Huntingtin-interacting Protein 1 (Hip1) and Hip1-related Protein (Hip1R) Bind the Conserved Sequence of Clathrin Light Chains and Thereby Influence Clathrin Assembly in Vitro and Actin Distribution in Vivo*

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Clathrin heavy and light chains form triskelia, which assemble into polyhedral coats of membrane vesicles that mediate transport for endocytosis and organelle biogenesis. Light chain subunits regulate clathrin assembly in vitro by suppressing spontaneous self-assembly of the heavy chains. The residues that play this regulatory role are at the N terminus of a conserved 22-amino acid sequence that is shared by all vertebrate light chains. Here we show that these regulatory residues and others in the conserved sequence mediate light chain interaction with Hip1 and Hip1R. These related proteins were previously found to be enriched in clathrin-coated vesicles and to promote clathrin assembly in vitro. We demonstrate Hip1R binding preference for light chains associated with clathrin heavy chain and show that Hip1R stimulation of clathrin assembly in vitro is blocked by mutations in the conserved sequence of light chains that abolish interaction with Hip1 and Hip1R. In vivo overexpression of a fragment of clathrin light chain comprising the Hip1R-binding region affected cellular actin distribution. Together these results suggest that the roles of Hip1 and Hip1R in affecting clathrin assembly and actin distribution are mediated by their interaction with the conserved sequence of clathrin light chains.

Clathrin-coated vesicles (CCVs) perform selective transport for receptor-mediated endocytosis and protein sorting during organelle biogenesis. The triskelion-shaped clathrin molecule is composed of trimerized clathrin heavy chains (HCs), each with a bound light chain (LC) subunit. Triskelia assemble into polyhedral lattice-coated cellular membranes. Most known regulatory and adaptor proteins influence coat assembly, membrane association, membrane fission, and uncoating by binding to sites on the HCs, which themselves can self-assemble into a lattice. The LC subunits suppress spontaneous lattice assembly in vitro and thus allow cellular clathrin assembly to be controlled and subjected to regulation by additional proteins. Other roles for the LCs have not yet been clearly defined, despite the fact that LCs appear to be composed of multiple functional domains. In this study, we establish that the function of a conserved sequence in vertebrate LCs is to bind to the CCV-enriched proteins, Hip1 and Hip1R (referred to henceforth as Hip1/R). The potential roles for this LC-Hip1/R interaction in the network of CCV regulation are characterized.

In vertebrate clathrin, there are two types of LCs, LCa and LCb, encoded by different genes. These LCs are expressed in all tissues at varying relative levels and are heterogeneously distributed in clathrin triskelia. Near the N terminus of LCs there is a 22-residue sequence, shared by LCa and LCb and completely conserved in all vertebrate HCs, which is also highly conserved in LCs from non-vertebrate species. The first three residues of this conserved sequence are a triplet of negatively charged residues (EEQ) that regulate clathrin assembly. If these residues are neutralized by mutation, the LCs no longer suppress in vitro clathrin assembly. Residues to the N-terminal side of the conserved sequence in LCb are the target for casein kinase phosphorylation and residues to the C-terminal side in LCa constitute a site that can be bound by Hsc70, a protein involved in CCV uncoating. Central to both LCs is a helical region that associates with HCs. C-terminal to this region are sites for short inserted sequences that are present because of alternate splicing of LC RNA in neurons. These sequences influence calmodulin binding to the C termini of LCs. LCs bind to the triskelion hub, a domain formed by the C-terminal third of the HCs through trimerization near their C termini. Thus LCs have the potential to regulate molecular interactions in the central region of the triskelion, whereas the majority of known clathrin-binding proteins interact with HC terminal domains at the ends of the triskelion legs.

