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Interaction of Huntingtin-Associated Protein with Dynactin P150^{Glued}

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Huntingtin is the protein product of the gene for Huntington's disease (HD) and carries a polyglutamine repeat that is expanded in HD (>36 units). Huntingtin-associated protein (HAP1) is a neuronal protein and binds to huntingtin in association with the polyglutamine repeat. Like huntingtin, HAP1 has been found to be a cytoplasmic protein associated with membranous organelles, suggesting the existence of a protein complex including HAP1, huntingtin, and other proteins. Using the yeast two-hybrid system, we found that HAP1 also binds to dynactin P150^{Glued} (P150), an accessory protein for cytoplasmic dynein that participates in microtubule-dependent retrograde transport of membranous organelles. An *in vitro* binding assay showed that both huntingtin and P150 selectively bound to a glutathione transferase (GST)–HAP1 fusion protein. An immunoprecipitation assay demonstrated that P150 and huntingtin coprecipitated with HAP1 from rat brain cytosol. Western blot analysis revealed that HAP1 was enriched in rat brain microtubules and comigrated with P150 and huntingtin in sucrose gradients. Immunofluorescence showed that transfected HAP1 colocalized with P150 and huntingtin in human embryonic kidney (HEK) 293 cells. We propose that HAP1, P150, and huntingtin are present in a protein complex that may participate in dynein–dynactin-associated intracellular transport.

Key words: Huntington's disease; huntingtin; dynactin; microtubule; intracellular transport; targeting

The N terminus of huntingtin contains a polyglutamine repeat that is expanded (>36 units) in patients with Huntington's disease (HD) (HD Collaborative Research Group, 1993). It has been postulated that the expanded polyglutamine repeat causes huntingtin to interact abnormally with other cellular proteins (Perutz, 1994; Albin and Tagle, 1995; Ross 1995). HAP1 is a huntingtin-associated protein that binds more tightly to huntingtin with an expanded polyglutamine repeat than to normal huntingtin (Li et al., 1995) and is exclusively expressed in neurons (Li et al., 1996). Like huntingtin, HAP1 is a cytoplasmic protein associated with microtubules, membranous organelles, and synaptic vesicles (Li et al., 1996; Gutekunst et al., 1997; Martin et al., 1997; Sharp et al., 1997), suggesting that HAP1 may bind to other proteins. Identification of proteins that bind to HAP1 and have known function will help to uncover the biological role of HAP1 and possibly the cellular consequences related to the interaction of huntingtin and HAP1. To this end, we used a yeast two-hybrid screen to identify proteins potentially associated with the HAP1–huntingtin complex.

We found that HAP1 interacts with dynactin P150^{Glued} (P150), an accessory protein for the microtubule motor protein dynein. Cytoplasmic dynein is a microtubule motor protein involved in a wide range of intracellular motile events, including retrograde vesicle transport in axons, membrane trafficking, nuclear migration, and both the positioning and anaphase movement of the mitotic spindle (Paschal and Vallee, 1987; Pfarr et al., 1990; Steuer et al., 1990; Corthesy-Theulaz et al., 1992; Xiang et al., 1994; Saunders et al., 1995). Retrograde transport carries large, varied membrane-bound organelles such as presumptive lysosomes, multivesicular bodies, mitochondria, and exogenous material to the cell body (Brady, 1985; Vale et al., 1985; Brady et al., 1990; Hirokawa et al., 1991). The intracellular transport mediated by cytoplasmic dynein involves a variety of accessory proteins that may participate in targeting dynein motor proteins to intracellular organelles (Vallee and Sheetz, 1996). Among these accessory proteins is the dynactin complex, a 20S protein heteromultimer that consists of various polypeptides ranging in molecular mass from 24 to 150 kDa (Paschal and Vallee, 1987; Gill et al., 1991; Schroer and Sheetz, 1991; Paschal et al., 1993; Allan, 1994). The dynactin complex is required by dynein to move vesicles along microtubules *in vitro* (Gill et al., 1991; Schroer and Sheetz, 1991). The largest component of the dynactin complex is P150, a homolog of the product of the *Drosophila* gene Glued (Holzbaur et al., 1991). The null mutation of Glued is embryonically lethal (Harte and Kankel, 1982), suggesting that the P150 polypeptide has a role in an essential cell function.

Using *in vitro* binding, coimmunoprecipitation, and cotransfection assays, we demonstrate that HAP1 binds to P150. The presence of a protein complex containing HAP1, P150, and huntingtin is further supported by a sucrose gradient assay that shows comigration of these proteins at the 20 S position. We propose that HAP1 and normal huntingtin may be involved in microtubule and dynein–dynactin-associated intracellular transport in the neuron.

MATERIALS AND METHODS

Yeast two-hybrid system. All experiments were performed with the yeast strain Y190. The yeast two-hybrid screen was conducted as described

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previously (Fields and Song, 1989; Li et al., 1995) to isolate HAP1-associated proteins (HAPAs). Full-length HAP1-A, an isoform of HAP1, fused to the GAL-4 DNA-binding domain was used as a bait to screen a rat brain cDNA library (Li et al., 1995). Transformed yeast cells were grown in Trp^-, Leu^-, and His^- synthetic medium containing 25 mM 3-aminotriazole (Sigma, St. Louis, MO) that can reduce the leaky expression of the His^+ phenotype. Positive colonies were identified by filter assays of β-galactosidase (β-gal) activity (Li et al., 1995). cDNAs from these positive colonies appearing within 120 min were rescued for retransformation of fresh yeast cells and confirmation of the interactions of these cloned proteins with HAP1.

Three HAP1 constructs containing different fragments between the middle region and the C terminus (amino acids 278–599) were fused to the GAL-4 activation domain in pPC86 vector to examine their interactions with huntingtin, DRPLA, and c-Jun proteins in pPC97 vector (Chevray and Nathans, 1992). These HAP1 fragments were also fused to the GAL-4 DNA-binding domain in pPC97 vector to test their interactions with P150 and other cloned HAPA proteins in pPC86 vector. The N-terminal fragment (amino acids 1–253) of huntingtin containing 23 glutamine repeats was used. DRPLA is a glutamine-repeat protein product of the gene for dentatorubral and pallidoluysian atrophy (Nagafuchi et al., 1994) with a 21 glutamine repeat and was used as a control in a previous study (Li et al., 1995). c-Jun (amino acids 250–334) is a DNA-binding protein used as an irrelevant control.

For assessment of protein–protein interactions in yeast, filter assays of β-galactosidase activity were performed by transferring the yeast colonies onto Whatman filters. The yeast cells were partially lysed by submerging the filters in liquid nitrogen for 15–20 sec. Filters were allowed to dry at room temperature for at least 5 min and placed onto filter paper presoaked in Z buffer (100 mM sodium phosphate, pH 7.0, 10 mM KCl, and 1 mM $MgSO_4$) supplemented with 50 mM β-mercaptoethanol and 0.07 mg/ml 5-bromo-4-chloro-3-indolyl β-D-galactoside. Filters were placed at 37°C for up to 3 hr.

