

# Huntingtin Interacting Protein 1 (HIP1) Regulates Clathrin Assembly through Direct Binding to the Regulatory Region of the Clathrin Light Chain\*

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**Huntingtin interacting protein 1 (HIP1) is a component of clathrin coats. We previously demonstrated that HIP1 promotes clathrin assembly through its central helical domain, which binds directly to clathrin light chains (CLCs). To better understand the relationship between CLC binding and clathrin assembly we sought to dissect this interaction. Using C-terminal deletion constructs of the HIP1 helical domain, we identified a region between residues 450 and 456 that is required for CLC binding. Within this region, point mutations showed the importance of residues Leu-451, Leu-452, and Arg-453. Mutants that fail to bind CLC are unable to promote clathrin assembly *in vitro* but still mediate HIP1 homodimerization and heterodimerization with the family member HIP12/HIP1R. Moreover, HIP1 binding to CLC is necessary for HIP1 targeting to clathrin-coated pits and clathrin-coated vesicles. Interestingly, HIP1 binds to a highly conserved region of CLC previously demonstrated to regulate clathrin assembly. These results suggest a role for HIP1/CLC interactions in the regulation of clathrin assembly.**

Endocytosis via clathrin-coated pits (CCPs)<sup>1</sup> and vesicles (CCVs) is a major pathway for the internalization of plasma membrane cargo (1). The formation of CCPs and CCVs is initiated by the assembly of clathrin triskelia into polyhedral cages that drive membrane curvature directly or through the recruitment of proteins that influence membrane dynamics (2). Clathrin triskelia are composed of three copies of the clathrin

heavy chain (CHC) linked together through a trimerization domain at their C termini (3). Extending from the trimerization domain are proximal and distal leg segments that end in a globular N-terminal domain (3). Clathrin assembly occurs through multiple weak interactions between the leg segments (4). There are a variety of proteins that regulate clathrin assembly during endocytosis, most notably the clathrin adaptor protein 2 (AP-2). Additional assembly proteins include AP180, epsin, HIP1, and its family member HIP12/HIP1R (5). Each of these proteins bind directly to clathrin triskelia as well as membrane phospholipids and these properties contribute to their ability to stimulate clathrin assembly at specific sites on the plasma membrane (6–14).

Within triskelia, each CHC is generally thought to be bound to one of two clathrin light chains (CLCs), CLCa and CLCb. There is an established role for CLCs in inhibiting clathrin assembly (15, 16), which may be explained by the orientation of CLCs on the CHC. Through a domain in the central region, CLCs bind to the proximal leg of the CHC with their C termini oriented toward the trimerization domain (17, 18). The N termini may make a U-turn halfway along the proximal leg folding back toward the trimerization domain (19) or they may extend along the proximal leg (20). In either scenario, the N-terminal region could be oriented on the proximal leg in a manner that could inhibit the self-association of CHCs necessary for assembly. Interestingly, the N termini of the CLCs contain a domain that is conserved among all isoforms and throughout evolution (21) and this domain has been implicated in the ability of CLCs to regulate clathrin assembly (22).

The identification of binding partners for CHC and CLCs has allowed for an increased understanding of the regulation of clathrin assembly but the mechanistic details of this process remain poorly defined. HIP1 is a newly described component of the coats of CCVs that binds to the N-terminal domain of the CHC through a clathrin box sequence LMDMD and to AP-2 through sequences that match consensus AP-2-binding motifs DPF and FXDXF (10, 23, 24). Through a central helical domain, HIP1 binds directly to CLCs and promotes clathrin assembly *in vitro* (14). The helical region also mediates HIP1 homodimerization and its heterodimerization with HIP12/HIP1R (14). Like HIP1, HIP12/HIP1R binds to CLCs through its central helical region and promotes clathrin assembly *in vitro* (11, 14). Electron microscopic analysis of the cages formed by HIP12/HIP1R-mediated assembly reveals that 85% are 60–80 nm in diameter similar to the cages formed with other assembly proteins such as AP-2 and AP180 (11). Together, these properties demonstrate a role for HIP1 and HIP12/HIP1R in the regulation of clathrin assembly.

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<sup>1</sup> The abbreviations used are: CCPs, clathrin-coated pits; AP-2, adaptor protein 2; CHC, clathrin heavy chain; CLC, clathrin light chain; CCVs, clathrin-coated vesicles; GST, glutathione S-transferase; GFP, green fluorescent protein; HIP1, Huntingtin interacting protein 1; HIP12/HIP1R, Huntingtin interacting protein 12/Huntingtin interacting protein 1 related; MES, 2-(4-morpholino)-ethanesulfonic acid; PBS, phosphate-buffered saline; WT, wild-type.