Hip1/R proteins were recently shown to bind LCs and were therefore candidates for interacting proteins that might regulate CCV formation and function through the hub domain. Hip1 was identified as a protein that loses binding to mutant huntingtin and has thus been implicated in Huntingdon’s disease. If Hip1R and the related Hip1R protein (47% sequence identity) are highly expressed in neuronal tissue. They have different developmental expression patterns and are also expressed in non-neuronal cells. Hip1/R proteins belong to the yeast Sla2/End4 family and all these proteins are implicated in actin binding and membrane traffic. They have an N-terminal ANTH domain for phospholipid interaction, a central helical domain for dimerization and clathrin interaction, and a C-terminal talin-like actin binding domain.
module (10, 13). Both Hip1R are enriched in CCVs and promote clathrin assembly in vitro (10, 13, 18–20). We had previously shown that transfection of cells with the hub fragment caused both LC and Hip1R dissociation from clathrin, suggesting that Hip1R was binding to LCs associated with the overexpressed hubs (11). Furthermore, hub overexpression disrupted the characteristic offset alignment of clathrin-coated pits with the actin cytoskeleton (11). We consequently hypothesized that the CCV link to actin might be mediated by Hip1R binding to the LC subunits of clathrin. Recent Hip1R RNA silencing experiments showed that decreasing Hip1R levels caused clathrin-coated pits to become more tightly associated with actin instead of being offset from actin filaments (21), further corroborating a role for Hip1R in linking CCVs to actin dynamics.

Here we map Hip1R binding to the conserved sequence of clathrin LCs. Mutations in this LC sequence abolished interaction with Hip1R and abolished the ability of Hip1R to promote clathrin assembly in vitro, suggesting that the Hip1R effects on assembly result from binding the regulatory residues of LCs. In vivo overexpression a LC fragment comprising the conserved Hip1R binding sequence affected the cellular distribution of actin. These results indicate that the conserved sequence of LCs bind Hip1R and coordinate Hip1R action on clathrin and the actin cytoskeleton.

**EXPERIMENTAL PROCEDURES**

**Yeast Two-hybrid Assays—**LCb fragments were amplified from bovine brain cDNAs (22) and cloned into the BamH I site of pACT2 (Clontech). Hip1R-(124–1037) was amplified from a human brain cDNA library (Clontech, provided by Suwon Kim, University of California, San Francisco) and cloned into the SalI/Smal sites of pGTB9 (Clontech) or Smal/Xhol sites of pACT2. Mouse Hip1R cDNA, provided by David Drubin, University of California, Berkeley, was amplified and cloned into the SalI site of pGBT9 or the BamHI/XhoI sites of pACT2. Mouse Hip1R-(1–655) was cloned into the EcoRI/Sall sites of pFlag-CMV2 (Stratagene) for expression as an N-terminal FLAG-tagged fusion protein. Human 293T cells were transfected by Lipofectamine 2000 (Invitrogen) with both HA-tagged LCs (or mutants) and FLAG-tagged Hip1R (or the empty vector) for 2 days. The lysates were collected in lysis buffer (phosphate-buffered saline, 0.75% Nonidet P-40, 50 mM EDTA, 1 mM dithiothreitol). Immunoprecipitation was carried out with a monoclonal antibody to the FLAG tag (M2, Sigma) and protein G-Sepharose (Amersham Biosciences). After a 2–3 h incubation, the resins were washed 3 times with the same buffer. The bound proteins were resolved by SDS-PAGE and immunoblotted with monoclonal antibodies to the FLAG and HA (HA.11, Covance) tags.

**Subcellular Fractionation of Assembled Clathrin—**293T cells transfected for 2 days were homogenized in buffer D (10 mM HEPES, pH 7.5, 150 mM KCl, 1 mM MgCl₂, 0.02% phenylmethylsulfonyl fluoride, and protease inhibitors) as described previously (25). Briefly, the cells were washed twice with the buffer, and lysed using a Dounce homogenizer (10 strokes) and by passing twice through 28.5-gauge needles. Unbroken cells were spun away at 1,000 × g for 1 min. The lysates were fractionated at 1,000 × g for 15 min into low-speed pellets (P1, enriched with nuclei, plasma membrane) and supernatant, which which was spun at 100,000 × g for 1 h to high-speed pellets (P2, enriched with Golgi, endocytic compartments and CCVs), and supernatants (S, cytosolic components). Equivalent proportions of each fraction were resolved by SDS-PAGE and immunoblotted with monoclonal antibodies to HC (TD.1), Hip1R (BD Biosciences), and actin (AC-40, Sigma) or rabbit antisera against green fluorescent protein (GFP) (BD Biosciences).