Liquid β-galactosidase assays were performed as described previously (Li et al., 1995). Briefly, yeast colonies were introduced into appropriate synthetic media and grown to an optical density at 600 nm (OD_{600}) of 0.6–0.8. Five milliliters of culture was pelleted, washed once with 1 ml of Z buffer, and then resuspended in 500 μl of Z buffer supplemented with 38 mM β-mercaptoethanol. Acid-washed glass beads were added to each sample and vortexed for 3 min on ice. Each sample lysate (50–150 μg of protein) was taken in triplicate for the liquid β-galactosidase assay. These samples were incubated with 900 μl of Z buffer in a 30°C water bath for 30 sec, and then 200 μl of 4 mg/ml *o*-nitrophenyl β-D-galactopyranoside solution was added to each tube. The reaction was allowed to continue for 15–30 min at room temperature and stopped by the addition of 500 μl of 1 M Na_2CO_3. The OD_{420} was taken to calculate the β-galactosidase activity using the equation: $1000 \times OD_{420}/(t \times mg)$, where t is the elapsed time (in minutes). The β-galactosidase activity was thus expressed as units per minute per milligram of yeast protein.

In vitro *binding. In vitro* binding assays were performed essentially as described previously (Li et al., 1995). The HAP1-A fragment (amino acids 278–599) was fused to the pGEX-4T vector. Glutathione *S*-transferase (GST)–HAP1 fusion protein was produced in bacteria BL21 (Pharmacia, Piscataway, NJ). GST–HAP1 fusion protein was then purified with glutathione–Sepharose beads (Sigma) and used for binding to full-length P150 and the N-terminal huntingtin. The cDNA encoding rat P150 was isolated by reverse transcriptase (RT)-PCR from rat brain RNA with two pairs of primers (S275, GTAGAGTCCGGGTGAGCAACATGGCC and A1570 GCATCTCCACCATCTCCTCA; S1503, AGCAGCGTGAGCGTCTTCAGG and A4229 ACCGAATTCACGGAAGTAGCAGAACC). These primers were derived from published sequences (Holzbaur et al., 1991) and allowed us to construct a full-length P150 cDNA in pCIS vector (Li et al., 1995). The full-length P150 was used to synthesize [^{35}S]methionine-labeled P150 with the *in vitro* translation TNT kit (Promega, Madison, WI). The N terminus of huntingtin (amino acids 1–253) containing 23 (23Q) or 44 (44Q) glutamine repeats (Li et al., 1995) was also used to synthesize radiolabeled huntingtin with [^{35}S]methionine. ^{35}S-labeled proteins (5–10 μl) were incubated with glutathione-agarose beads containing the GST protein or GST–HAP1 fusion protein (200 ng) in 0.2 ml of binding buffer (0.5% Triton X-100 in PBS) for 2 hr. After the beads were washed three times with the binding buffer, proteins bound to the beads were resolved by 8% SDS-PAGE and visualized by autoradiography. Quantitative assessment of protein in the gel was performed using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). In some cases, the gel was stained with Coomassie brilliant blue to visualize the GST fusion proteins present in each track.

Antibody production. Two isoforms of HAP1, termed HAP1-A and HAP1-B, have been isolated. They differ in the sequences at their C termini; HAP1-A has 21 amino acids at the C terminus that differ from the 51 amino acids at the C terminus of HAP1-B (Li et al., 1995). Anti-peptide antibodies for HAP1 (EM31 and EM32) were raised against the C-terminal sequences of these two rat HAP1 isoforms (Li et al., 1995), SRRGHPPASGTSYRSSTL for HAP1-A and ATHSPSAREEEGPSGAT for HAP1-B. These peptides were conjugated with BSA to serve as immunogens for Covance Inc. (Denver, PA) to produce rabbit antisera. Anti-peptide antibodies were purified using affinity columns linked with immunogen (Li and Snyder, 1995). A fragment of rat P150 (amino acids 1023–1223) was used to produce GST–P150 fusion protein that served as immunogen to produce rabbit antibodies (EM49). The anti-P150 antibody was purified by incubation of whole serum with a nitrocellulose strip containing electrophoretically purified GST–P150. After multiple washes, antibodies were eluted with 0.2 M Gly, pH 2.15, for 10 min and immediately neutralized by addition of 1.5 M Tris-HCl, pH 8.8. The specificity of purified EM49 was found to be comparable with that of the well characterized anti-P150 antibody UP235 (provided by Dr. Holzbaur, University of Pennsylvania; Tokito et al., 1996). Affinity-purified anti-HAP1 antibodies (EM31 and EM32) were mixed together (1:1) to allow recognition of both HAP1-A and HAP1-B isoforms. These antibodies and anti-P150 antibody (EM49) were used at 1:1000 dilution for Western blotting. The following antibodies were also used in the study: anti-huntingtin antibodies (1:500 dilution) that were rabbit polyclonal antibodies described previously (Gutekunst et al., 1995); anti-HA (human influenza hemagglutinin) epitope antibody 12CA5 (1:100–500 dilution; Boehringer Mannheim, Indianapolis, IN); and rabbit polyclonal antibodies to Rab2 (Santa Cruz Biotechnology, Santa Cruz, CA), to neuronal nitric oxide synthase (nNOS) (Transduction Laboratories, Lexington, KY), or to ubiquitin (Dako, Carpenteria, CA).

Immunoprecipitation. Rat brain cytosolic extracts were obtained by homogenizing 2 gm of rat brain tissue in 4 ml of PIPES buffer (100 mM Na-PIPES, 50 mM Na-HEPES, 1 mM EDTA, 1 mM $MgSO_4$, pH 6.9, and the following protease inhibitors: 100 μg/ml PMSF, 1 μg/ml leupeptin, 1 μg/ml aprotinin, and 1 μg/ml pepstatin A). Homogenates were centrifuged at 18,000 × *g* for 15 min at 4°C, and the supernatant was then clarified by centrifugation at 120,000 × *g* for 30 min at 4°C. The clarified supernatant (600 μl) was preincubated with protein A-Sepharose beads (50 μl of 1:1 slurry; Pharmacia) for 30 min at 4°C. After the beads were pelleted, the supernatant was incubated with 50 μl of protein A-agarose beads linked with 5 μl of affinity-purified anti-HAP1 antibodies for 1 hr at 4°C. Controls involved immunoprecipitations with 20 μg/ml of unconjugated peptides and HAP1 antibodies that had been preadsorbed with the peptides overnight. In addition, protein A-agarose beads alone or linked with 5 μl of rabbit IgG (Sigma) were also used as controls. The beads containing immunocomplexes were pelleted and washed twice with 1 ml of 0.4% Triton X-100 in PBS. Precipitated proteins were resuspended in 100 μl of SDS-PAGE sample buffer and boiled for 5 min. Thirty microliters of brain extracts and immunoprecipitates were resolved by 6% SDS-PAGE and detected by Coomassie blue staining and Western blotting.