TABLE I  
List of primers used to generate different constructs

Construct	PCR product generated	Forward primer sequence	Reverse primer sequence
GST-HIP1-AAA	Megaprimer	5'-CCACGCTGCCGCGCGCGGAAGAATG-3'	5'-GCGCCCGGGTCATTCTTCAAGCTGGTTCAGG-3'
	Insert	5'-GCGGGATCCAAGGATGAGAAGGGACCAC-3'	
GST-HIP12-ELL-AAA	Megaprimer	5'-GGAGGCAGCCGCGCGCGCGCGCAG-3'	5'-GCGGAATTCTCAGTCGTCAGCTTGCTCAGC-3'
	Insert	5'-GCGGGATCCAAGGACGACAGGGACCTCC-3'	
GST-HIP12-AAA-DLL	Megaprimer	5'-GCACGCGCGCGCGCGCGAAAGAAGC-3'	5'-GCGGAATTCTCAGTCGTCAGCTTGCTCAGC-3'
	Insert	5'-GCGGGATCCAAGGACGACAGGGACCTCC-3'	
GST-HIP1-(336-546)	Insert	5'-GCGGGATCCAAGGATGAGAAGGGACCAC-3'	5'-GCGCCCGGGTTCACAGGCTTGCTCCGCTCC-3'
GST-HIP1-(336-463)	Insert	5'-GCGGGATCCAAGGATGAGAAGGGACCAC-3'	5'-GCGCCCGGGTTCAGGACACTGTTTGGTCACC-3'
GST-HIP1-(336-456)	Insert	5'-GCGGGATCCAAGGATGAGAAGGGACCAC-3'	5'-GCGCCCGGGTTCATGCATTCTTCCGACG-3'
GST-HIP1-(336-449)	Insert	5'-GCGGGATCCAAGGATGAGAAGGGACCAC-3'	5'-GCGCCCGGGTTCAGCGTGGTTCGAACC-3'
GST-HIP1-(336-442)	Insert	5'-GCGGGATCCAAGGATGAGAAGGGACCAC-3'	5'-GCGCCCGGGTTCAGCTGTACTTCTCCTTTAGC-3'
GST-HIP1-(336-435)	Insert	5'-GCGGGATCCAAGGATGAGAAGGGACCAC-3'	5'-GCGCCCGGGTTCAGCTATATATCGCTGTTTCATTGG-3'
GST-HIP1-(436-463)	Insert	5'-GCGGGATCCAAGCTAAAGGAGAAGTACAGC-3'	5'-GCGCCCGGGTTCAGGACACTGTTTGGTCACC-3'
GST-HIP1-D450A	Megaprimer	5'-CCACGCTGCCCTGCTGCG-3'	5'-GCGCCCGGGTTCATTCTTCAAGCTGGTTCAGG-3'
	Insert	5'-GCGGGATCCAAGGATGAGAAGGGACCAC-3'	
GST-HIP1-L451A	Megaprimer	5'-CGCTGACGCGCTGCGGAAG-3'	5'-GCGCCCGGGTTCATTCTTCAAGCTGGTTCAGG-3'
	Insert	5'-GCGGGATCCAAGGATGAGAAGGGACCAC-3'	
GST-HIP1-L452A	Megaprimer	5'-TGACCTGGCGCGGAAGAATG-3'	5'-GCGCCCGGGTTCATTCTTCAAGCTGGTTCAGG-3'
	Insert	5'-GCGGGATCCAAGGATGAGAAGGGACCAC-3'	
GST-HIP1-R453A	Megaprimer	5'-CCTGCTGCGGAAGAATGCAG-3'	5'-GCGCCCGGGTTCATTCTTCAAGCTGGTTCAGG-3'
	Insert	5'-GCGGGATCCAAGGATGAGAAGGGACCAC-3'	
GST-HIP1-K454A	Megaprimer	5'-GCTGCGGGCGAATGCAGAG-3'	5'-GCGCCCGGGTTCATTCTTCAAGCTGGTTCAGG-3'
	Insert	5'-GCGGGATCCAAGGATGAGAAGGGACCAC-3'	
GST-HIP1-N455A	Megaprimer	5'-GCGGAAGGCTGCAGAGG-3'	5'-GCGCCCGGGTTCATTCTTCAAGCTGGTTCAGG-3'
	Insert	5'-GCGGGATCCAAGGATGAGAAGGGACCAC-3'	
GST-CLCb-(1-228)	Insert	5'-GCGGAATTCTCAAGCGATCCGGTTG-3'	5'-GCGGAATTCTCAGCGGGACAGTGGCG-3'
GST-CLCb-(1-165)	Insert	5'-GCGGAATTCTCAAGCGATCCGGTTG-3'	5'-GCGGAATTCTCATGGCTGCTGGTAG-3'
GST-CLCb-(77-228)	Insert	5'-GCGGGATCCCTTTCAGGAGGCCAACG-3'	5'-GCGGAATTCTCAGCGGGACAGTGGCG-3'
GST-CLCb-(90-157)	Insert	5'-GCGGGATCCATTGCCAGCTGACAG-3'	5'-GCGGAATTCTCAAGCGATCCGGTTG-3'
GST-CLCb-(EED/QQN)	Megaprimer	5'-GCGGAATTCTCAAGCGATCCGGTTG-3'	5'-GCGCCCGGGTTCATTCTTCAAGCTGGTTCAGG-3'
	Insert	5'-GCGGAATTCTCAAGCGATCCGGTTG-3'	5'-GCGGAATTCTCAGCGGGACAGTGGCG-3'
GST-CLCb-(Asp-20-41)	Template (1-19)	5'-GCGGAATTCTCAAGCGATCCGGTTG-3'	5'-GCGCCCGCTCCGGG-3'
	Template (42 + -228)	5'-GGAGGCAGCCGCGCGCGCGCTTCGGGC-3'	5'-GCGGAATTCTCAGCGGGACAGTGGCG-3'
	Insert	5'-GCGGAATTCTCAAGCGATCCGGTTG-3'	5'-GCGGAATTCTCAGCGGGACAGTGGCG-3'

To further understand the relationship between the function of HIP1 to assemble clathrin *in vitro* and its direct binding to CLCs, we have identified the sites in HIP1 that mediate CLC interactions and have generated point mutations that block CLC binding and *in vitro* clathrin assembly. Moreover, we have determined that HIP1 binds to the N-terminal domain of the CLCs that has been implicated in the regulation of clathrin assembly. These data further support a role for HIP1 in the formation of CCPs and CCVs.

#### EXPERIMENTAL PROCEDURES

**Antibodies and Reagents**—A rabbit polyclonal antibody against HIP12 and a mouse monoclonal antibody against HIP1 were described previously (10, 25). Monoclonal antibodies against AP-2, CHC, and the FLAG and His<sub>6</sub> epitope tags were from Affinity Bioreagents, Transduction Labs, Sigma, and Qiagen, respectively.