**Indirect Immunofluorescence—**Bovine brain LCb (1–44) (or mutant) was amplified and cloned into the BglII/EcoRI sites of pEGFP-C2 (Clontech) so that the LC fragment was expressed at the C terminus of GFP. Human 293T cells grown on coverslips were transfected with the GFP construct for 2 days, and prepared as described (26). Hip1R and cortactin were labeled, respectively, with monoclonal anti-Hip1R (BD Biosciences), and anti-cortactin (4F11, Upstate Biotechnology) antibodies, followed by Alexa Fluor 647-conjugated goat anti-mouse IgG (Molecular Probes). Alexa Fluor 568 phalloidin (Molecular Probes) was used to stain F-actin. Images were collected by an API DeltaVision DV3 Restoration microscope using a MicroMax 5 MHz cooled CCD camera (Roper Scientific), and deconvolution was carried out using the API SoftWoRx software.

**RESULTS**

**Clathrin Assembly Assays—**Hip1R-(346–655) was prepared by cleaving the GST tag off from the fusion construct using factor Xa following the manufacturer’s protocol (Novagen). The assays were modified according to Ref. 24. His tag hub was preincubated with excess LCb at 4°C for at least 1 h to saturate the hub with the LC subunits. The mixture was loaded onto Nanosep 30,000 concentrators (Fäll Corp.) and eluted with 10 mM Tris, pH 7.7, to wash away the uncleared LCb. After a 5-min spin to remove aggregates, indicated proteins were loaded in mini-dialysis units (Slide-A-Lyzer, 10,000, Pierce) and dialyzed overnight at 4°C against assembly buffer (100 mM MES, pH 6.7, 1 mM EDTA, 0.5 mM MgCl₂, 1 mM Tris (2-carboxyethyl)phosphine hydrochloride). The mixtures were then centrifuged at 109,000 × g for 45 min to separate the assembled lattices (pellets) from the free forms (supernatant). Equivalent portions of each fraction were analyzed by SDS-PAGE and Coomassie Blue staining. For quantification of band intensities, gels were scanned and analyzed using NIH Image software.

**Immunoprecipitation—**Bovine brain LCb was cloned into the EcoRI/Xhol sites of pCDNA3 (Invitrogen) with the HA tag incorporated immediately subsequent to the ATG codon. Mouse Hip1R-(1–655) was cloned into the EcoRI/Sall sites of pFlag-CMV2 (Sigma) for expression as an N-terminal FLAG-tagged fusion protein. Human 293T cells were transfected by Lipofectamine 2000 (Invitrogen) with both HA-tagged LCs (or mutants) and FLAG-tagged Hip1R (or the empty vector) for 2 days. The lysates were collected in lysis buffer (phosphate-buffered saline, 0.75% Nonidet P-40, 50 mM EDTA, 1 mM dithiothreitol). Immunoprecipitation was carried out with a monoclonal antibody to the FLAG tag (M2, Sigma) and protein G-Sepharose (Amersham Biosciences). After a 2–3 h incubation, the resins were washed 3 times with the same buffer. The bound proteins were resolved by SDS-PAGE and immunoblotted with monoclonal antibodies to the FLAG and HA (HA.11, Covance) tags.

**Clathrin Light Chain and Hip1/Hip1R Interaction**
Presumably, this is because of the presence of the histidine and adenine after 4–6 days. Transformants expressing interacting constructs grew in the absence of His or without (negative control) histidine (His) and adenine (Ade).

Plate growth assay. Yeast AH109 cells were co-transformed with the bait and prey vectors, with (positive control) or without (negative control) histidine (His) and adenine (Ade). Transformants expressing interacting constructs grew in the absence of His and adenine after 4–6 days. Filter assays were performed following the manufacturer’s protocol (Clontech). The blue color indicates a positive interaction, developed within 12 h following substrate application. The assays shown are typical of three independent experiments.

This result did not seem to have a strong binding site. In contrast, Hip1/R interacted robustly with both LCs (LCb in Fig. 1 and not shown for LCa), consistent with conclusions of earlier protein binding and genetic studies of Hip1/R, Hip1, and yeast Sla2p (10, 12, 13). Dimerization and LC interaction were detected for the central region of Hip1R (residues 346–655, Fig. 1). Hip1/R bound weakly to the hinge ear fragment (residues 616–937) of the β-subunit of the AP2 adaptor molecule, but Hip1R did not bind LC mutants I35A, E39A (not shown), or the double mutant comprising I35A and E39A (Fig. 2D). The interaction between wild-type and mutant LC proteins and Hip1/R was then tested in a cellular context. FLAG-tagged Hip1R (residues 1–655) and HA-tagged LCa (wild-type or mutant) were co-transfected into human 293T cells, and they were tested for co-immunoprecipitation. Antibody to the FLAG epitope isolated wild-type HA-tagged LCb bound FLAG-tagged Hip1R, confirming that Hip1R interacts with LCa (Fig. 2E). Single mutations in HA-tagged LCd, D22V, I38A, or E42A (corresponding to D22V, I35A, or E39A in LCb) disrupted the interaction with FLAG-Hip1R and the mutants were not co-immunoprecipitated (Fig. 2E and not shown). These results confirm that several residues in the conserved sequences of both LCs and LCb are critical for interacting with Hip1/R.