Sucrose gradient fractionation of S3 cytosol. This experiment was performed as described previously (Paschal et al., 1993). Rat brain homogenate (1:4 w/v) in PIPES buffer was first centrifuged at 15,000 × *g* for 15 min and then at high speed (120,000 × *g*) for 45 min. The resulting S3 supernatant (3.5 ml) was layered onto 8 ml of 5–20% linear sucrose density gradient in PIPES buffer that was composed of 16 fractions (0.5 ml/fraction). After centrifugation at 120,000 × *g* at 4°C for 16 hr in a Ti SW-41 rotor (Beckman), each fraction (0.5 ml) was collected, and 60 μl samples were analyzed by Western blotting with antibodies to HAP1, huntingtin, and P150.

Microtubule sedimentation. Microtubule pellets were prepared from rat brain homogenates by a standard method (Paschal et al., 1991). Rat brain was homogenized in PIPES buffer (1:1 w/v). The homogenates were clarified by centrifugation at 18,000 × *g* for 20 min and then at 120,000 × *g* for 45 min. The resulting supernatant (S3) was treated with 1 mM GTP and 20 μM taxol (Sigma) at room temperature for 30 min to polymerize microtubules. The polymerized microtubules were pelleted by centrifugation at 120,000 × *g* for 30 min. The pellets were washed once with PIPES buffer and then resuspended in 4 ml of PIPES buffer containing 1 mM GTP and 20 μM taxol. For ATP extraction, MgATP was added to the resuspended pellets at 10 mM concentration, and the extraction was

Table 1. Interactions of HAP1 with associated proteins determined by filter assay of β-galactosidase activities

HAP1 constructs	Huntingtin (1–253)	P150 (1023–1223)	HAPA-10 (1–340)	Jun (250–334)	DRPLA (450–712)
HAP1 (278–599)	(+)	(+)	(+)	(−)	(−)
HAP1 (278–445)	(+)	(+)	(−)	(−)	(−)
HAP1 (278–370)	(+)	(−)	(−)	(−)	(−)

Filter assay of β-galactosidase activities of yeast Y190 containing respective HAP1 and other protein constructs. For plasmid constructs, see Materials and Methods. (+) indicates a positive reaction, whereas (−) indicates a negative reaction. Numbers in parentheses indicate amino acid positions.

rocked for 15 min at room temperature. The control was the microtubule pellets without ATP extraction. The extracted microtubules were then pelleted again, and the pellets were resuspended in 2 ml of PIPES buffer. The supernatant (100 μl) and pellet suspension (40 μl) were analyzed by 7.5% SDS-PAGE.

Coexpression and double labeling of transfected cells. Human embryonic kidney (HEK) 293 cells were used for cotransfection studies. To double label HAP1 and huntingtin that were coexpressed in HEK 293 cells, we tagged huntingtin (amino acids 1–253) containing 23 glutamine repeats with an HA epitope (YPYDVPDYA) at its C terminus so the mouse anti-HA antibody 12CA5 could recognize the transfected huntingtin, whereas rabbit polyclonal antibody to HAP1 could recognize transfected HAP1-A. To double label coexpressed HAP1-A and full-length P150, we tagged the C terminus of HAP1-A with the HA epitope to allow recognition by mouse antibody 12CA5. The addition of the HA epitope to HAP1-A did not alter the subcellular localization of the expressed HAP1-A in transfected cells. HEK 293 cells in chamber slides (Nalge Nunc., Naperville, IL) were cotransfected with HAP1-A and huntingtin or P150 (1–2 μg of cDNA for each) using lipofectin for 24–36 hr. The cells were then fixed with 4% paraformaldehyde in PBS for 15 min, permeabilized with 0.4% Triton X-100 in PBS for 30 min, preincubated with PBS containing 5% normal goat serum for 1 hr, and then incubated with specific primary antibodies in PBS containing 2% normal goat serum overnight. In general, rabbit polyclonal antibodies were used at 1:1000 dilution, and the mouse monoclonal antibody 12CA5 was used at 1:100 dilution for incubation with transfected cells. After the cells were washed with PBS three times, fluorescent FITC- or rhodamine-conjugated secondary antibodies (1:200 dilution; Jackson ImmunoResearch, West Grove, PA) were then added to the cells, and the expressed proteins were localized using fluorescence microscopy.

RESULTS

Interaction of HAP1 with P150 and huntingtin in yeast

To investigate whether HAP1 binds to proteins of known function, we fused full-length HAP1-A to the GAL-4 DNA-binding domain and screened a rat brain cDNA library using the yeast two-hybrid system. HAP1-A is a HAP1 isoform and has 21 amino acids at the C terminus that differ from those (51 amino acids) of HAP1-B (Li et al., 1995). Seventeen positive colonies were isolated from 1.5 million yeast transformants by selection of His^+- and β-galactosidase-positive transformants. We chose to study the clones that displayed strong and specific interaction with HAP1. Among the 10 clones that displayed specific β-galactosidase activity within 2 hr, three clones contained cDNAs encoding partial amino acid sequences of HAP1, suggesting that HAP1 binds to itself in yeast. Two clones carried cDNAs encoding 200 amino acids of rat P150 (from amino acids 1023–1223). Two other clones encoded 340 amino acids of unknown function that were termed HAPA-10. The remaining clones are being characterized. Because P150 is a microtubule motor-binding protein and because huntingtin and HAP1 associate with microtubules and various intracellular organelles (Gutekunst et al., 1995, 1997; Li et al., 1996; Martin et al., 1997; Sharp et al., 1997), the interaction between HAP1 and P150 was chosen for further characterization.

The yeast two-hybrid screen suggests that HAP1 binds to several proteins. Analysis of the HAP1 protein using the Coils program (Lupas et al., 1991) predicted a coiled coil structure in HAP1 between amino acids 120 and 380. The coiled coil is also present in various microtubule-binding proteins including P150 from amino acids 200 to 550 and from 920 to 1020. To verify the specificity of the interactions between HAP1 and associated proteins in yeast, we examined the interactions of different HAP1 fragments (between amino acids 278 and 599) with human huntingtin (amino acids 1–253), P150 (amino acids 1023–1223), HAPA-10, c-Jun (amino acids 250–334), and DRPLA (amino acids 450–712) (Table 1). The N-terminal huntingtin contained 23 glutamine repeats. c-Jun is a leucine zipper protein containing a coiled coil, and DRPLA is a polyglutamine (21 glutamine) protein that also contains a coiled coil. The result showed that all HAP1 fragments interacted with huntingtin, suggesting that the shortest HAP1 fragment (amino acids 278–370) contains the site for binding to huntingtin. P150 did not interact with this shortest fragment but did bind to two other HAP1 fragments (amino acids 278–445 and 278–599). Thus, P150 seems to bind to a different region of HAP1, which may be between amino acids 370 and 445. HAPA-10 only interacted with the longest HAP1 fragment that included the C terminus of HAP1-A. In contrast, we observed no interaction of any of these HAP1 fragments with c-Jun or DRPLA. These results suggest that the binding of HAP1 to huntingtin and P150 is selective.

