

# HIP1 and HIP12 Display Differential Binding to F-actin, AP2, and Clathrin

IDENTIFICATION OF A NOVEL INTERACTION WITH CLATHRIN LIGHT CHAIN\*

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Published, JBC Papers in Press, March 11, 2002, DOI 10.1074/jbc.M112310200Valerie Legendre-Guillemain<sup>‡§¶</sup>, Martina Metzler<sup>§||</sup>, Martine Charbonneau<sup>‡</sup>, Lu Gan<sup>||</sup>,  
Vikramjit Chopra<sup>||</sup>, Jacynthe Philie<sup>‡</sup>, Michael R. Hayden<sup>||</sup>, and Peter S. McPherson<sup>‡\*\*</sup>From the <sup>‡</sup>Department of Neurology and Neurosurgery, Montreal Neurological Institute, McGill University, Montreal, Quebec H3A 2B4, Canada and the <sup>||</sup>Centre for Molecular Medicine and Therapeutics, Department of Medical Genetics University of British Columbia, Vancouver, British Columbia V5Z 4H4, Canada

**Huntingtin-interacting protein 1 (HIP1) and HIP12 are orthologues of Sla2p, a yeast protein with essential functions in endocytosis and regulation of the actin cytoskeleton. We now report that HIP1 and HIP12 are major components of the clathrin coat that interact but differ in their ability to bind clathrin and the clathrin adaptor AP2. HIP1 contains a clathrin-box and AP2 consensus-binding sites that display high affinity binding to the terminal domain of the clathrin heavy chain and the ear domain of the AP2  $\alpha$  subunit, respectively. These consensus sites are poorly conserved in HIP12 and correspondingly, HIP12 does not bind to AP2 nor does it demonstrate high affinity clathrin binding. Moreover, HIP12 co-sediments with F-actin in contrast to HIP1, which exhibits no interaction with actin *in vitro*. Despite these differences, both proteins efficiently stimulate clathrin assembly through their central helical domain. Interestingly, in both HIP1 and HIP12, this domain binds directly to the clathrin light chain. Our data suggest that HIP1 and HIP12 play related yet distinct functional roles in clathrin-mediated endocytosis.**

Huntington disease, a neurological disorder characterized by selective loss of striatal and cortical neurons and manifest clinically by chorea and intellectual decline, results from polyglutamine expansion of huntingtin into the pathologic range of beyond 35 repeats. In an effort to understand the disease mechanism, several groups have identified proteins that associate with huntingtin. One such protein, HIP1, was identified in a yeast two-hybrid screen (1, 2). Binding of HIP1 to huntingtin is dramatically reduced following polyglutamine expansion, strongly implicating this interaction in the disease process. Subsequent to the identification of HIP1, a highly related pro-

tein (HIP12/HIP1R) was identified based on its homology to HIP1 (3–5). In contrast to HIP1, HIP12 does not bind directly to huntingtin (5).

HIP1 and HIP12 are orthologues of yeast Sla2p, a protein that functions in both endocytosis and regulation of the actin cytoskeleton (6–9). Each of these proteins contains an ENTH (epsin N-terminal homology) domain that is thought to be involved in clathrin-mediated endocytosis through binding to phosphatidylinositol 4,5-bisphosphate-containing membranes (10–13). The central portion of these proteins consists of a helical domain with a high probability to form coiled-coil interactions, followed by a C-terminal talin-homology domain. The talin-homology domain has been shown to bind F-actin in Sla2p and HIP12 suggesting a function in linking membrane attachment and clathrin-coated vesicle (CCVs)<sup>1</sup> formation with actin dynamics (4, 14).

Recent studies have shown that both HIP1 and HIP12 are enriched on clathrin-coated pits and CCVs and co-localize with markers of endocytosis including clathrin, the clathrin adaptor AP2 and Rab5 (4, 15–17). HIP1 binds directly to the terminal domain of the clathrin heavy chain through a type I clathrin box with the sequence LMDMD and to the ear domain of the  $\alpha$  subunit of AP2 (15–17). Moreover, a fragment of HIP1 containing the clathrin and AP2-binding sites blocks clathrin-mediated endocytosis (15, 17).

To further characterize the roles of HIP1 and HIP12 in clathrin-mediated endocytosis, we analyzed and compared the protein interaction and functional properties of these two proteins. Here, we report that HIP1 strongly associates with CCVs but unlike HIP12, does not bind directly to F-actin. In contrast, HIP12 does not demonstrate high affinity binding to clathrin and AP2 as seen for HIP1. However, HIP1 and HIP12 both bind directly to the clathrin light chain through their central helical domain that interestingly, stimulates clathrin assembly. These results suggest related but distinct functions for HIP1 and HIP12 in clathrin-mediated endocytosis and identify a novel interaction with clathrin light chain that appears to contribute to clathrin assembly.

## EXPERIMENTAL PROCEDURES

**Antibodies and Reagents**—Rabbit polyclonal antibodies against HIP1 (HIP1FP) and HIP12 (HIP12FP) and a mouse monoclonal antibody against HIP1 (mAb HIP no. 9) were previously described (5, 15). Mono-

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§ Both authors contributed equally to this work.

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\*\* Canadian Institutes of Health Research Investigator and a McGill University William Dawson Scholar. To whom correspondence should be addressed: Dept. of Neurology and Neurosurgery, Montreal Neurological Institute, McGill University, 3801 rue University, Montreal, QC, H3A 2B4, Canada. Tel.: 514-398-7355; Fax: 514-398-8106; E-mail: peter.mcpherson@mcgill.ca.

<sup>1</sup> The abbreviations used are: CCVs, clathrin coated vesicles; AP2, adaptor protein 2; CHC, clathrin heavy chain; ENTH, Epsin N-terminal homology; GST, glutathione S-transferase; HIP1, huntingtin interacting protein 1; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PtdIns(4,5)P<sub>2</sub>, phosphatidylinositol (4,5)-bisphosphate; HA, hemagglutinin; MES, 4-morpholineethanesulfonic acid.

clonal antibodies against the clathrin heavy chain and  $\alpha$ -adaptin were purchased from Transduction Laboratories. Monoclonal antibody against the clathrin light chain was purchased from Santa Cruz. Monoclonal antibodies against the FLAG, HA, and His<sub>6</sub> epitopes were purchased from Sigma, Roche Molecular Biochemicals, and Qiagen, respectively.