Because the residues identified were spread over the conserved sequence of the LCs, we investigated the possibility that the whole conserved sequence was involved in Hip1/R binding. A peptide comprising the conserved LC sequence (residues 20–41 and LCa residues 23–44, EEDPAAALQQESELAGIEND, 22-mer) was tested for its ability to compete with the whole conserved sequence of LCb for binding to GST-Hip1R (Fig. 2D). As increasing concentration of the peptide was added, the amount of LCb binding to GST-Hip1R declined (Fig. 2D). A peptide with mutations affecting LC-Hip1/R interactions (EE and DPF motifs) failed to compete with LCb for binding to GST-Hip1R (Fig. 2). Single mutations in HA-tagged LCa, D25V, I38A, or E42A (corresponding to D22V, I35A, or E39A in LCb) disrupted the interaction with FLAG-Hip1R and the mutants were not co-immunoprecipitated (Fig. 2E and not shown). These results confirm that several residues in the conserved sequences of both LCs and LCb are critical for interacting with Hip1/R.

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**Fig. 2.** Hip1 and Hip1R bind to the conserved sequence of clathrin light chains LCa and LCb.

**A.** Mapping the binding region of Hip1 and Hip1R in bovine brain LCb. Yeast SFY526 cells were co-transformed with human Hip1-(124–1037) or mouse Hip1R-(1–1068) (in pGBT9) and the indicated LCb fragments (in pACT2). Quantitative β-galactosidase assays were performed using o-nitrophenyl β-D-galactopyranoside as substrate and results are shown in β-galactosidase units. Units are defined as the amount that hydrolyzes 1 μmol of o-nitrophenyl β-D-galactopyranoside per min per cell, as described in the Clontech yeast protocols handbook. Above the fragments tested, a diagram of full-length LCb is delineated with the conserved and HC binding regions shaded. Similar results (not shown) were obtained with the equivalent fragments of bovine brain LCa.

**B.** Sequences of the mutant fragments of bovine brain LCb-(1–77) that were unable to bind Hip1. AH109 cells harboring Hip1-(124–1037) (in pGBT9) were co-transformed with mutated PCR products of LCb-(1–77) and a gapped prey vector, pACT2. The inserts of the recombinant clones (numbered mutants) unable to interact with Hip1-(124–1037) were sequenced. Mutated residues from the screens are in bold (black and color). Mutation sites tested individually by site-directed mutagenesis are in color: green, no effect on Hip1 binding; orange, a mild effect; and red, eliminated Hip1 binding. Residues of the conserved sequence (20–41) are highlighted with a yellow background.

**C.** Interaction between the different LCb mutants and full-length Hip1R. The individual mutations generated in the LCb mutants were based on the screen results (Fig. 2B), except for changing residues 32–34 to alanine as part of an initial alanine mutation scan. Quantitative β-galactosidase (β-gal) assays were performed in SFY526 cells harboring bovine brain LCb or mutants (in pACT2) and mouse Hip1R-(1–1068). The results are shown in β-galactosidase units as the mean, and error bars indicate the standard deviations of triplicate determinations. Asterisks (*) indicate that these mutants showed very weak interaction with Hip1R in a more sensitive filter assay.

**D.** Peptide competition assay. The GST Hip1R-(346–655) fragment was first bound to glutathione-Sepharose resins. The indicated concentration of conserved peptide (wild-type, WT; or mutant with changes D22V, I35A, and E39A, MT) was added for 30 min, followed by incubating with recombinant LCb for 1 h. Proteins bound to the affinity resins were assayed by immunoblotting with a monoclonal antibody to GST to detect GST-Hip1R or a rabbit polyclonal antiserum raised against the conserved region of LCs. As a control, full-length LCb with mutations I35A and E39A were combined with GST-Hip1R and its binding was not detected.