Table 2. Interactions of HAP1 with associated proteins determined by liquid assay of β-galactosidase activities

DNA activation hybrid	DNA binding hybrid	β-Galactosidase $U \cdot min^{-1} \cdot mg^{-1}$
HAP1 (278–370)	c-Jun (250–334)	12 ± 4
HAP1 (278–370)	Huntingtin 23Q	119.6 ± 20.2
HAP1 (278–370)	Huntingtin 44Q	237.2 ± 33.8
HAP1 (278–370)	DRPLA (450–712)	8 ± 2

Liquid assay of β-galactosidase activities of yeast Y190 harboring HAP1 (amino acids 278–370) fused to the GAL-4 activation domain and other protein constructs fused to the GAL-4 binding domain. Numbers in parentheses indicate amino acid positions. β-Galactosidase activities of cell extracts were determined in triplicate from three independent experiments. Data shown are units of β-galactosidase activity per minute per milligram of protein ± SD after background subtraction.

Previous studies using HAP1 (1–445) demonstrated that HAP1 bound more huntingtin 44Q than 23Q (Li et al., 1995; Kalchman et al., 1997). As the shorter fragment of HAP1 (278–370) was sufficient to bind to huntingtin, we quantitatively tested the binding of this fragment to huntingtin containing 23 or 44 glutamine repeats using a liquid assay. We observed that the interaction of HAP1 (278–370) with huntingtin 44Q yielded more β-galactosidase activity (237.2 units/min/mg of protein) than did the interaction with huntingtin 23Q (119.6 units/min/mg of protein) (Table 2). Western blot analysis showed that huntingtin 44Q and 23Q were expressed at similar levels in yeast (data not shown). In the controls, the liquid assay showed background levels (8 and 12 units/min/mg of protein) of β-galactosidase activities for the interaction of HAP1 with DRPLA and c-Jun.

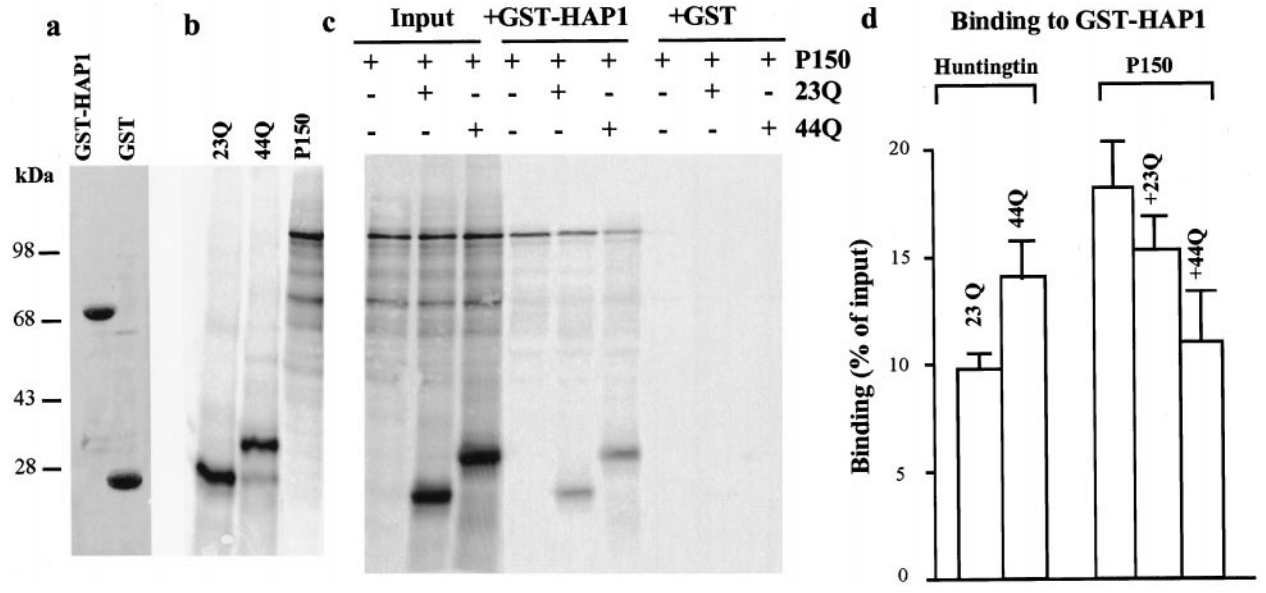

Figure 1. *In vitro* binding of HAP1 to P150 and huntingtin. *a*, Coomassie blue staining of a gel containing purified GST–HAP1 and unconjugated GST. *b*, Autoradiograph of ^{35}S-labeled, N-terminal huntingtin with 23Q or 44Q and the ^{35}S-labeled full-length P150 that were produced by *in vitro* translation. *c*, The binding of the GST–HAP1 fusion protein to lysates containing ^{35}S-labeled P150 and huntingtin. *Input* was lysates containing ^{35}S-labeled proteins before incubation with GST and GST–HAP1. Proteins bound to the agarose beads containing GST–HAP1 or GST alone were resolved by SDS-PAGE and visualized by autoradiography. *d*, Quantitative assessment by phosphorimaging of huntingtin bound to GST–HAP1 (*23Q* and *44Q*) and P150 bound to GST–HAP1 in the absence (*no label*) and presence of huntingtin (*+23Q* and *+44Q*). Values were obtained from three independent experiments and expressed as percent of the respective input.

In vitro binding of HAP1 to P150 and huntingtin

Because yeast two-hybrid assays are prone to artifacts, we performed an *in vitro* binding assay with GST–HAP1 (amino acids 278–599) (Fig. 1*a*). We used [^{35}S]methionine-labeled full-length P150 in the binding assay so we could test the direct interaction between these proteins. To examine whether huntingtin and P150 could simultaneously bind to HAP1, we also generated ^{35}S-labeled, N-terminal huntingtin containing 23Q or 44Q (Fig. 1*b*) and used them in the binding assay along with P150. P150 alone or mixed with huntingtin 23Q or 44Q was incubated with GST–HAP1 or GST (Fig. 1*c*). By autoradiography, we observed that both P150 and huntingtin bound to GST–HAP1 but not to GST alone. Quantitative assessment of the amounts of proteins bound to GST–HAP1 was then performed using a phosphorimager (Fig. 1*d*). In comparison with the input of proteins before the binding, more huntingtin 44Q (14.2% of input) than 23Q (9.8% of input) appeared to bind to GST–HAP1. The amount of P150 bound to the GST–HAP1 (17.6% of input) seemed to be slightly higher than that of huntingtin. However, this binding was decreased in the presence of huntingtin 23Q (15.1% of input) and huntingtin 44Q (11.2% of input). Equal amounts of GST–HAP1 were used in each reaction. Thus the results suggest that these three proteins could form a protein complex *in vitro* and perhaps huntingtin, particularly with 44 glutamine repeats, may inhibit the binding of GST–HAP1 to P150.