**DNA Constructs and Recombinant Proteins**—Mammalian expression constructs encoding full-length HIP1 and HIP12 and the talin homology domain of HIP1, each with a FLAG epitope tag at the C terminus, were previously described (5, 15). A construct encoding human full-length HIP12 with a C-terminal HA epitope tag was generated by insertion of a cDNA sequence, 5'-GGAGGTGGATATCCCTATGATGCCCGATT-ATGCC, encoding a linker of 3 glycines followed by the HA tag. The integrity of the HA-tagged HIP12 construct was verified by DNA sequencing. Bacterial fusion proteins encoding His<sub>6</sub>-tagged terminal domain of the clathrin heavy chain and the amino acids 276–335 of HIP1 fused to GST (GST-HIP1-(276–335)) were previously described (15). A His<sub>6</sub>-tagged construct encoding full-length clathrin light chain b was generated by subcloning the GFP-clathrin light chain b cDNA (kindly provided by Dr. Juan Bonifacino) into the pTrcHisB bacterial expression vector (Qiagen). The following HIP1 and HIP12 GST fusion proteins were created by PCR amplification from either full-length HIP1 or HIP12 cDNAs with subsequent cloning into pGEX-2T or pGEX-4T vectors (Amersham Biosciences): GST-HIP12-(302–348), GST-HIP1-(336–610), GST-HIP12-(349–644), GST-HIP1-(731–1003) (GST-HIP1-talin), and GST-HIP12-(765–1068) (GST-HIP12-talin).

**Pull-down Assays**—Rat brains were homogenized in buffer A (10 mM HEPES-OH, pH 7.4, 0.83 mM benzamidine, 0.23 mM phenylmethylsulfonyl fluoride, 0.5  $\mu$ g/ml aprotinin, and 0.5  $\mu$ g/ml leupeptin) and the homogenates were centrifuged at 205,000  $\times$   $g_{max}$  for 30 min at 4 °C. The supernatant (cytosolic extract) was collected and Triton X-100 was added to 1% final concentration. Aliquots of the extract (2 mg) were incubated overnight at 4 °C with GST fusion proteins pre-coupled to glutathione-Sepharose beads. After incubation, samples were washed three times in buffer A containing 1% Triton X-100. For binding experiments, purified His<sub>6</sub>-tagged clathrin terminal domain or clathrin light chain b fusion proteins (15  $\mu$ g) in buffer A were incubated overnight at 4 °C with various GST fusion proteins pre-coupled to glutathione-Sepharose beads. After incubation, samples were washed three times in buffer A. In all cases, proteins specifically bound to the beads were eluted with gel sample buffer and analyzed by SDS-PAGE and Western blot.

For pull-downs from transfected cells, HeLa cells were grown on 10-cm<sup>2</sup> dishes and transfected with 6  $\mu$ g of HIP1 or HIP12 cDNA using FuGENE (Roche Molecular Biochemicals). Twenty-four h post-transfection, cells were washed twice with ice-cold phosphate-buffered saline (PBS) and lysed in buffer A (containing 1% Triton X-100 and 10  $\mu$ M Pefa (Roche Molecular Biochemicals)). Samples were incubated overnight at 4 °C with GST fusion proteins pre-coupled to glutathione-Sepharose beads. After incubation, samples were washed three times in buffer A containing 1% Triton X-100 and 10  $\mu$ M Pefa. Bound proteins were analyzed by SDS-PAGE and transferred to nitrocellulose for Western blot analysis.

**Immunoprecipitation Assays**—HEK-293T cells were grown on 10-cm<sup>2</sup> plates and transfected with 4  $\mu$ g of HIP12 cDNA by CaPO<sub>4</sub> precipitation. Transfected and nontransfected cells were washed 24 h post-transfection with ice-cold PBS and lysed in ABL buffer (20 mM Tris-Cl, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100) supplemented with a 1:100 dilution of aprotinin (Sigma), 20 ng/ml leupeptin (Roche Molecular Biochemicals), and 10  $\mu$ M Pefa. The cell lysates were sonicated and centrifuged at 245,000  $\times$   $g_{max}$  for 15 min. The supernatants were then incubated for 6 h at 4 °C with mouse monoclonal antibody HIP1 no. 9 pre-bound to protein G-Sepharose (Amersham Biosciences). Subsequently, samples were washed 6 times in ABL buffer and specifically bound proteins were analyzed by SDS-PAGE and Western blot.

**Immunofluorescence**—Cells were plated onto gelatinized glass slides in 6-well tissue culture plates and transfected with 1  $\mu$ g of HIP1 or HIP12 cDNA. Twenty-four h post-transfection, the cells were washed in PBS, fixed in PBS containing 4% paraformaldehyde for 15 min at room temperature, and permeabilized in PBS containing 0.3% Triton X-100 and 1% paraformaldehyde for 5 min. The cells were then incubated in PBS containing 3% normal goat serum for 30 min to block nonspecific binding. Depending on the experiment, the cells were then incubated with monoclonal antibodies against the FLAG or HA epitopes for 1 h at room temperature or overnight at 4 °C with a polyclonal antibody against HIP1, followed by a 1-h incubation at room temperature with appropriate secondary antibodies in PBS containing 2% normal goat serum. Staining of the actin cytoskeleton was achieved by incubation

with Texas Red-phalloidin. Cells were then extensively washed in PBS, mounted, and observed on a BioRad Radiance Plus confocal microscope (BioRad, Hercules, CA) using Laser Sharp software (BioRad).

**Actin-binding Assays**—Purified human non-muscle monomeric actin (Cytoskeleton Inc.) was polymerized as described previously (4) and incubated with purified GST-HIP1-talin and GST-HIP12-talin (20  $\mu$ g each) for 1 h at room temperature in a final volume of 50  $\mu$ l. The samples were then centrifuged for 30 min at 362,000  $\times$   $g_{max}$ . Protein components of the pellets and supernatants were analyzed by SDS-PAGE followed by Coomassie Blue staining. Gels were scanned on a ScanJet 6300 (Hewlett Packard) and protein amounts were determined using Quantity One (Bio-Rad) software. Statistical analysis was done by the Student's *t* test.