**DNA Constructs and Recombinant Proteins**—GST-HIP1-(276-335), GST-HIP12-(302-348), GST-HIP1-(336-610), and GST-HIP12-(349-644) were described previously (10, 14). The following HIP1 and CLCb GST fusion proteins were created by PCR amplification from full-length HIP1 cDNA or CLCb cDNA with the appropriate primers (see Table I) with subsequent cloning into pGEX-4T-1 vectors (Amersham Biosciences): GST-HIP1-(336-546), GST-HIP1-(336-463), GST-HIP1-(336-456), GST-HIP1-(336-449), GST-HIP1-(336-442), GST-HIP1-(336-435), GST-HIP1-(436-463), GST-CLCb-(1-228), GST-CLCb-(1-165), GST-CLCb-(77-228), and GST-CLCb-(90-157). The GST fusion proteins GST-HIP1-AAA, GST-HIP12-ELL-AAA, GST-HIP12-AAA-DLL, GST-HIP1-D450A, GST-HIP1-L451A, GST-HIP1-L452A, GST-HIP1-R453A, GST-HIP1-K454A, GST-HIP1-N455A, and GST-CLCb-(EED/QQN) were generated by the megaprimer technique (26). A first step of PCR amplification from either full-length HIP1, HIP12, or CLCb cDNAs was performed with appropriate primers (see Table I) to generate the megaprimer for each mutant. A second step of PCR amplification from either full-length HIP1, HIP12, or CLCb cDNAs was performed to generate the fragment using the megaprimer generated in the first PCR and appropriate primers (see Table I), with subsequent cloning into pGEX-2T or pGEX-4T-1 (Amersham Biosciences). The GST-CLCb-(Δ20-41) was created by 2 steps of PCR amplification. The

first step generates the templates CLCb-(1-19) and CLCb-(42-228) with appropriate primers (see Table I) using the full-length CLCb cDNA. The second step generates the fragment, using the two templates generated in the first PCR and the appropriate primers (see Table I) with subsequent cloning into pGEX-4T-1 (Amersham Biosciences). Mammalian expression constructs encoding full-length FLAG-HIP1 or full-length HA-HIP12 were described previously (10, 14, 25). The DLL to AAA mutation in the FLAG-tagged HIP1 construct was introduced by subcloning of the appropriate fragment digested from GST-HIP1-AAA. The GFP-CLCb construct was a generous gift from Dr. Juan Bonifacio. His<sub>6</sub>-HIP1-(336-610) was generated by subcloning of the appropriate fragment from GST-HIP1-(336-610) into pTrcHisA (Invitrogen). His<sub>6</sub>-CLCb was described previously (14). All constructs were confirmed by sequencing.

**Binding Assays**—HEK-293 cells or HeLa cells were transfected by calcium phosphate. After 48 h, cells were washed in phosphate-buffered saline (PBS: 154 mM NaCl, 58 mM NaPO<sub>4</sub>, pH 7.4) and then lysed in buffer A (10 mM HEPES-OH, pH 7.4, 0.83 mM benzamide, 0.23 mM phenylmethylsulfonyl fluoride, 0.5 μg/ml aprotinin, and 0.5 μg/ml leupeptin) and Triton X-100 was added to a final concentration of 1%. Lysates were centrifuged at 245,000 × *g*<sub>max</sub> for 15 min and the supernatant was incubated overnight at 4 °C with various GST fusion proteins pre-coupled to glutathione-Sepharose. The beads were subsequently washed 3 times in buffer A containing 1% Triton X-100. For other binding assays, purified His<sub>6</sub>-tagged fusion protein (15 μg) in buffer A was incubated for 3 h at 4 °C with GST fusion proteins pre-coupled to glutathione-Sepharose. The beads were subsequently washed 3 times in buffer A. For all assays, proteins specifically bound to the beads were eluted with sample buffer and analyzed by SDS-PAGE and Western blot. Overlays of enriched fractions of coat proteins prepared from adult rat brain CCVs were performed as described (14, 27).

**CCV Purification and Clathrin Assembly Assays**—Clathrin from brain CCVs and CCVs from transfected HEK-293 cells were purified as described previously (14). Clathrin assembly assays were performed with 0.5 μM purified clathrin and different concentrations (0.5-2 μM) of fusion proteins in a final volume of 90 μl of clathrin assembly buffer (10 mM Tris-Cl, pH 8.5). Assembly was conducted at physiological pH and was initiated at 4 °C by addition of 10 μl of 1 M MES, pH 6.5-6.7. The

mixture was kept on ice for 45 min and then centrifuged at  $400,000 \times g_{\max}$  for 6 min. The proteins in the supernatant and pellet were separated by SDS-PAGE and were detected by Coomassie Blue staining.

**Immunofluorescence**—HeLa cells were plated on poly-L-lysine-coated coverslips, transfected with 0.8  $\mu\text{g}$  of DNA with Genejuice (Novagen) and were processed for indirect immunofluorescence using standard protocols.