Coimmunoprecipitation of HAP1, huntingtin, and P150

To examine whether HAP1 associates with P150 and huntingtin *in vivo*, we conducted immunoprecipitations with antibodies to HAP1 and P150. Because the middle portion of HAP1 was found to bind to both huntingtin and P150, we generated anti-peptide antibodies against the C-terminal regions of HAP1 and expected that these antibodies might more efficiently precipitate the HAP1 complex. Western blots showed that these antibodies specifically reacted with both HAP1 isoforms (75 kDa for HAP1-A and 85 kDa for HAP1-B) in rat brain and in transfected cells (Fig. 2*a*). A rabbit polyclonal antibody (EM49) to P150 was also produced and found to react specifically with transfected P150 in HEK 293 cells and with a doublet at ~150/135 kDa in rat brain (Fig. 2*b*). The doublet represented isoforms of P150 that have been revealed by other anti-P150 antibodies (Tokito et al., 1996). Immunoprecipitation of rat brain cytosol with anti-HAP1 antibodies was then conducted, and the precipitated proteins were separated by SDS-PAGE. By Coomassie blue staining of the gel, we observed two weak bands of molecular weights corresponding to those of HAP1-A and HAP1-B in the sample precipitated by anti-HAP1 antibodies (Fig. 2*c*). However, it is difficult to define the other weak bands in the HAP1 precipitate. We then performed immunoblots and demonstrated the coprecipitation of HAP1, P150, and huntingtin by anti-HAP1 antibodies (Fig. 2*c*). Precipitation of P150 and huntingtin apparently depended on the presence of HAP1 because preincubation of these anti-peptide antibodies with the peptides (20 μg/ml) eliminated the precipitation for HAP1 as well as P150 and huntingtin. Controls using protein A-Sepharose beads alone or beads linked with rabbit IgG did not show any significant precipitation of HAP1, P150, or huntingtin. We also probed the blot with antibodies to other proteins, including nNOS, GTP-binding protein Rab2, and ubiquitin, but did not find these proteins in the immunoprecipitates. These results indicate a specificity of the coprecipitation of HAP1, P150, and huntingtin.

Comigration of HAP1, huntingtin, and P150 in sucrose gradients

Immunoprecipitation suggested that soluble forms of these proteins are involved in a protein complex in the cytosol. We therefore performed sucrose gradient fractionation of rat brain cytosol. This method separates protein complexes based on their densi-

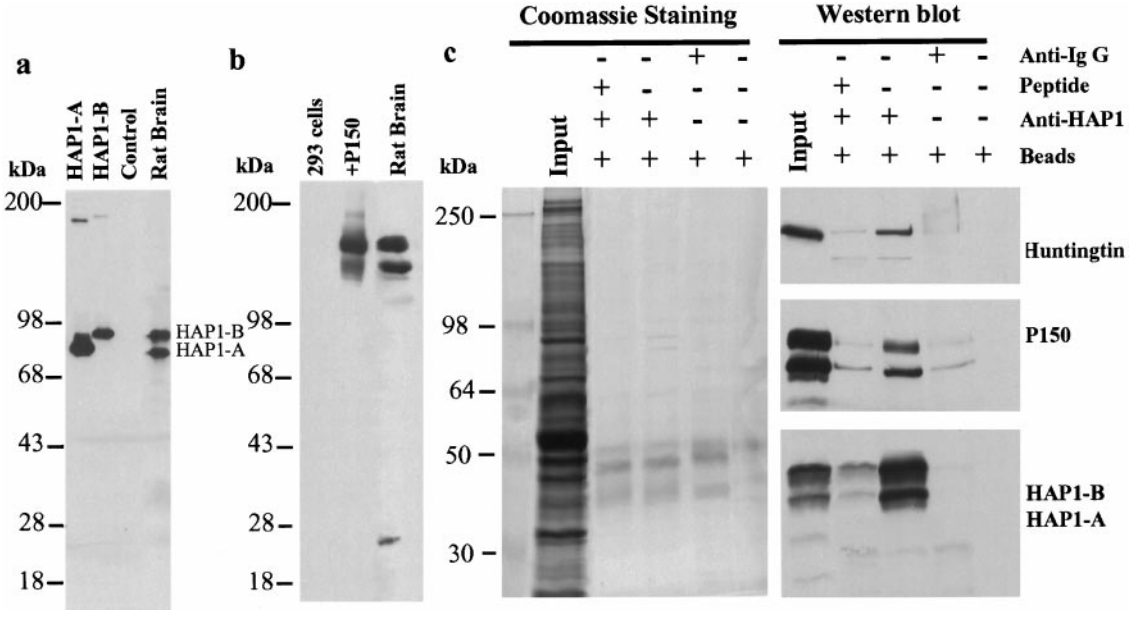

Figure 2. Immunoprecipitation of rat brain proteins with anti-HAP1 antibodies. *a*, Anti-HAP1 antibodies specifically recognized transfected HAP1 (*HAP1-A* and *HAP1-B*) in HEK 293 cells. The control was untransfected cells. The antibodies also reacted with two bands (75 and 85 kDa) in rat brain that corresponded to *HAP1-A* and *HAP1-B*, respectively. *b*, Rabbit polyclonal antibody to P150 specifically reacted with transfected P150 in HEK 293 cells and with polypeptides of 150 and 135 kDa in rat brain. The control was untransfected HEK 293 cells. *c*, Rat brain cytosolic extracts were immunoprecipitated using anti-HAP1 antibodies (+ *anti-HAP1*). A Coomassie blue-stained SDS-polyacrylamide gel (8%) containing immunoprecipitates is shown. The same immunoprecipitates were also resolved by 6% SDS-PAGE, and the blot was cut to strips that were probed with antibodies to huntingtin, P150, and HAP1. Lysates were brain extracts before immunoprecipitation. The controls were immunoprecipitations with immunogen-preadsorbed HAP1 antibody (+ *peptide*), rabbit IgG (+ *anti-IgG*), and protein A-agarose beads alone (+ *beads*).