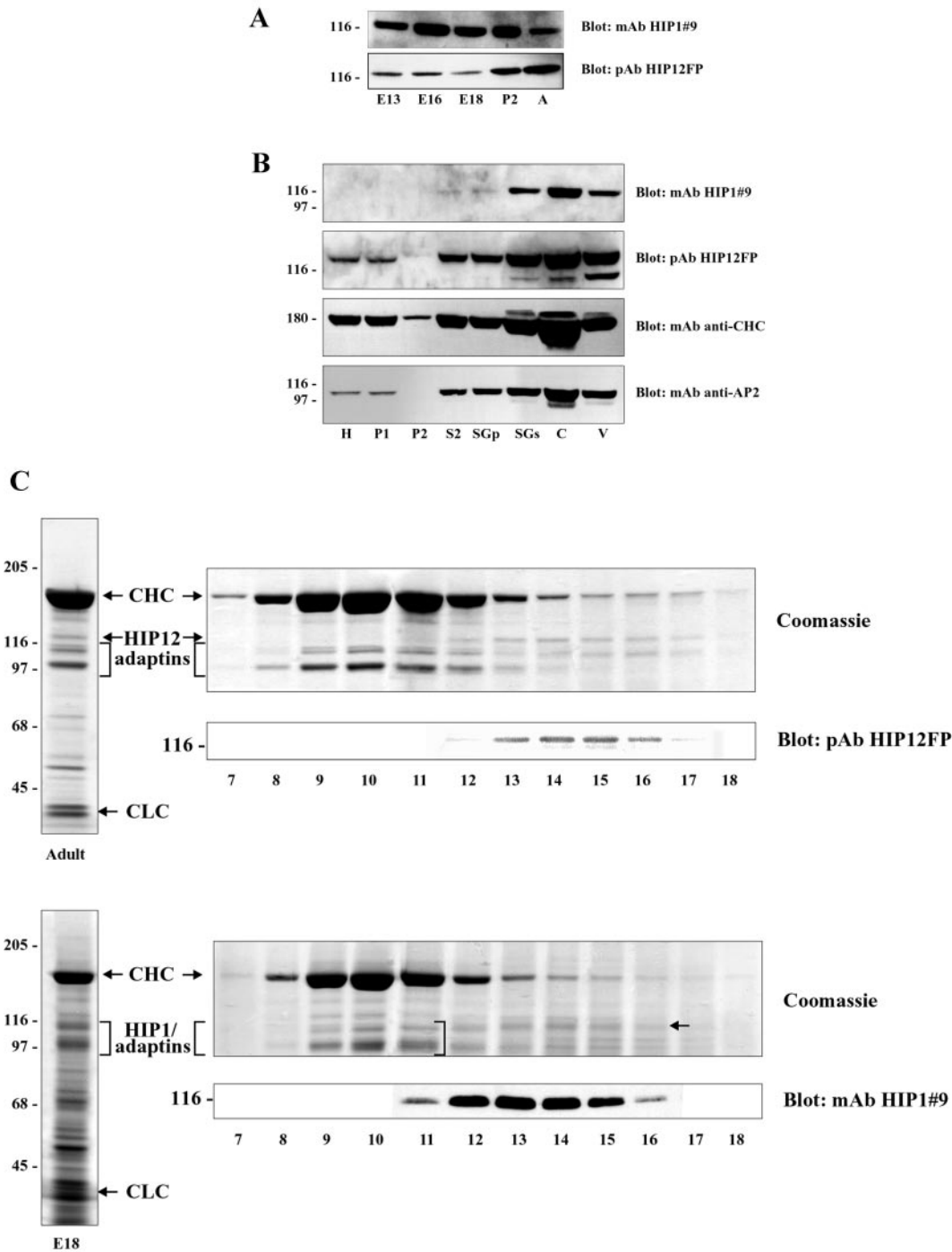
**Clathrin Purification and Clathrin Assembly Assays**—CCVs were purified from adult rat brains as described (18). To purify clathrin, coats were stripped from the vesicles by incubation in buffer B (0.5 M Tris, pH 7.0, 2 mM EDTA, 0.83 mM benzamidine, 0.23 mM phenylmethylsulfonyl fluoride, 0.5  $\mu$ g/ml aprotinin, and 0.5  $\mu$ g/ml leupeptin) for 15 min on ice. The samples were then centrifuged for 15 min at 245,000  $\times$   $g_{max}$  with the purified coat fraction remaining in the supernatant. The stripping and centrifugation steps were repeated a second time and the supernatants were pooled. The pooled supernatants were loaded onto a continuous 5–20% sucrose gradient made in buffer B and then centrifuged for 3.5 h at 195,000  $\times$   $g_{max}$  in a Sorvall Step Saver vertical rotor. Fractions of 2 ml were collected and analyzed by SDS-PAGE and Coomassie Blue staining. Peak clathrin fractions were pooled and dialyzed overnight in clathrin assembly buffer (10 mM Tris-Cl, pH 8.5) and subsequently used for clathrin assembly assays.

Clathrin assembly assays were performed with 0.5  $\mu$ M purified clathrin and different concentrations (0.12–2  $\mu$ M) of fusion proteins in a final volume of 90  $\mu$ l of clathrin assembly buffer. Assembly was initiated at 4 °C by addition of 10  $\mu$ l of 1 M MES, pH 6.7. The mixture was kept on ice for 45 min and then centrifuged at 400,000  $\times$   $g_{max}$  for 6 min. The supernatant (80  $\mu$ l) was loaded on a SDS-PAGE and the clathrin assembly was quantified by Coomassie Blue staining.