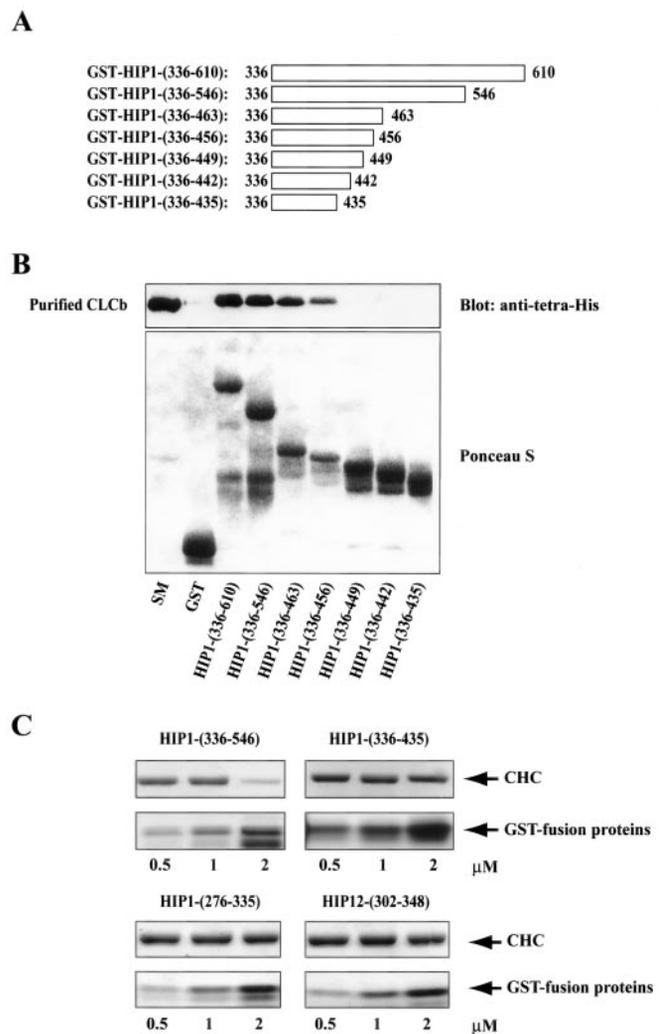
## RESULTS

**A Motif within the Helical Domain of HIP1 Is Necessary for CLC Binding and Clathrin Assembly**—Through its helical domain, HIP1 promotes clathrin assembly *in vitro* and binds directly to the CLC (14). To better understand the relationship between these two activities, we sought to identify the CLC-binding site by designing a series of HIP1 helical domain deletion constructs that were expressed as GST fusion proteins (Fig. 1A) and tested for binding to CLCb in pull-down assays. Sequential deletion of residues from 610 to 456 does not markedly affect the binding of HIP1 to CLCb (Fig. 1B). Although slightly lower levels of binding were observed for GST-HIP1-(336–463) and GST-HIP1-(336–456) compared with the full-length helical domain, this is likely because of the lower levels of fusion protein expressed for these constructs (Fig. 1B). In contrast, deletion to residues 449, 442, or 435 abolishes CLCb interactions (Fig. 1B). These data suggest a CLC-binding site in HIP1 between residues 449 and 456. Surprisingly, GST-HIP1-(436–463) containing this site does not bind to CLCb (data not shown), suggesting that a region upstream of residue 436 is also necessary for CLCb binding.

We next tested whether HIP1 deletion constructs promote clathrin assembly *in vitro*. GST-HIP1-(336–546) and GST-HIP1-(336–463), which bind to CLCb stimulate clathrin assembly, whereas GST-HIP1-(336–435) neither binds to CLCb nor promotes assembly (Fig. 1C and data not shown). We also performed assembly assays with GST-HIP1-(276–335) and GST-HIP12-(302–348), two fusion proteins that bind to the terminal domain of the CHC (10, 14). Neither construct promotes assembly (Fig. 1C), demonstrating that GST fusion protein binding to clathrin is by itself not sufficient to cause clathrin sedimentation. These results provide a correlation between the ability of HIP1 to bind to CLC and stimulate clathrin assembly *in vitro*.

The region between residues 450 and 456 in HIP1 contains the tripeptide <sup>450</sup>DLL. DLL motifs have been implicated in the clathrin assembly activity of AP180 although this appears to involve binding to the CHC (28). Sequence alignment of HIP1 with HIP12/HIP1R revealed an ELL tripeptide in HIP12/HIP1R at position 463 that aligns with the HIP1 <sup>450</sup>DLL motif, as well as a DLL tripeptide starting at position 569. We thus generated GST-HIP1- and GST-HIP12-helical domain fusion proteins in which the DLL or ELL tripeptides were replaced with AAA (Fig. 2A). In contrast to GST-HIP1-(336–610) and GST-HIP12-(349–644), which bind directly to CLCb, mutation of <sup>450</sup>DLL in HIP1 and <sup>463</sup>ELL in HIP12 abolish CLCb binding (Fig. 2B). Mutation of <sup>569</sup>DLL in HIP12 has no effect on this interaction (Fig. 2B). These data suggest that the DLL and ELL sequences in HIP1 and HIP12/HIP1R, respectively, contribute to CLC binding. To support that the fusion proteins were properly expressed, we performed pull-down assays with wild-type and mutant fusion proteins using lysates from cells transfected with HIP1 or HIP12. Mutation in the CLC-binding site on HIP1 and HIP12 does not affect the ability of these fusion proteins to form heterodimers or homodimers (Fig. 2C).

We next tested for the ability of the mutated fusion proteins to promote clathrin assembly *in vitro*. GST-HIP1-AAA does not promote assembly as compared with reactions performed in the absence of fusion protein, whereas HIP12-AAA-DLL shows slight assembly activity (Fig. 2D). Both of these constructs



**FIG. 1. A region between residues 450 and 456 in the HIP1 helical domain is necessary for CLCb binding and clathrin assembly.** A, schematic representation of GST fusion proteins of the helical domain of HIP1 used for binding and clathrin assembly assays. B, purified His<sub>6</sub>-CLCb was incubated with GST or GST-HIP1 fusion proteins bound to glutathione-Sepharose beads. The starting material (SM) equals 1/10th of the amount of His<sub>6</sub>-CLCb added to the beads. The amount of His<sub>6</sub>-CLCb specifically bound to the beads was analyzed by Western blot using an anti-tetra-His antibody and the level of GST fusion proteins was revealed by Ponceau S staining. C, clathrin assembly assays were performed with GST-HIP1 and GST-HIP12 fusion proteins at increasing concentrations. The samples were centrifuged at high-speed and CHC and GST fusion proteins remaining in the supernatant were analyzed by SDS-PAGE and Coomassie Blue staining.

show significantly less assembly activity than GST-HIP12-ELL-AAA, for which clathrin appears in the pellet fraction at the expense of the supernatant (Fig. 2D). These results provide further evidence that HIP1 and HIP12/HIP1R promote clathrin assembly through interactions with CLCs.

**CLC Interactions Target HIP1 to CCPs and CCVs**—We previously observed that a HIP1 construct lacking the helical domain failed to enrich on CCVs isolated from transfected HEK-293 cells (10). To test if the localization of HIP1 is dependent on CLC interactions, we generated full-length HIP1-<sup>450</sup>AAA mutants tagged with FLAG. In pull-down assays, Flag-HIP1-<sup>450</sup>AAA failed to bind to GST-CLCb, whereas binding was readily detected for the WT protein (Fig. 3A) and both proteins bound equally well to a GST construct encoding the  $\alpha$ -ear of AP-2 (data not shown). As described previously, Flag-HIP1-WT, like CHC, is enriched on CCVs isolated from transfected HEK-293 cells (Fig. 3B). In contrast, Flag-HIP1-<sup>450</sup>AAA is not