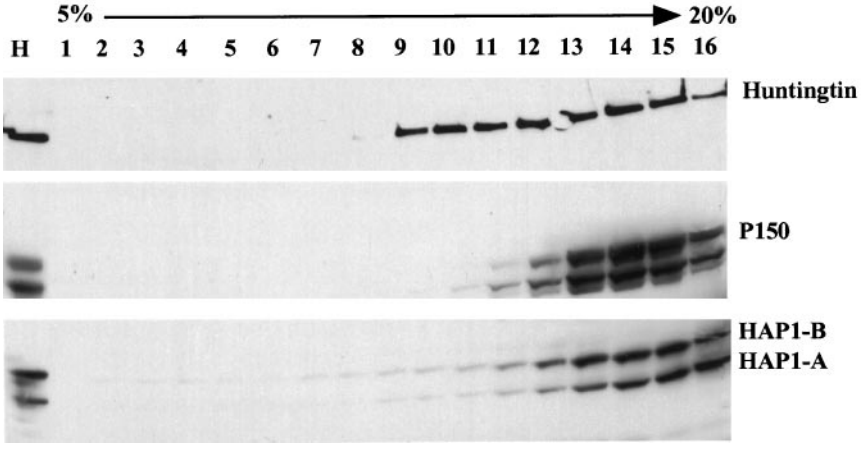

Figure 3. Comigration of HAP1 with P150 in a sucrose gradient. Fractionation of rat brain cytosolic fraction (S3) through a 5–20% sucrose density gradient. Sixty microliter samples from each of 16 fractions were resolved by 7.5% SDS-PAGE. The blot with transferred proteins was probed with antibodies to huntingtin, P150, and HAP1. *H* is rat brain cytosolic extract before the gradient fractionation.

ties. Rat brain cytosol was fractionated on a linear sucrose gradient (5–20%) and analyzed by Western blotting (Fig. 3). P150 has been found to peak at 20S (from fraction 13 to 15) in a 5–20% sucrose gradient (Paschal et al., 1993). We found that both P150 and HAP1 peaked in fractions 13–15 with nearly identical profiles. The distribution of huntingtin covered a relatively broad region from fraction 9 to 16. However, the highest concentration of huntingtin was also found between fractions 12 and 15. The comigration of these proteins in the gradient supports the idea that these proteins may be involved in the same protein complex in the cytosol.

Sediment of HAP1 in microtubule pellets

Because the dynactin complex can be purified by ATP extraction of microtubules and because the binding of dynactin P150 to microtubules is decreased by ATP (Waterman-Storer et al., 1995; Tokito et al., 1996), we examined whether HAP1 is associated with microtubules and whether this association is also regulated by ATP. We prepared microtubule pellets by sedimentation of polymerized microtubules from rat brain cytosol. Both HAP1 isoforms (HAP1-A and HAP1-B) cosedimented with endogenous rat brain microtubules (Fig. 4*a,b*). HAP1 and P150 were enriched in microtubules compared with huntingtin that was more concentrated in the eluate than in the pellets. Of two major isoforms of dynactin (150 and 135 kDa), only the 150 kDa polypeptide was preferentially associated with microtubules, consistent with a recent report (Tokito et al., 1996). However, much less HAP1 than P150 was released into the eluate after extraction of microtubule pellets with 10 mM ATP (Fig. 4). Thus, although HAP1 also associates with microtubules, this association seemed to be less sensitive to ATP extraction than was that of P150.

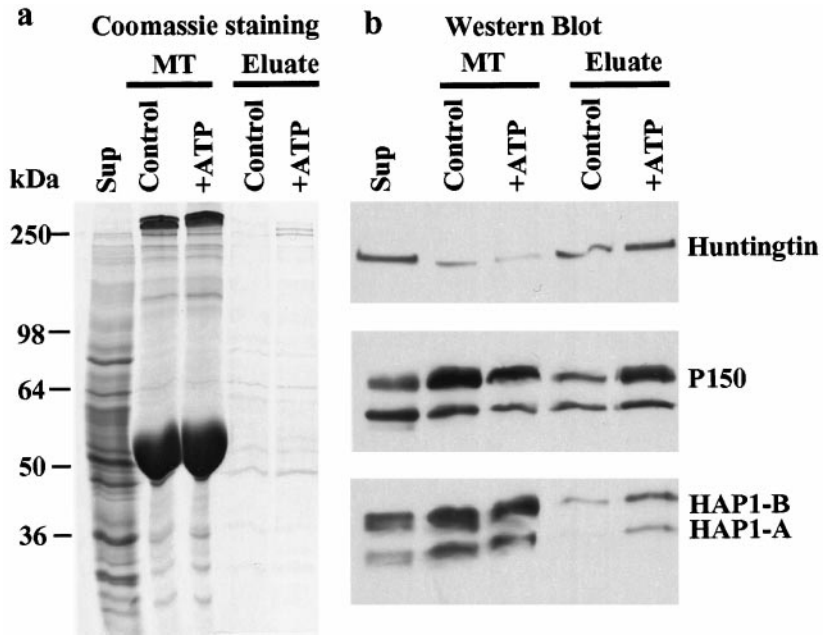

Figure 4. Cosedimentation of HAP1 and P150 with rat brain microtubules. *a*, Coomassie blue staining of a gel containing polymerized rat brain microtubules. *Sup* was a high speed supernatant before the polymerization of microtubules. Microtubule pellets (*MT*) were extracted with the normal PIPES buffer (*MT + control*) and with 10 mM ATP (*MT + ATP*). The respective eluates (*control* and *+ ATP*) were also resolved in the gel. *b*, The same samples analyzed by Western blotting with antibodies to huntingtin, P150, and HAP1.

Colocalization of HAP1 with huntingtin or P150 in transfected cells

To eliminate the possibility that HAP1 and P150 associate with each other only *in vitro* or after homogenization, we examined protein association *in vivo* by imaging the localization of HAP1, P150, and huntingtin in the cell. Because the antibodies we used were all rabbit polyclonal antibodies, we could not examine the colocalization of these proteins in the brain. Therefore we expressed HA-tagged HAP1 or huntingtin in HEK 293 cells so a mouse monoclonal antibody to the HA epitope could be used for immunofluorescent double labeling. Expressed N-terminal huntingtin (Fig. 5*a*) or full-length P150 (Fig. 5*b*) were diffusely distributed in the cytoplasm in transfected HEK 293 cells. In addition, transfection of P150 into the cells produced P150 decoration on thick, wavy bundle-like structures similar to those observed by Waterman-Storer et al. (1995) in the Rat-2 cell line. These bundle-like structures are thought to be formed by grouped microtubules (Waterman-Storer et al., 1995). Transfection of HAP1-A isoform alone into HEK 293 cells, however, resulted in HAP1-immunoreactive granular structures in the cytoplasm (Fig. 5*c*). Overexpression of HAP1-B or other proteins under the same conditions did not display such structures (data not shown). Similar HAP1 immunoreactive structures (0.5–5 μm in diameter) in the rat brain were also observed and appeared to be cytoplasmic inclusions (C. A. Gutekunst, S.-H. Li, X.-J. Li, S.M. Hersch, unpublished observations). While the nature of these structures is being studied, the unique and granular shapes of these structures allowed examination of the colocalization of HAP1 and its associated proteins in transfected cells.