## RESULTS

**HIP1 and HIP12 Are Abundant Coat Proteins of CCVs of Adult and Embryonic Rat Brains**—Both HIP1 and HIP12 have been implicated in clathrin-mediated endocytosis (4, 15–17, 19). To further characterize the endocytic functions of these proteins, we sought to directly compare their biochemical and functional properties. Both HIP1 and HIP12 are predominantly expressed in brain (1, 2). Within brain, however, the two proteins demonstrate distinct developmental expression profiles (Fig. 1A). Specifically, HIP1 is most highly expressed during early development, with the highest detectable levels at embryonic day 13 (E13) and E16, whereas HIP12 expression is only weakly detectable at E13 through E18 but increases in postnatal day 2 rats with high levels of expression in adult brain (Fig. 1A). Interestingly, both HIP1 and HIP12 are highly enriched on the coats that are stripped from purified CCVs prepared from E18 brains (data not shown) and adult brains (Fig. 1B). HIP1 and HIP12 show a comparable enrichment in the CCV fractions to both each other and to the coat proteins clathrin and AP2 (data not shown and Fig. 1B). Moreover, both proteins appear to be major components of the clathrin coats (Fig. 1C). To make this determination, coat proteins stripped from adult or E18 CCVs where fractionated on linear 5–20% sucrose gradients, which readily separated HIP1 and HIP12 from the large subunits of the adaptin complexes (Fig. 1C). Coomassie Blue staining of the fractions revealed that HIP1 and HIP12 were present on E18 or adult rat brain CCVs, respectively, at near stoichiometric levels with adaptin subunits (Fig. 1C).

**HIP1 and HIP12 Co-localize and Interact**—Earlier work in our laboratory has shown that HIP1 and HIP12 interact in the yeast two-hybrid system (5). To confirm this interaction, co-immunoprecipitation studies were performed in HEK-293T cells. Immunoprecipitation of endogenous HIP1 with a monoclonal antibody led to co-immunoprecipitation of endogenous HIP12 whereas no precipitation of HIP12 was seen in the

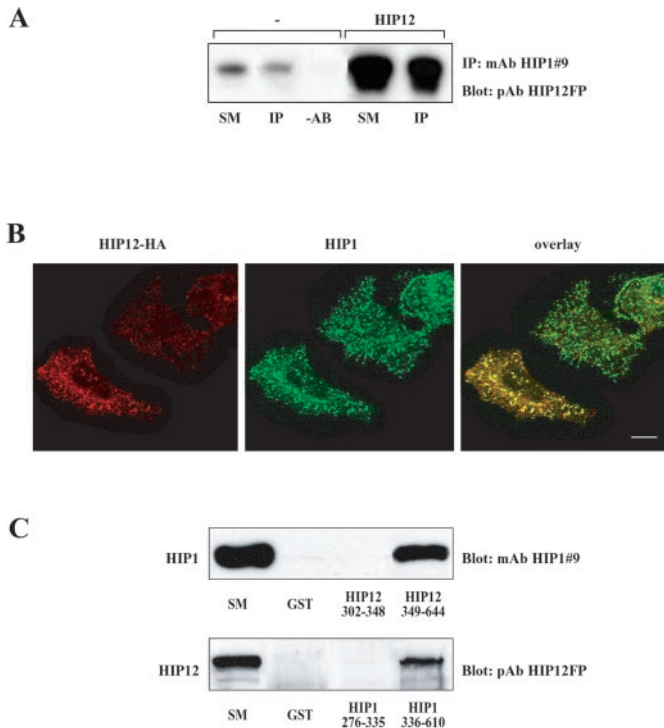


**FIG. 1. HIP1 and HIP12 are coat proteins of CCVs and display distinct developmental expression profiles.** *A*, post-nuclear supernatants (100  $\mu$ g) from brain extracts of various rat developmental stages were analyzed by Western blot with anti-HIP1 or anti-HIP12 antibodies as indicated. *E*, embryonic day; *P*, post-natal day; *A*, adult. *B*, purified CCVs were obtained by subcellular fractionation of adult rat brain homogenates. Aliquots (100  $\mu$ g) of the various fractions were analyzed by Western blot with antibodies against clathrin heavy chain (*CHC*), the  $\alpha$ -adaptin subunit of AP2 (*AP2*), HIP1 and HIP12, as indicated. *H*, homogenate; *P*, pellet; *S*, supernatant; *SGp*, sucrose gradient pellet; *SGs*, sucrose gradient supernatant; *C*, coats; *V*, vesicles. The coats and vesicles correspond to the supernatant and pellet fractions, respectively, following incubation of the purified CCVs with 0.5 M Tris, pH 9.0. *C*, purified CCVs from adult and E18 brains were stripped of their coats in 0.5 M Tris, pH 7.0, 2 mM EDTA. Aliquots of the coat fractions were analyzed on SDS-PAGE and Coomassie Blue staining (*panels at left*). The coat proteins were then fractionated on continuous 5–20% sucrose gradients. The gradients were collected in 20 equal fractions and aliquots from fractions 7–18 were analyzed by SDS-PAGE with Coomassie Blue staining (*Coomassie*). The migration of HIP1 and HIP12 was determined by Western blot as indicated. The *bracket* on the E18 gradient demonstrates the migratory positions of the large subunits of the adaptins, whereas the *arrow* indicates the migratory position of HIP1.

absence of HIP1 antibody (Fig. 2A). Transfection of the cells with HIP12 cDNA led to a significantly greater immunoprecipitation of HIP12 by endogenous HIP1 (Fig. 2A). We next analyzed the intracellular localization of HIP1 and HIP12 in neuronal NT2 cells following transfection of HA-tagged HIP12.

Confocal microscopy analysis of the expression of HIP12-HA shows punctate staining throughout the cell and extensive co-localization with endogenous HIP1 (Fig. 2B). These punctae correspond predominantly to clathrin-coated pits and CCVs as previously described (4, 15).