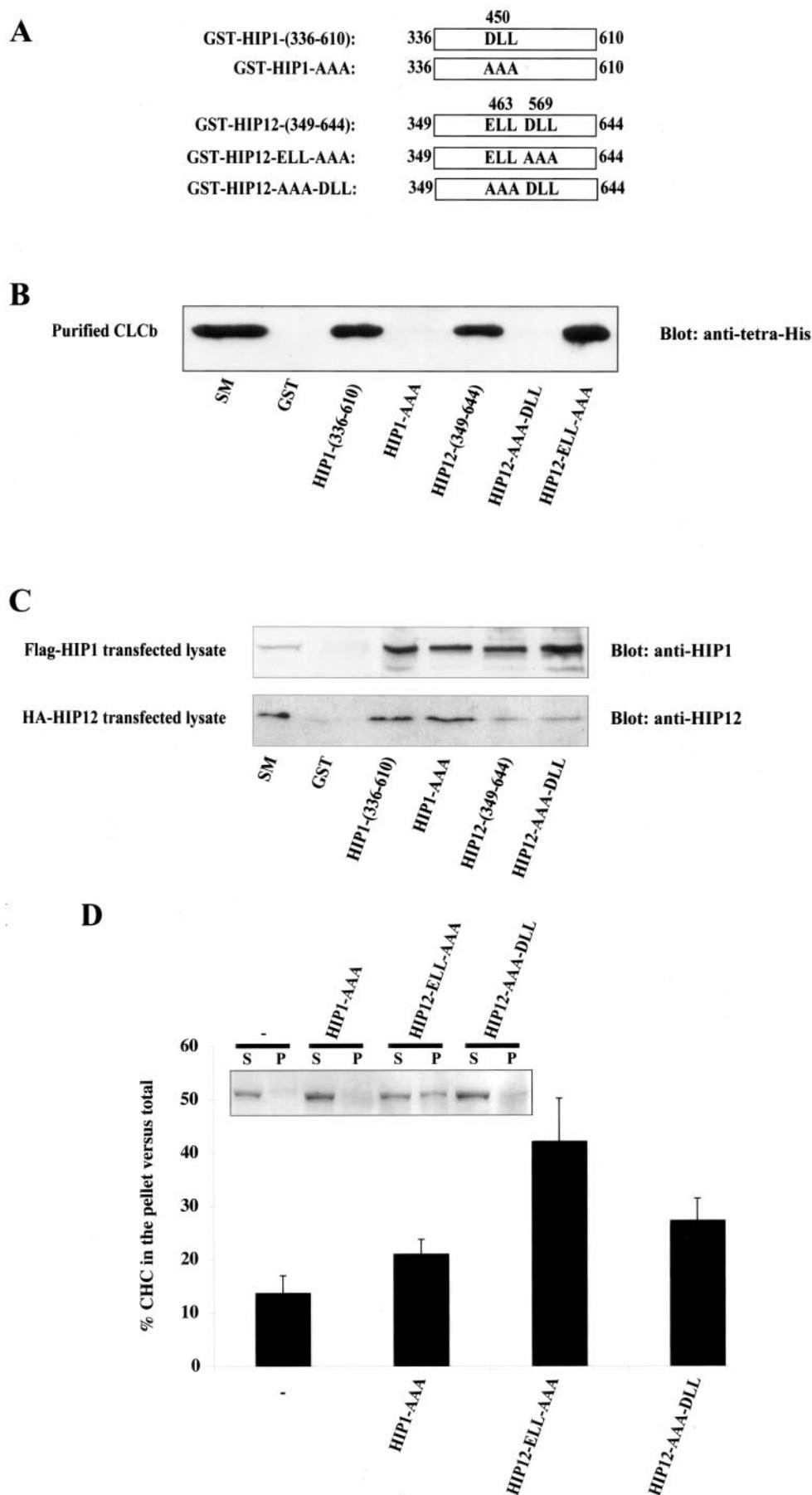
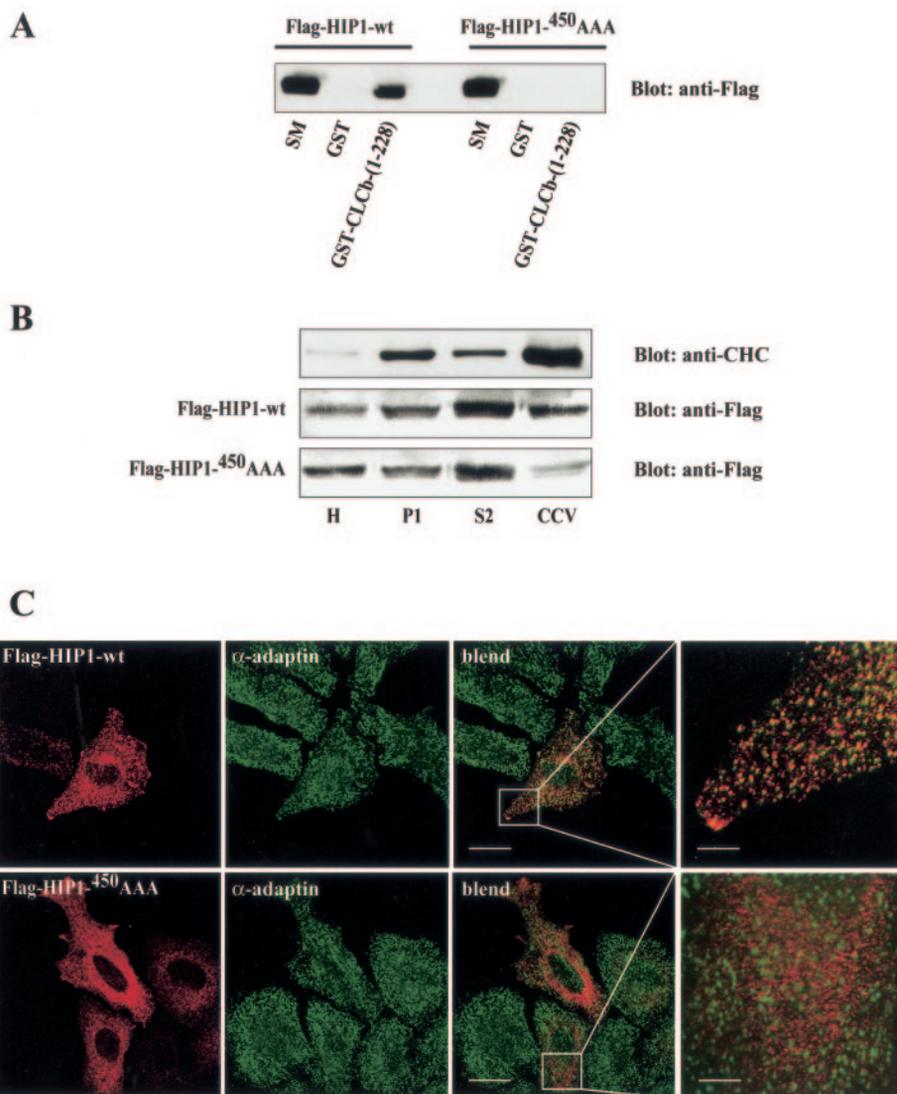