We cotransfected HAP1-A with HA-tagged huntingtin into HEK 293 cells. The expressed huntingtin was precisely colocalized with HAP1-A to the granular structures (Fig. 5*d*). Both proteins were also colocalized to dots or punctate-like structures. Similarly, the N-terminal huntingtin or full-length huntingtin with 44 glutamine repeats was also localized to these structures when coexpressed with HAP1-A (data not shown). When P150 was expressed with the HA-tagged HAP1-A in HEK 293 cells, its subcellular localization was changed, and it was colocalized with HAP1-A to the granular or punctate-like structures (Fig. 5*e*). To confirm the specificity of these colocalizations, we cotransfected HAP1-A with HA-tagged DRPLA, another glutamine-repeat protein that does not interact with HAP1 (Li et al., 1995). DRPLA did not colocalize with HAP1-A on the cytoplasmic structures (Fig. 5*f*). We also coexpressed HAP1-A with GST protein or with another polyglutamine-repeat protein, ataxin-1 (Orr et al., 1993). None of them was found to localize with HAP1-A to these granules (data not shown).

DISCUSSION

The present study demonstrates that HAP1 interacts with dynactin P150 and huntingtin, thus implying the presence of a protein complex that includes huntingtin, HAP1, and P150. This conclusion is supported by the following findings: (1) P150 and huntingtin specifically interact with HAP1 in yeast; (2) P150 and huntingtin simultaneously bind to GST–HAP1 *in vitro*; (3) a protein complex containing these three proteins is precipitated by anti-HAP1 antibody and is also present in the same fractions of sucrose gradients; and (4) HAP1 colocalizes with P150 and huntingtin in transfected cells, suggesting that they do associate *in vivo*. Because dynactin P150 participates in dynein-mediated intracellular organelle or vesicle transport, we propose that this protein complex is involved in the coupling of the dynein–dynactin complex to intracellular organelles or structures and that the function of huntingtin may be associated with intracellular trafficking.

Consistent with the above idea, huntingtin has been found to associate with a variety of membranous organelles and synaptic vesicles (DiFiglia et al., 1995; Gutekunst et al., 1995; Sharp et al., 1995). Huntingtin has also been found to bind to various proteins including glyceraldehyde phosphate dehydrogenase (GAPDH) (Burke et al., 1996), an unidentified calmodulin-associated protein (Bao et al., 1996), a ubiquitin-conjugating protein (HIP2) (Kalchman et al., 1996), epidermal growth factor (EGF) receptor-signaling complexes (Liu et al., 1997), and a protein homologous to the yeast cytoskeleton-associated protein Sla2p (HIP1) (Kalchman et al., 1997; Wanker et al., 1997). Therefore,

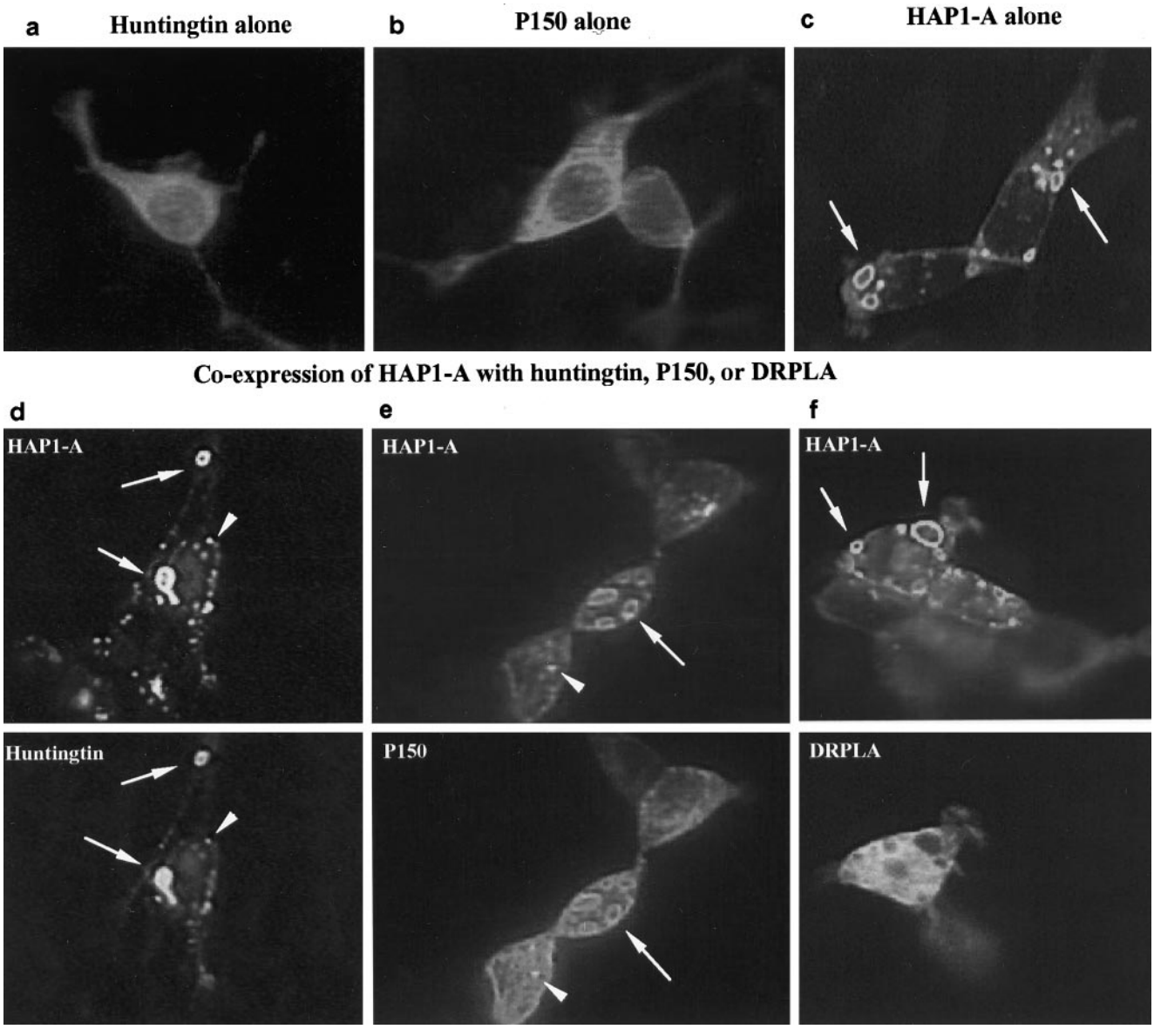

Figure 5. Colocalization of transfected HAP1-A with huntingtin and P150 in HEK 293 cells. *a, b*, Transfection of (*a*) huntingtin (amino acids 1–253) with 23 glutamine repeats or (*b*) full-length P150 resulted in a diffuse distribution of the expressed proteins in the cytoplasm. *c*, Transfection of HAP1-A construct alone resulted in HAP1-immunoreactive granular (*arrows*) and punctate-like structures. *d–f*, Coexpression of HAP1-A and (*d*) huntingtin or (*e*) P150 resulted in protein colocalization on the granular (*arrows*) and punctate-like (*arrowheads*) structures, whereas coexpression of (*f*) HAP1-A and DRPLA did not. Immunofluorescent double labeling used rabbit antibodies to the respective proteins and mouse monoclonal antibodies to HA-tagged proteins.