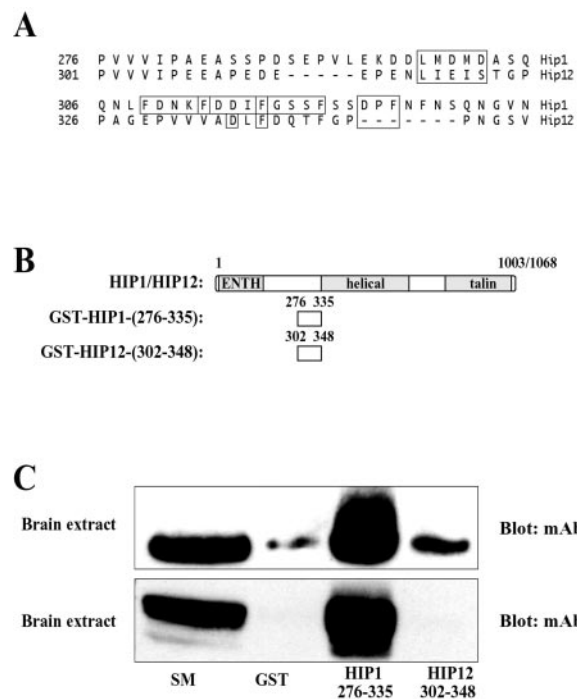




**FIG. 2. HIP1 and HIP12 interact via their helical domains.** *A*, HEK 293T cells were transfected with full-length HIP12 cDNA (HIP12) or were left untransfected (–). Cell lysates were prepared and immunoprecipitations were carried out in the presence or absence (–AB) of mouse mAb HIP1#9. Cell lysate (SM) and immunoprecipitated protein (IP) were analyzed by SDS-PAGE and Western blot with anti-HIP12FP antibody. *B*, co-localization of transfected HIP12-HA (red) and endogenous HIP1 (green) was analyzed in neuronal NT2 cells. Overlays of the images are shown in the panel on the right (overlay). Scale bar = 10  $\mu$ M. *C*, HeLa cells were transfected with either HIP1 or HIP12 full-length cDNA as indicated. Cell extracts were prepared and tested for binding to GST alone or various HIP12 and HIP1 GST fusion proteins. Equal volumes of unbound and bound proteins were analyzed by SDS-PAGE and Western blot with anti-HIP1 and anti-HIP12 antibodies as indicated.

To determine the regions of HIP1 and HIP12 that mediate their interaction, pull-down experiments were performed. Previous studies have shown that a small fragment of HIP12 encompassing the helical domain interacts with HIP1 in the yeast two-hybrid system (5). Therefore, we analyzed whether HIP1 and HIP12 interact through their helical domains. In fact, the helical domains of both HIP1 and HIP12 are sufficient to specifically pull-down HIP12 and HIP1, respectively (Fig. 2C). These studies suggest that the HIP1-HIP12 interaction could affect specific functions of each protein.

**HIP1 and HIP12 Display Differential Binding to Clathrin and AP2**—Between its ENTH and helical domains, HIP1 contains a clathrin-box consensus sequence, LMDMD, which binds to the terminal domain of the clathrin heavy chain (15). This sequence is found in close association with the sequences FDNKF, FDDIF, FGSSF, and DPF, which match consensus sequences for binding to AP2 (17). Upon sequence alignment, the LMDMD motif in HIP1 aligns with the sequence LIEIS in HIP12 (Fig. 3A). The LIEIS sequence resembles a clathrin-box motif similar to that found in the clathrin-binding proteins ACK1 and ACK2 (20, 21). In contrast, the DPF motif is absent from HIP12 and the FDNKF, FDDIF, and FGSSF sequences, which match the consensus FX(N/D/S)X(F/L) (17), are not conserved (Fig. 3A). We thus designed a HIP1-GST fusion protein, GST-HIP1-(276–335), encompassing these sites (Fig. 3B) and tested for its ability to bind clathrin and AP2 in comparison with a GST fusion protein encoding the corresponding region of

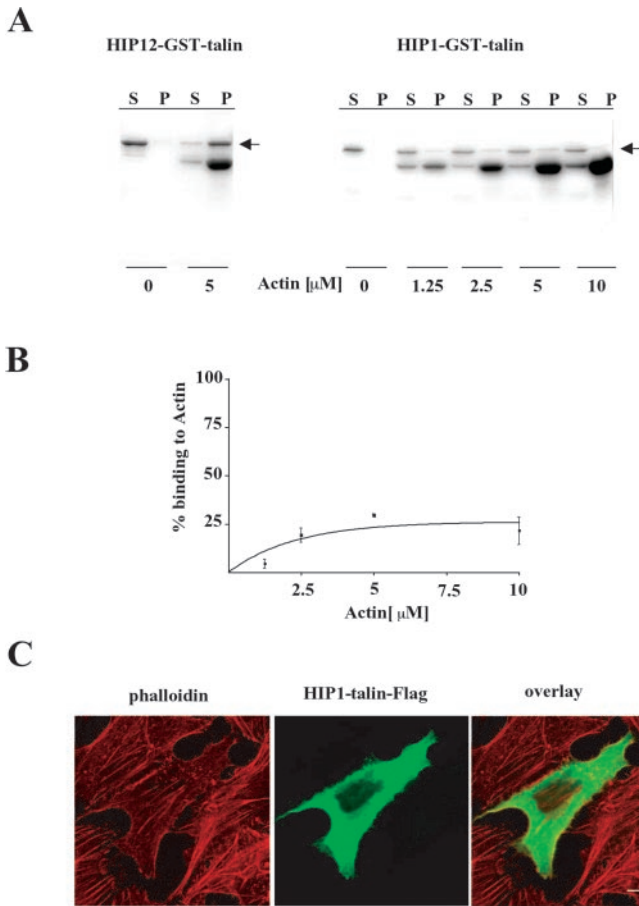


**FIG. 3. HIP1 and HIP12 display differential binding to clathrin and AP2.** *A*, sequence alignment of a region of HIP1 and HIP12 demonstrating consensus clathrin and AP2-binding sites. *B*, schematic representation of various GST fusion proteins used for the binding assays. *C*, soluble proteins from brain extracts were affinity purified with equal amounts of HIP1 or HIP12-GST fusion proteins bound to glutathione-Sepharose beads. Proteins specifically bound to the beads were analyzed by Western blot with antibodies against CHC or the  $\alpha$ -adaptin subunit of AP2 (AP2) as indicated.

