein (600 μg) was loaded in the SM1 lane to facilitate the visualization of the mutant allele. Solid arrow indicates normal huntingtin and open arrow indicates mutant huntingtin.

tions (12). Huntingtin may also be associated with microtubules as indicated by immunogold electron microscopy (14). Thus, it has been proposed that huntingtin may be involved in transport processes as well as vesicle function or vesicle recycling. Many of these processes are calcium dependent and alterations in the regulation or protein interactions in these events could have deleterious consequences.

Perutz and colleagues have hypothesized that glutamine repeats act as a polar zipper and expansion of polyGln leads to increased affinity or altered protein–protein interactions. Consistent with this hypothesis are our observations that mutant huntingtin binds more avidly to CAM-Sepharose and that the calcium-mediated regulation of this interaction is lost. It is not known how the expansion in polyGln alters the interaction with CAM-Sepharose. It could conceivably occur through an altered direct interaction with a calcium/CAM-binding protein or with another binding protein within the calcium/CAM- huntingtin protein complex. The recent observation of the selective recognition of a monoclonal antibody to huntingtin with expanded polyGln indicates that there is an altered conformation of huntingtin that might account for altered protein interactions (26).

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