**E.** Co-immunoprecipitation of LCa with Hip1R. Human 293T cells were transfected with FLAG-tagged Hip1R-(1–655) (or empty vector) and HA-tagged LCa (or mutants with changes I38A or D25V) and cell lysates were immunoprecipitated with antibody to the FLAG tag. Immunoprecipitates were separated by SDS-PAGE and immunoblotted with anti-HA antibody.
was bound to Hip1R (Fig. 3A, lane 4). To establish the binding preferences of Hip1R for free LCb and hub-bound LCb further competition experiments were performed (Fig. 3B). Bound and free LCb were preincubated with GST-Hip1R followed by further binding of bound or free LCb. One, three, or six molar ratios of LCb were incubated with Hip1R and increasing amounts were bound (Fig. 3B, lanes 2–4). However, when these were exposed to subsequent incubation with a fixed amount of hub-bound LCb at three molar ratios to the Hip1R, equal amounts of hub and LCb were bound and the excess free LCb was displaced (Fig. 3B, lanes 5–7). On the other hand, when increasing molar ratios of hub-bound LCb were first bound to Hip1R and then incubated with three molar ratios of free LCb (lanes 11–13), the increasing amounts of hub and LCb bound to Hip1R remained unchanged (compared with lanes 8–10), showing no displacement of hub-bound LCb by free LCb. These results indicate that Hip1R interacts with LCs that are bound to HCs in preference to unbound LCs. This observation, however, appears to contradict the fact that less LCb is bound by Hip1R when hub is present (Fig. 3A, lane 5). One explanation is that free LCs binds Hip1R stoichiometrically but weakly. Then, when bound to hub, LCs fold better and bind more strongly to Hip1R but can no longer bind stoichiometrically. Taken together, these observations unequivocally indicate that Hip1R does not displace LC from its HC-binding site and demonstrate that Hip1R can bind the LC-hub complex, so both clathrin subunits can bind Hip1R simultaneously.

**Hip1R Promotes the Assembly of Clathrin Hub through Light Chain Binding**—Because LC displacement is not likely to play a role in Hip1R effects on clathrin, we hypothesized that binding to the regulatory residues in the conserved LC sequence might account for stimulation of clathrin assembly in vitro. To address this, we analyzed the activity of a recombinant fragment of Hip1R (residues 346–655) in promoting assembly of recombinant hub fragments with bound LCs (wild-type or with the Hip1R-binding residues mutated). Under weakly acidic conditions, pH 6.7, recombinant hub assemblies into large pseudodotracodomes (23, 24), which can be fractionated from unassembled protein by ultracentrifugation (Fig. 4, lanes 1 and 2). Note that this assembly is reversible (data not shown). At pH 6.7, recombinant hub fully occupied by recombinant LCb does not extensively assemble because of the presence of the negatively charged residues at the N terminus of the LC conserved sequence (4), so most protein remains in the supernatant (14–34% in the pellet versus 73% in the pellet without LCs) (Fig. 4, lanes 3–8). When recombinant Hip1R (346–655, cleaved from GST) was added to hub with bound wild-type LCb, it increased the amount of assembled protein in the pellet to 62% (Fig. 4, lanes 11 and 12). Note that the Hip1R fragment did not appear in the pellet of assembled hub without LCb (lanes 9 and 10). Hip1R promoted hub assembly in the presence of LCb (lanes 11 and 12) but did not fully induce assembly to the levels observed in the absence of LCb (lanes 1 and 2 and 9 and 10). This could be explained if Hip1R is not very efficient at promoting assembly, which would be expected if Hip1R cannot bind stoichiometrically to hub-LC complexes, as suggested by the results shown in Fig. 3. When the Hip1R fragment was added to hub occupied by mutant LCb (single I35A or double I35A,E39A mutants), it did not effectively promote assembly (only 22–43% in the pellet) (Fig. 4, lanes 13–16). The results were statistically significant (p < 0.05, for the comparison between lanes 11 and 12).