FIG. 2. Conserved residues in HIP1 and HIP12/HIP1R are necessary for CLCb binding and clathrin assembly. *A*, schematic representation of GST fusion proteins containing point mutations in the helical domain of HIP1 and HIP12 used for binding and clathrin assembly assays. *B*, purified His<sub>6</sub>-CLCb was incubated with GST or with GST-HIP1 and GST-HIP12 fusion proteins bound to glutathione-Sepharose beads.

**FIG. 3. HIP1 is targeted to CCPs and CCVs through binding to CLC.** *A*, lysates from HEK-293 cells transfected with full-length FLAG-tagged-HIP1-WT or full-length FLAG-tagged-HIP1-<sup>450</sup>AAA were incubated with equal amounts of GST or GST-CLCb-(1-228) fusion protein bound to glutathione-Sepharose. The starting material (*SM*) equals 1/10th of the amount of lysate added to the beads. Proteins specifically bound to the beads were analyzed by Western blot with an anti-FLAG antibody. *B*, crude CCVs were isolated from HEK-293 cells transfected with full-length FLAG-tagged-HIP1-WT or full-length FLAG-tagged-HIP1-<sup>450</sup>AAA. Aliquots of various fractions (*H*, homogenate; *PI*, pellet 1; *S2*, supernatant 2; and *CCV*s) from the CCV isolation procedure were analyzed by Western blot with anti-CHC or anti-FLAG antibodies. *C*, HeLa cells were transfected with full-length FLAG-tagged-HIP1-WT or full-length FLAG-tagged-HIP1-<sup>450</sup>AAA. Expression of the constructs was revealed by immunofluorescence using an anti-FLAG antibody in *red*. The localization of endogenous AP-2 was revealed by  $\alpha$ -adaptin antibody in *green*. Overlays of the images and 5 times magnification of specific regions are shown. Scale bars represent 10 and 2  $\mu$ m in the magnified images.

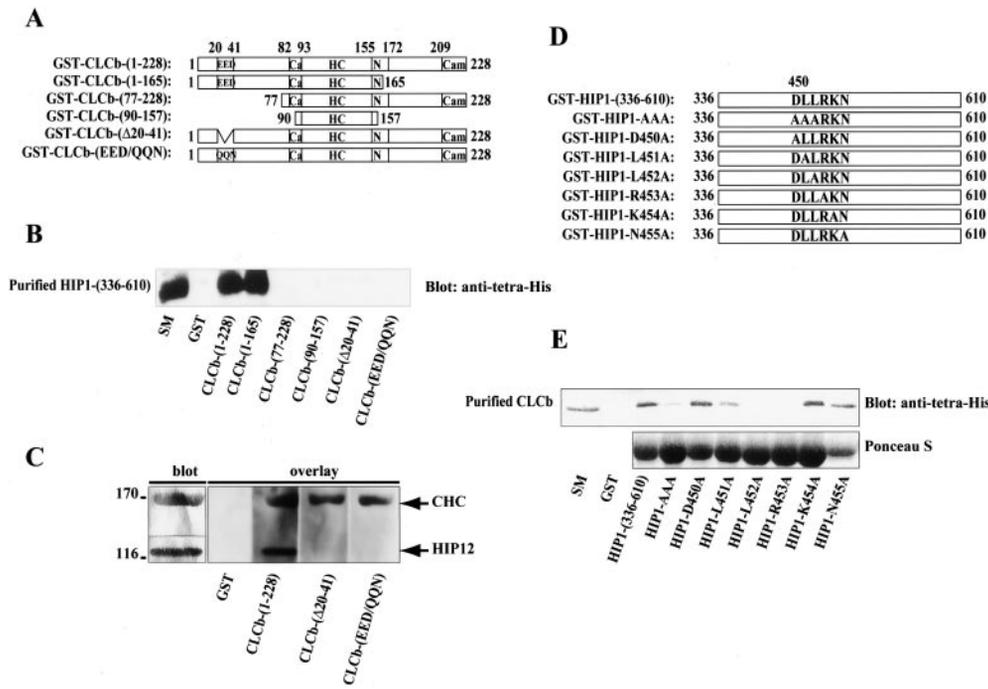


enriched on CCVs compared with the homogenate. We next examined the intracellular localization of these constructs in HeLa cells by immunofluorescence. Flag-HIP1-WT shows a discrete punctuate staining that is similar to that seen for endogenous HIP1 (10) and which is highly co-localized with endogenous AP-2 ( $\alpha$ -adaptin) at CCPs (Fig. 3C). In contrast, Flag-HIP1-<sup>450</sup>AAA has a diffuse distribution displaying little co-localization with  $\alpha$ -adaptin (Fig. 3C). Essentially identical results are obtained using Ds-Red-tagged HIP1 constructs (data not shown). A slight enrichment of both Flag-HIP1-WT and Ds-Red-HIP1-WT in a perinuclear pool likely corresponding to the trans-Golgi network was observed in a proportion of transfected cells (29) and this staining was also lost in <sup>450</sup>AAA mutants (data not shown). These data suggest that the binding of HIP1 to CLC is necessary for its targeting to CCPs and CCVs.