HIP12, GST-HIP12-(302–348). As previously demonstrated (15), GST-HIP1-(276–335) binds strongly to clathrin (Fig. 3C, top panel). In contrast, GST-HIP12-(302–348) demonstrates much weaker clathrin binding (Fig. 3C, top panel). In both cases, the binding is mediated through the terminal domain of the clathrin heavy chain (15) (data not shown). Moreover, GST-HIP1-(276–335) binds strongly to AP2 whereas no binding of GST-HIP12-(302–348) to AP2 was detected (Fig. 3C, bottom panel).

**In Contrast to HIP12, HIP1 Does Not Bind Actin**—HIP12 binds actin through its talin-homology domain (4). To determine whether the talin homology domain of HIP1 also mediates actin binding, a GST-HIP1-talin homology domain fusion protein was analyzed for co-sedimentation with F-actin. As a control, the talin homology domain of HIP12 was expressed as a GST fusion protein and analyzed in parallel. Surprisingly, in contrast to HIP12, HIP1 bind only very weakly to actin *in vitro* (Fig. 4A). Specifically, in the presence of 5  $\mu$ M F-actin, 87.6%  $\pm$  6.0 (mean  $\pm$  S.E.,  $n = 3$ ) of GST-HIP12-talin homology domain was bound to F-actin. In contrast, only 19.3%  $\pm$  3.7 (mean  $\pm$  S.E.,  $n = 3$ ) of the GST-HIP1-talin homology domain was bound to the same amount of polymerized actin (Fig. 4B). A number of different parameters such as pH and ion concentrations were tested for their potential effect on the binding of HIP1-talin to actin. However, none of these alterations allowed for increased binding (data not shown). Consistent with this observation is the lack of co-localization between the HIP1-talin homology domain and the cortical actin cytoskeleton following overexpression of HIP1-talin in cultured cells (Fig. 4C). This is in contrast to the observation that the talin homology domain of HIP12 shows enrichment along cortical actin filaments (4).

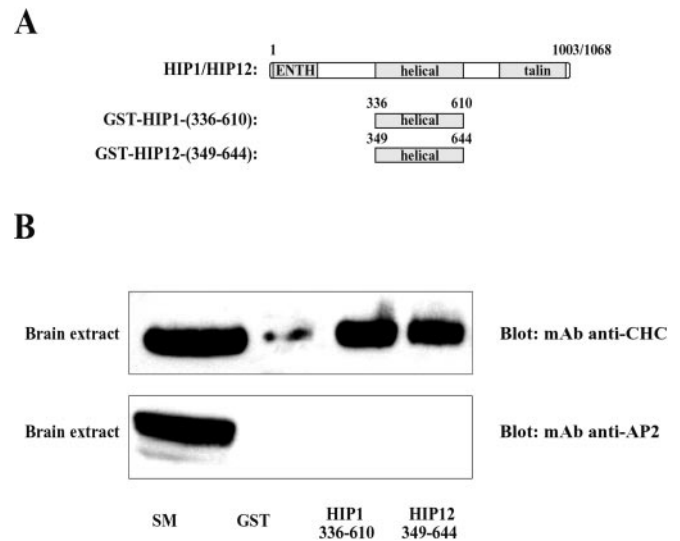
**HIP1 and HIP12 Contain a Second Clathrin-binding Site in Their Helical Domain**—We previously demonstrated that GST-



**FIG. 4. HIP1 does not bind actin in contrast to HIP12.** *A*, binding of the HIP1-talin homology domain expressed as a GST fusion protein (*HIP1-talin*) to filamentous actin was analyzed by cosedimentation in the presence of various concentrations of assembled actin (0–10  $\mu$ M). Binding of the HIP12-talin homology domain GST fusion protein (*HIP12-talin*) was used as positive control. The arrows indicate HIP1 or HIP12 fusion proteins in the supernatant (S) and pellet (P) fractions. *B*, the percentage of GST-HIP1-talin bound to actin is shown as mean  $\pm$  S.E. of three independent experiments. *C*, HeLa cells expressing a FLAG-tagged construct encoding the HIP1-talin homology domain (*HIP1-talin-Flag*) were immunostained with an anti-FLAG antibody shown in green and with Texas Red-phalloidin shown in red to reveal F-actin. Overlays of the confocal images are shown in the right. Scale bar = 10  $\mu$ M.

HIP1-(219–616), encompassing the clathrin box motif and the helical domain, demonstrated stronger binding to clathrin than GST-HIP1-(276–335) alone, encompassing only the clathrin box consensus site (15). This observation led us to suggest the presence of a second clathrin-binding site in the helical domain of HIP1 (15). Moreover, the helical domains of HIP1 and HIP12 were recently reported to mediate binding to clathrin (16, 19). We thus designed fusion proteins encompassing the helical domains of HIP1, GST-HIP1-(336–610) and HIP12, GST-HIP12-(349–644) (Fig. 5A) and tested their ability to bind clathrin and AP2. Both fusion proteins show specific binding to clathrin from brain extracts (Fig. 5B, top panel) but not to AP2 (Fig. 5B, bottom panel).

**HIP1 and HIP12 Promote Clathrin Assembly through Their Helical Domains**—Full-length HIP12 stimulates clathrin assembly (19). Moreover, both HIP1 and HIP12 contain a DLL motif in their helical domains that contributes to the clathrin assembly activity of other clathrin adaptor proteins (22). We thus tested the ability of both of the helical domains to promote clathrin assembly *in vitro*. Interestingly, both GST-HIP1-(336–610) and GST-HIP12-(348–644) fusion proteins stimulated



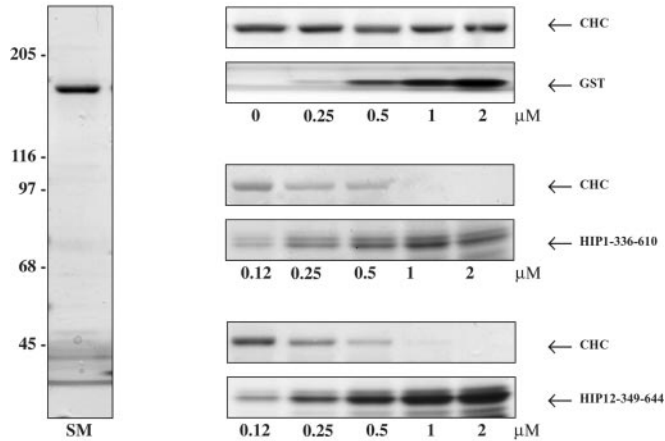
**FIG. 5. The helical domains of HIP1 and HIP12 bind to clathrin but not AP2.** *A*, schematic representation of various GST fusion proteins used for the binding assays. *B*, soluble proteins from brain extracts were affinity purified with equal amounts of HIP1 or HIP12 GST fusion proteins bound to glutathione-Sepharose beads. Proteins specifically bound to the beads were analyzed by Western blot with antibodies against CHC or the  $\alpha$ -adaplin subunit of AP2 (AP2) as indicated.