were analyzed for the presence of Hip1R and LCs by immunoblotting, respectively, with monoclonal antibodies to the FLAG and HA tags. The absence of the proteins in the transfected lysates was confirmed by immunoblotting the left-hand lanes. Note that LCs' residue numbers are different from LCb, so that the mutations tested were equivalent to those shown in C and D.
Hip1R promotes the assembly of recombinant hub via its interaction with LCs. Hub-LCb complexes were formed by mixing Hub with wild-type LCb or LCb bearing single (355A) or double (355A,E399A) mutations in the conserved sequence of LCb that affect Hip1R binding. Hub alone or Hub complexed to LCs were dialyzed overnight at 4 °C against assembly buffer (100 mM MES, pH 6.7, 1 mM EGTA, 0.5 mM MgCl2, 1 mM Tris(2-carboxyethyl)phosphine hydrochloride) with or without addition of the recombinant fragment Hip1R-(346–655). The dialyzed mixtures were centrifuged at 109,000 g for 45 min to separate the assembled proteins (P, pellets) from the unassembled proteins (S, supernatant). Equivalent proportions of the P and S fractions were resolved by SDS-PAGE, which was then stained by Coomassie Blue. Bars below the bands represent the percentage of hub in the pellet (versus that in both pellet and supernatant) averaged from at least three independent experiments.

12 and lanes 15 and 16). Thus the mutant LCs retained the regulatory residues that inhibited hub assembly, but were defective in Hip1R binding through mutations in other residues of the conserved LC sequence. The implication is that Hip1R binding to the conserved sequence can reverse suppression of hub assembly by LCs.

Overexpression of the Hip1R Binding Fragment of Clathrin Light Chains Affects Actin Distribution in Vivo—To test the role of the Hip1R-LC interaction in vivo, we transfected 293T cells with a fragment of LCb (residues 1–44) comprising the Hip1R-binding region, expressed as a fusion protein with GFP. Protein expression and clathrin assembly state were analyzed in the transfected cells. The amount of Hip1R was noticeably decreased in the cells expressing GFP-LCb-(1–44) (Fig. 5A), suggesting that the LCb fragment was blocking the binding of Hip1R to cellular proteins and thereby decreasing its stability. This effect on Hip1R stability was not observed upon transfection with a GFP fusion protein comprising LCb-(1–44) bearing three mutations in the Hip1R binding sequence (D22Y, 355A, E399A) or upon transfection with GFP alone (Fig. 5A). The pools of assembled versus unassembled clathrin were then compared in cells transfected with the different GFP constructs. The amounts of HC in the assembled (P2) and cytosolic (S) fractions were similar for cells expressing any of the GFP constructs (Fig. 5B). However, the amount of Hip1R in cells expressing GFP-LCb-(1–44) was still significantly lower than the level in cells expressing GFP alone or the GFP construct with mutant LCb-(1–44) (Fig. 5B). Thus, overexpression of the LC fragment containing the Hip1R-binding region does not appear to affect the degree of clathrin assembly, despite causing a reduction in Hip1R level.

The morphology of the transfected cells was examined by immunofluorescence. Hip1R staining appeared diminished in cells expressing GFP-LCb-(1–44), compared with those expressing GFP alone (Fig. 5C). However, localization of clathrin HC or the AP2 adaptor or internalized transferrin seemed to be unchanged by transfection with either GFP construct, as assessed by immunofluorescence (not shown). Most of the actin staining around the cell cortex was the same in cells transfected with GFP, GFP-LCb-(1–44), or GFP-mutant LCb-(1–44). However, effects on distribution of cortical actin were observed at the interface region where cells were in contact with coverslips (Fig. 5, C and D). In cells expressing GFP-LCb-(1–44), abnormal actin projections in globular-spherical (Fig. 5D) or parallel-oblong (Fig. 5C) shapes were observed at focal contact sites. These actin structures were rarely seen in cells expressing GFP alone or the GFP mutant LCb-(1–44). The abnormal actin projections were then analyzed for the presence of other proteins. HC, AP2, or vinculin (a protein involved in focal adhesion) partially co-localized with the projections. However, cortactin, a protein recently reported to bind Hip1R (21), was consistently localized to the tips of the actin protrusions in cells expressing GFP-LCb-(1–44) (Fig. 5C). These observations suggest that disruption of Hip1R binding to LCs results in Hip1R instability and has a consequent effect on cellular actin dynamics.