**HIP1 Binds Directly to the Regulatory Region of the CLCb**—We next sought to identify the binding site for HIP1 on

the CLC. Based on the work of Brodsky and colleagues (17), we designed various deletion constructs of the CLCb (Fig. 4A) and tested them for binding to the purified His<sub>6</sub>-tagged HIP1 helical domain. As expected, full-length GST-CLCb binds to His<sub>6</sub>-HIP1-(336–610) (Fig. 4B). Similar binding is observed for a C-terminal deletion construct, GST-CLCb-(1–165) (Fig. 4B). In contrast, a construct with an N-terminal deletion, GST-CLCb-(77–228), and a construct encoding the CHC binding site, GST-CLCb-(90–157), fail to bind to HIP1 (Fig. 4B). The N-terminal segment of CLCb(1–77) contains a region encompassing residues 20–41, which has been described previously to be a regulatory domain for clathrin assembly activity (22). Importantly, a full-length CLCb construct with a deletion of this region, GST-CLCb( $\Delta$ 20–41), fails to bind HIP1 (Fig. 4B). Within this domain are 3 acidic residues critically important for the regulatory activity (22). Interestingly, a GST construct encoding full-length CLCb, in which these residues are mutated to uncharged residues (GST-CLCb-(EED/QQN)), fails to

The starting material (*SM*) equals 1/10th of the amount of His<sub>6</sub>-CLCb added to the beads. The amount of CLCb specifically bound to the beads was analyzed by Western blot using an anti-tetra-His antibody. *C*, lysates from HeLa cells transfected with full-length FLAG-tagged-HIP1 or full-length hemagglutinin (*HA*)-tagged-HIP12 were incubated with equal amounts of GST or GST-HIP1 and GST-HIP12 fusion proteins bound to glutathione-Sepharose beads. Proteins specifically bound to the beads were analyzed by Western blot with anti-HIP1 or anti-HIP12 antibodies. The starting material equals 1/10th of the amount of lysate added to the beads. *D*, clathrin assembly assays were performed with GST or GST-HIP1 and GST-HIP12 fusion proteins as indicated. The samples were centrifuged at high-speed and CHC in the supernatant and pellet were analyzed by SDS-PAGE and Coomassie Blue staining. The percentage of CHC in the pellet *versus* the total (supernatant and pellet) was determined by densitometric scanning for three independent experiments and is expressed as a mean  $\pm$  S.E.



**FIG. 4. HIP1 binds to the regulatory region of CLCb.** *A*, schematic representation of GST fusion proteins of CLCb used for binding assays. *EED* denotes three acidic residues previously shown to be involved in the regulation of clathrin assembly. *Ca*,  $\text{Ca}^{2+}$ -binding site; *HC*, CHC-binding site; *N*, neuronal-specific insert; *Cam*, calmodulin-binding site. *B*, purified His<sub>6</sub>-HIP1-(336–610) was incubated with equal amounts of various GST-CLCb fusion proteins bound to glutathione-Sepharose beads. The amount of HIP1 specifically bound to the beads was analyzed by Western blot using an anti-tetra-His antibody. The starting material (*SM*) equals 1/10th of the amount of His<sub>6</sub>-HIP1-(336–610) added to the beads. *C*, purified coat components, chemically stripped from adult rat brain CCVs, were separated by SDS-PAGE and proteins were revealed either by Western blot with an anti-CHC or anti-HIP12 antibodies or by overlay using GST, GST-CLCb-(1–228), GST-CLCb-(Δ20–41), and GST-CLCb-(EED/QQN) fusion proteins. *D*, schematic representation of GST fusion proteins containing point mutations in the helical domain of HIP1 used for binding assays. *E*, purified His<sub>6</sub>-CLCb was incubated with equal amounts of GST or GST-HIP1 fusion proteins bound to glutathione-Sepharose beads. The amount of CLCb specifically bound to the beads was analyzed by Western blot using an anti-tetra-His antibody and the level of GST fusion proteins was revealed by Ponceau S staining. The starting material equals 1/10th of the amount of His<sub>6</sub>-CLCb added to the beads.

bind HIP1 (Fig. 4*B*). To ensure that the CLCb constructs used in these experiments are functional, we tested them in overlay assays on purified coat fractions from adult rat brain CCVs. As shown in Fig. 4*C*, GST-CLCb-(1–228) recognizes a band that corresponds to the CHC and a second band that corresponds to HIP12/HIP1R, the major HIP isoform expressed in adult rat brain CCVs (14). In contrast, GST-CLCb-(Δ20–41) and GST-CLCb-(EED/QQN) recognize only the CHC and not HIP12/HIP1R (Fig. 4*B*). GST alone does not recognize any proteins (Fig. 4*B*). Together, these results suggest that HIP1 and HIP12/HIP1R bind directly to the regulatory region of the CLC and are in fact the first binding partners described for this important domain.

The demonstration that acidic residues in the regulatory region of CLCb are necessary for HIP1 binding is not obviously compatible with the observation that DLL residues in HIP1 contribute to CLCb interactions (Fig. 2). We thus decided to further examine the CLC-binding site in HIP1 by performing an alanine scan between residues 450 and 456 (DLLRKN). These mutants were generated as GST fusion proteins in the context of the helical domain (Fig. 4*D*) and were tested for binding to His<sub>6</sub>-tagged CLCb. Mutation to alanine of residues Asp-450, Lys-454, and Asn-455 have no effect on CLCb binding (Fig. 4*E*). In contrast, mutation of residues Leu-452 and Arg-453 abolish and residue Leu-451 reduces CLCb binding (Fig. 4*E*). Thus, we have identified the residues LLR in the region of HIP1 from 450 to 456 as important for binding to the CLC. The sequence <sup>450</sup>DLRKN in HIP1 aligns with the sequence <sup>463</sup>ELLRKN in HIP12/HIP1R and mutation in HIP12 of Arg-466 to alanine also abolishes CLCb binding (data not shown).