clathrin assembly in a dose-dependent manner with full activity detected in the presence of 0.5  $\mu$ M fusion protein (Fig. 6). In contrast, GST alone did not stimulate clathrin assembly (Fig. 6). These results demonstrate that like HIP12 (19), HIP1 stimulates clathrin assembly, and that for both proteins, assembly activity is localized to their helical domains, which demonstrate weak clathrin binding.

**HIP1 and HIP12 Bind Directly to Clathrin Light Chain**—Inspection of the helical domains of HIP1 and HIP12 failed to reveal any type I or type II clathrin box sequences (23) that could mediate interactions with the terminal domain of the clathrin heavy chain. This suggested the possibility of a novel form of clathrin interaction mediated by the helical domains. To explore this further, we performed additional binding assays with GST-HIP1-(336–610) and GST-HIP12-(348–644). Consistent with the lack of consensus clathrin terminal domain-binding sites, neither helical domain fusion protein bound to purified His<sub>6</sub>-tagged clathrin terminal domain (Fig. 7A, bottom panel), whereas the same fusion proteins bound to clathrin heavy chain from brain extracts (Fig. 7A, top panel). Clathrin exists in the cytosol of cells as a triskelion, in which three molecules of clathrin heavy chain are associated with three molecules of clathrin light chain (24). Thus, it is possible that the helical domain fusion proteins bind to clathrin light chain leading to an indirect interaction with the heavy chain. This idea is supported by the recent observation that addition of light chains to truncated clathrin cages restores their ability to bind HIP12 (19). To directly test if the helical domains of HIP1 and HIP12 bind to clathrin light chain, we generated a His<sub>6</sub>-tagged fusion protein encoding full-length clathrin light chain b. Interestingly, this purified fusion protein bound strongly to the HIP1 and HIP12 helical domain GST fusion proteins (Fig. 7B). Taken together, these studies demonstrate that the helical domains of HIP1 and HIP12 do not interact with the clathrin heavy chain via its terminal domain, although we cannot rule out the possibility that they could interact with the proximal or distal legs of the heavy chain. In contrast, HIP1 and HIP12 bind directly to the clathrin light chain through the helical domains, which also stimulate clathrin assembly.

## DISCUSSION

Many proteins involved in clathrin-mediated endocytosis harbor a facet of protein interaction modules that enable them to function in the recruitment of clathrin triskelia to sites of

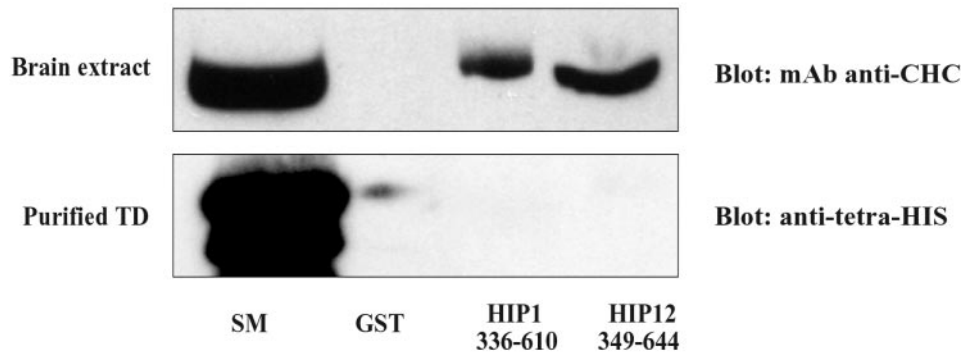


**FIG. 6. HIP1 and HIP12 promote clathrin assembly through their helical domains.** An aliquot of the purified clathrin used as a starting material (*SM*) for the clathrin assembly assays was resolved on SDS-PAGE and stained with Coomassie Blue. Clathrin assembly assays were performed with increasing amounts of fusion proteins GST, GST-HIP1-(336–610), and GST-HIP12-(348–644) as indicated. The GST fusion proteins and the clathrin remaining in the supernatant after high-speed centrifugation following initiation of clathrin assembly were analyzed by SDS-PAGE and Coomassie Blue staining.

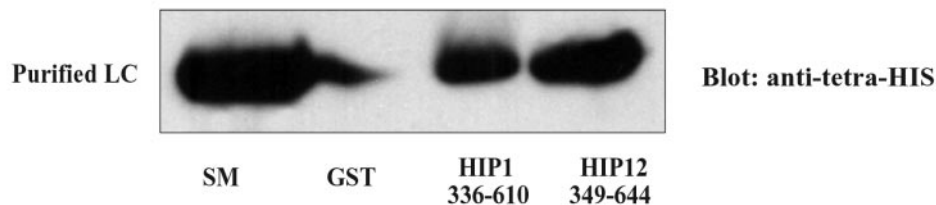
endocytosis and in the subsequent formation of CCVs, shedding of coat proteins, and intracellular transport of the uncoated vesicle (25, 26). In the present work, we show that two family members, HIP1 and HIP12, are major components of CCVs. As such, they are comparable in their enrichment on purified vesicles to the coat proteins  $\alpha$ -adaptin, clathrin, and AP180. In contrast, accessory proteins of clathrin-mediated endocytosis such as dynamin, synaptojanin, amphiphysin I and II, epsin, endophilin, and Eps15 are far less enriched on CCVs (27). Moreover, HIP1 and HIP12 are present on CCVs at near stoichiometric levels with the adaptor proteins. Finally, both proteins can be stripped from purified vesicles by treatment with Tris buffer, which is well established to remove coat components from vesicles (28). Thus, HIP1 and HIP12 should be considered as novel members of the clathrin coat.