huntingtin may be involved in various protein complexes. The cellular and subcellular localization of HAP1 has been found to be similar to that of huntingtin (Gutekunst, Li, Hersch, unpublished observations). Immunogold electron microscopy showed that HAP1 is associated with microtubules and many types of membranous organelles, including mitochondria, endoplasmic reticulum, tubulovesicles, endosomal/lysosomal organelles, and synaptic vesicles (Gutekunst, Li, Hersch, unpublished observations). HAP1 has also been found to associate with the mitotic spindle apparatus (Martin et al., 1997) and large dense-core vesicles in pheochromocytoma (PC12) cells (Sharp et al., 1997). Yeast two-hybrid assays suggest that HAP1 interacts with P150 and other unknown proteins. The interaction of HAP1 with dynactin has also been observed by other investigators (Engelender et al., 1997). Binding of HAP1 to various proteins may be necessary for HAP1 to associate with intracellular organelles such as microtubules and the granular structures seen in transfected cells. However, the HAP1 construct used in the binding assay contains a partial α-helical coiled coil that could also mediate nonspecific protein interactions in yeast. By testing the binding of HAP1 to various proteins that contain coiled coil structures, we observed that HAP1 did not interact with c-Jun and DRPLA that also contain a coiled coil structure. In addition, HAP1 was not found to interact with c-Fos (amino acids 132–211), another leucine zipper protein that also contains a coiled coil structure (Li et al., 1995). Moreover, different regions of HAP1 mediated the binding of HAP1 to huntingtin, P150, or other proteins in yeast. Therefore, it is unlikely that the binding

of HAP1 to P150 results from a nonspecific interaction of the coiled coils. Instead, HAP1 may be a multifunctional polypeptide with distinct domains for interacting with various proteins.

Because yeast two-hybrid assays suggest that N-terminal huntingtin (amino acids 1–253) binds to the region of HAP1 (amino acids 278–370) that was unable to interact with P150, huntingtin and P150 may bind to different regions of HAP1 and thus form a stable protein complex. The N terminus of huntingtin was used to characterize its binding to HAP1 because it contains the polyglutamine repeat and could be expressed in yeast and *in vitro*. Moreover, the N-terminal human huntingtin containing an expanded glutamine repeat (>115 units) was sufficient to induce a progressive neurological phenotype in transgenic mice (Mangiarini et al., 1996). An *in vitro* binding assay showed that the N terminus of huntingtin and P150 could be precipitated by GST–HAP1. It is interesting to note that huntingtin, especially the huntingtin with 44 glutamine repeats, seemed to decrease the binding of P150 to HAP1. It remains to be shown whether an expanded polyglutamine repeat alters the association of HAP1 with P150 *in vivo*.

A protein complex containing HAP1, P150, and huntingtin *in vivo* is suggested by several lines of evidence. Immunoprecipitation showed that huntingtin and P150 were coprecipitated with HAP1 from rat brain. The nearly identical migrations of cytosolic HAP1 and P150 in a sucrose gradient further supports this suggestion. Because P150 associates with a dynein protein complex that can be isolated by ATP extraction of microtubules (Gill et al., 1991; Paschal et al., 1993; Schafer et al., 1994), we also examined the association of HAP1 and huntingtin with microtubules in the absence and presence of ATP. Although HAP1 was found to be as enriched as P150 in microtubules, ATP extraction of microtubules liberated less HAP1 than P150 in the ATP eluate. Therefore, unlike other substoichiometric components in the ATP-released dynactin complex (Gill et al., 1991; Paschal et al., 1993; Schafer et al., 1994), HAP1 may be a minor form in this complex. The association of HAP1 and P150 could mainly occur on membranous organelles, microtubules, and/or in the cytosol. The lower sensitivity of HAP1 to ATP extraction suggests that HAP1 may not directly or tightly bind to the dynein protein complex under these conditions. The enrichment of HAP1 in microtubules may be because of some direct binding of HAP1 to microtubules or to other molecules associated with microtubules.

Previous studies using immunocytochemistry demonstrated that huntingtin is associated with microtubules (DiFiglia et al., 1995; Gutekunst et al., 1995; Bhide et al., 1996). However, we found that huntingtin was not as enriched as HAP1 in microtubule pellets. It is possible that the association of huntingtin with microtubules is via its binding to HAP1 and is therefore not as stable as the binding of HAP1 and P150 to microtubules *in vitro*. Huntingtin may also be involved in protein complexes other than the HAP1–dynactin complex, as suggested by its relatively wide distribution in the sucrose gradient.

The distinct localization of P150 and huntingtin on HAP1 immunoreactive granular structures in transfected cells further suggests that these proteins may associate *in vivo*. Their colocalization was selective because another polyglutamine-repeat protein, DRPLA, did not colocalize with HAP1 on these structures. Overexpressed proteins in transfected cells may not display the same subcellular localization as they do *in vivo*; however, these HAP1-A-induced cytoplasmic structures in transfected cells enabled us to examine the protein colocalization in living cells. Although the nature of these structures remains to be defined, imaging the protein colocalization on these structures is especially helpful for confirming the binding results obtained from the yeast two-hybrid screen, *in vitro* binding, and immunoprecipitation. Because the definitive subcellular distribution of P150 *in vivo* has not been obtained, extensive studies are required to confirm whether HAP1 and P150 also colocalize in the brain.

The association of HAP1 with P150 and huntingtin provides a possible link between intracellular transport and the function of normal huntingtin. This is because the dynactin complex is required for dynein-mediated vesicle movement *in vitro* and the function of P150 is thought to be the targeting of dynein motor proteins to intracellular organelles (Gill et al., 1991; Schroer and Sheetz, 1991). Targeting of microtubule motor proteins to membranous organelles may involve a number of different proteins that dynamically associate with a variety of vesicles or organelles. In addition, huntingtin could be intracellularly transported by the HAP1–dynactin complex. Given that the dynactin P150 protein complex plays a role in the targeting or transporting of intracellular organelles or molecules, it is possible that HAP1 and normal huntingtin may have a role in intracellular trafficking.

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