## DISCUSSION

We have identified a short stretch of amino acids in the helical domains of HIP1 and HIP12/HIP1R that are critical for the interaction of these proteins with CLCs. Mutation of these residues eliminates CLC binding and the ability of the helical domains to stimulate clathrin assembly *in vitro*. This supports that the HIP proteins stimulate clathrin assembly through a mechanism involving CLC interactions. The *Saccharomyces cerevisiae* homologue of the HIPs, Sla2p, also interacts through its helical domain with CLC although the precise motif mediating binding is unknown (30). Alignment of the helical domains of mammalian HIPs with *S. cerevisiae* and *Schizosaccharomyces pombe* homologues fails to reveal an obvious sequence motif in the yeast proteins as that identified here. Thus, the ability of HIP proteins to bind through their helical domains to CLC is conserved in yeast and human although whether or not this represents a conserved function in the regulation of clathrin assembly is unknown.

The assembly of clathrin triskelia occurs through a series of weak interactions between adjacent proximal and/or distal leg segments (4). Adaptor proteins such as AP-2, AP180, and epsin appear to mediate assembly through binding to the terminal domain of the CHC (7, 9, 13, 28) and a model has been described in which these proteins cross-link the terminal domains of three independent triskelion bringing proximal and distal legs from adjacent CHCs into proximity (28, 31). It is also well established that CLCs inhibit clathrin assembly *in vitro* (15, 16). One model predicts that this occurs through the ability of CLCs to mask charge interactions needed for CHC self-interaction (22). One factor that appears to relieve this inhibitory

influence is  $\text{Ca}^{2+}$ , which at relatively high concentrations stimulates assembly (15).  $\text{Ca}^{2+}$  binds to the CLC through an EF-hand motif located between residues 85 and 96 and this induces a conformational change that affects CLC interaction with CHC (21). Our data and that of Chen and Brodsky (46) suggests that interactions of the HIP proteins with CLCs could also provide a mechanism to overcome CLC-mediated inhibition. HIP1 could induce a conformational change affecting the relationship between CLC and CHC or alternatively, it could mask the ability of CLCs to disrupt charge interactions between CHCs. Consistent with the second model, HIP1 binds to specific acidic residues in a highly conserved domain of the CLC previously implicated in charge neutralization (22). Arg-453 and Arg-466, which are required for interaction of HIP1 and HIP12/HIP1R, respectively, with CLC, could contribute to the neutralization of the overall acidic nature of the conserved CLC domain, preventing its ability to block CHC self-interactions leading to assembly.

*In vivo*, the formation of a clathrin coat is a complex process. Clathrin is recruited to the membrane where it assembles into a lattice and a series of dynamic rearrangements of clathrin triskelia occur as the lattice obtains progressively increased curvature (32). Assembly continues until the deeply invaginated CCP buds from the membrane and the CCV is uncoated through clathrin disassembly (5). Not surprisingly, there are multiple proteins that function in the formation of a clathrin coat and each may regulate different steps of assembly. Cells with decreased levels of AP-2 through RNA interference have a decrease in the number of CCPs at the cell surface and the pits that remain are smaller (33, 34), suggesting that AP-2 may be especially important for recruitment and initial lattice formation. AP180 also contributes to the early formation of a lattice (35) and it may additionally function to control the size of CCVs as a mutation affecting its *Drosophila* homologue, LAP results in larger synaptic vesicles generated through CCV-mediated membrane recycling (36). Epsin appears to couple the assembly of clathrin coats to the generation of membrane curvature (35, 37).

The stage at which HIP1 functions in clathrin assembly *in vivo* is not known. However, we demonstrate that CLC interactions are necessary for the subcellular targeting of HIP1 to CCPs and CCVs, consistent with the observation that CLCs are necessary for the ability of HIP12/HIP1R to interact with clathrin cages (11). Thus, it is unlikely that HIP1 functions in the early steps of clathrin recruitment and assembly. It is known that exchange of clathrin triskelia between cytosolic pools and clathrin coats, which necessitates ongoing cycles of assembly/disassembly, is a necessary aspect of the growth phase of clathrin coats (38, 39). Disruption of assembly/disassembly cycles through chemical cross-linking causes an abnormal lattice structure (40). It is possible that the HIP proteins function in assembly reactions related to this dynamic exchange process. Consistent with this notion, RNA interference studies reveal that CCPs still form at the plasma membrane in HIP12/HIP1R-depleted cells, and in fact, there appears to be a more stable association of clathrin coats with the membrane (41). HIP12/HIP1R also functions in clathrin-mediated budding at the trans-Golgi network and as for the plasma membrane, a more stable association of clathrin coats is seen on the trans-Golgi network membrane in HIP12/HIP1R-depleted cells (29). Thus, HIPs could regulate the assembly of clathrin involved in the growth of a CCP that is needed to make it competent to bud from the membrane.

The HIP proteins have roles in clathrin-mediated membrane budding in addition to their function in clathrin assembly. For example, HIP1 has been proposed to mediate the recruitment

of specific cargo molecules such as neuronal glutamate receptors into CCPs (42). Interestingly, it has been recently demonstrated that the presence of cargo in CCPs may drive an equilibrium between coated pit assembly and disassembly toward assembly with an increased probability of generating a mature CCV (43). Stimulation of the assembly activity of HIP1 upon interaction with cargo could provide a mechanism contributing to this phenomenon. In addition, HIP12/HIP1R and the yeast HIP homologue Sla2p are actin-binding proteins. Sla2p deletion in yeast and depletion of HIP12/HIP1R through RNA interference leads to an enhanced co-localization of actin and CCVs, suggesting that HIP12/HIP1R and Sla2p are necessary for a dynamic and productive association of CCVs with the actin cytoskeleton (29, 41, 44). Overexpression of a region of CLCb from residues 1 to 44, which contains the HIP12/HIP1R-binding domain, causes a similar phenotype (46), suggesting that HIP12/HIP1R could link actin to CCPs through CLC interactions. Given the multiple roles of the HIP proteins in clathrin-mediated trafficking, it is perhaps not surprising that their depletion through RNA interference or genetic approaches causes defects in clathrin-mediated endocytosis (41, 42, 44). Surprisingly, knockdown of both CLCs by RNA interference fails to block endocytosis of transferrin or epidermal growth factor receptor (45). However, because CLCs are normally inhibitory to assembly, their loss of function may not be manifest as an inhibition of endocytosis similar to what is seen for loss of HIP proteins. Future studies will examine these issues and the role of the HIPs in regulating clathrin assembly *in vivo*.

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