Several endocytic accessory proteins that participate in the formation of clathrin-coated pits and CCVs contain a tandem arrangement of high affinity clathrin and AP2 consensus-binding sites including amphiphysin I (29), epsin (30), and arrestins (31). This tandem arrangement of consensus sites is also found in HIP1 but is not conserved in the HIP1 family member HIP12. HIP12 does not contain the DPF/W or FX(N/D/S)X(F/L) consensus AP2-binding sites (17, 32, 33) and correspondingly does not show binding to AP2. Moreover, the clathrin box motif that is present in HIP12 binds clathrin with rather low affinity. Since the tandem arrangement of clathrin- and AP2-binding sites is thought to stabilize clathrin/adaptor interactions (30), HIP12 probably contributes less to the stabilization of this

A



B



**FIG. 7. HIP1 and HIP12 bind directly to the clathrin light chain through their helical domains.** A, soluble proteins from brain extracts or purified His<sub>6</sub>-tagged clathrin terminal domain (*TD*) fusion protein were affinity purified with equal amounts of HIP1 or HIP12 GST fusion proteins bound to glutathione-Sepharose beads. Proteins specifically bound to the beads were analyzed by Western blots with antibodies against CHC or the His epitope (tetra-HIS) as indicated. B, purified His<sub>6</sub>-tagged clathrin light chain b (*LCb*) fusion protein was affinity purified with equal amounts of HIP1 or HIP12 GST fusion proteins bound to glutathione-Sepharose beads. Proteins specifically bound to the beads were analyzed by Western blot with the anti-His epitope antibody (*tetra-HIS*).



protein complex. As such, HIP12 is comparable with amphiphysin II that interacts with clathrin through type I and type II clathrin box sequences but does not bind to AP2 (34, 35).

The formation of clathrin-coated pits does not occur randomly. Instead, defined sites of endocytosis exist that are demarcated by AP2 (36) with extended areas of the membrane not being involved in coated pit formation (37). It has been proposed that the underlying membrane cytoskeleton contains coated pit-nucleation sites and that actin may be involved in the spatial organization of endocytic components at the membrane (37, 38). Interestingly, both HIP1 and HIP12 contain an ENTH domain, a PtdIns(4,5)P<sub>2</sub>-binding motif that is found in several other coat and accessory proteins including AP180 and epsins (11, 12). Recent studies have demonstrated that PtdIns(4,5)P<sub>2</sub> is localized predominantly on the plasma membrane of quiescent neurons but that its levels are increased on endocytic membranes following stimulation of endocytic activity (39). In addition to potential PtdIns(4,5)P<sub>2</sub> binding, HIP1 and HIP12 contain a talin homology domain that in HIP12 binds directly to actin (4). Thus, it is intriguing to speculate that HIP1 and HIP12 may play a role in the coordination of clathrin, actin, and PtdIns(4,5)P<sub>2</sub> functions at the plasma membrane to demarcate sites of clathrin assembly. Surprisingly, however, HIP1 does not bind to actin. This appears to result from the observation that a helical segment at the N terminus of the talin homology domain of HIP1 can disrupt actin binding through an intrasteric interaction (40). Nevertheless, heterodimerization of HIP1 and HIP12 may be a mechanism that allows HIP1 to indirectly bind to F-actin and that also allows AP2 to be recruited to clathrin nucleation sites defined by the HIP proteins at the plasma membrane.

Consistent with a role for the HIP1-HIP12 complex in defining endocytic active zones, HIP12 has been demonstrated to stimulate clathrin assembly (19). Here, we have determined that HIP1 also functions in clathrin assembly and that for both proteins assembly activity is localized to the helical domain. GST fusion proteins encoding the helical domains of HIP1 and HIP12 are also able to affinity purify clathrin triskelia from brain extracts. Interestingly, the helical domains do not bind to the terminal domain of the clathrin heavy chain but do bind directly to the clathrin light chain, consistent with recent results demonstrating that light chains are necessary for HIP12 binding to clathrin hub domains (19). Clathrin triskelia, composed of three heavy chains, each with a bound light chain, assemble into a polyhedral lattice at physiological pH only in the presence of assembly proteins such as AP2 or AP180. Interestingly, it has been proposed that the light chains interact with the distal legs of the clathrin heavy chains to prevent clathrin assembly (41). Specifically, the recombinant hub fragments of the clathrin heavy chains assemble in the absence of light chains at physiological pH whereas addition of purified light chains blocks the assembly reaction (41). Moreover, Src-dependent phosphorylation of the clathrin heavy chain at tyrosine 1477, a site that appears to be involved in binding to light chain, stimulates clathrin assembly, possibly by releasing the inhibitory effect of the light chain (42). Thus, HIP1 and HIP12 may stimulate clathrin assembly by interacting with the clathrin light chain and through an unknown mechanism release light chain-mediated inhibition allowing the heavy chains to interact and assemble (41). This model is consistent with the observation that overexpression of HIP12 in cultured cells causes a mislocalization of the light chains without a major effect on the distribution of the heavy chains (19). HIP1 and HIP12 are the first assembly proteins demonstrated to interact directly with clathrin light chain and this interaction appears

to reveal a novel mechanism involved in the regulation of clathrin assembly.

Growing evidence suggests a role for the disease-associated protein huntingtin in clathrin-mediated endocytosis and intracellular vesicle transport. For example, huntingtin associates with the  $\alpha$ -adaptin C subunit of the AP2 complex (43) and with endophilin A3 (44) and it associates with vesicles and microtubules (44–47). The interaction of huntingtin with AP2 and endophilin suggests that huntingtin participates in the complex network of interactions regulating clathrin-mediated endocytosis and vesicle recycling. Huntingtin has been localized to endosomal and lysosomal membranes (48) and it is present but not enriched on purified CCVs (46) (data not shown). Intriguingly, the interaction of HIP1 with huntingtin is directly modulated by polyglutamine expansion in huntingtin, suggesting that disturbances in protein interaction and subsequent alterations in clathrin-mediated endocytosis could contribute to the pathogenesis of Huntington disease.

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