DISCUSSION

Role of the Conserved LC Sequence—All vertebrate clathrin LCs share a conserved sequence of 22 residues, located about 20 residues from the N terminus. Apart from this sequence the vertebrate LCs (LCa and LCb) have 60% sequence identity, although they do share other common features including the central location of the helical HC-binding domain, a calcium-binding site just N-terminal to this domain, and a calmodulin-binding site at the C terminus. Non-vertebrate species that have only one clathrin LC have a considerable range of sequence divergence.3 However, for many of these, the most conserved sequence besides the HC-binding domain corresponds to the conserved LC sequence in vertebrates. Thus the function of the conserved sequence must be fundamental to clathrin function. Previously we showed that the three negatively charged N-terminal residues of this sequence regulate clathrin assembly (4). Here we demonstrate that one of these residues and several in the remainder of the conserved sequence are critical for binding Hip1R proteins, and that the entire 22-residue segment serves as a Hip1R-binding site. Three hypotheses emerge from this observation. First, the co-localization of Hip1R binding with the assembly regulating residues is likely to play some role in controlling clathrin assembly. Second, Hip1R binding must be vital for CCV function because its binding site is so conserved. Third, a corollary of these first two hypotheses is that the regulation by Hip1R of actin dynamics relative to clathrin dynamics is likely to be critical for effective CCV formation or transport.

Role of Hip1R in Clathrin Assembly—The promotion of clathrin assembly by Hip1R in vitro can be explained if the binding of Hip1R to the conserved sequence blocks the inhibitory negative charges at the N terminus. Indeed, we show that the promotion of Hip1R of the assembly depends on its ability to bind the conserved LC sequence. Mutation of either the LC conserved sequence as shown here or the corresponding bind-
ported by the studies reported here, that Hip1/R acts to promote clathrin assembly through dual binding to clathrin via the LC binding site plus a predicted clathrin box motif. This motif, which is supposed to interact with the N-terminal domain of HCs (30), is 5' to the central region of Hip1 (LMDMD) or Hip1R (LIEIS). Although, no interaction with the relevant domain of HCs was detected by the yeast two-hybrid assays performed here, in vitro studies showed that that Hip1 bound HCs, but Hip1R did not (10, 13, 19, 20). Thus there could be some subtle interaction of HCs with the Hip1 clathrin box that is compromised in the yeast two-hybrid assay. Despite the apparent strength of the LC-Hip1R interaction, disruption of the LC-Hip1R interaction by transfection did not have a detectable effect on levels of intracellular clathrin assembly, although it did decrease the levels of Hip1R in cells. However, in a cell, clathrin assembly and disassembly is controlled by numerous accessory proteins. Whereas, LCs suppress the fundamental assembly reaction, this suppression is overcome by the binding of several different intracellular adaptor molecules, including AP1, AP2, GGAs, AP180/CALM, epsin, and Dab2 (2, 31). These factors presumably orient triskelia into anti-parallel alignment for assembly, increase the curvature of the bound membrane, and/or alter the thermodynamics to favor lattice formation (24, 32–34). Thus even if Hip1/R contributes to reducing the threshold of clathrin assembly by neutralizing the intrinsic regulation by LCs, loss of its assembly promoting activity might not be easily detectable in the cellular context where promotion of clathrin assembly is a redundant activity of a number of CCV-associated proteins.

**Role of Hip1/R-LC Binding in Connecting CCVs to Actin**—Hip1R is an actin-binding protein, as well as a clathrin-binding protein, and several studies have demonstrated that Hip1R can influence the orientation of actin with respect to CCVs (11, 21). Recently, reduction of Hip1R in mammalian cells by RNA in-
terference or of *slo2p* protein in yeast *slo2Δ* mutants was reported to enhance co-localization of actin with CCVs and to increase actin polymerization in the cell periphery (21, 35). We observed a similar phenotype in cells expressing LCB-(1–44), which displayed reduced Hip1R levels. The loss of interaction of Hip1/R with LCs appeared to destabilize Hip1R. Aberrant actin projections were present at the focal adhesion side of the plasma membrane. In some instances, clathrin-coated pits visualized by AP2 staining of transfected cells appeared to be tightly associated with actin structures. This contrasted with AP2 staining in wild-type cells that aligned with actin but did not have overlapping localization. However, no dramatic effects on transferrin uptake were observed in the LC fragment expressing cells (not shown), unlike inhibitory effects observed upon small interfering RNA reduction of Hip1R in cells (21). Whereas Hip1R was less stable in the presence of the interacting LC fragment, the reduction in Hip1R levels was not as great as observed when Hip1R was targeted by small interfering RNA, which could account for the differences in endocytic phenotype observed. Surprisingly, no effects on transferrin and epidermal growth factor uptake were observed when LC expression was inhibited by small interfering RNA (36). However, in these experiments the fate of Hip1/R was not established and could be different from its fate when bound to an LC fragment.

Interestingly, cortactin was consistently observed at the tips of the aberrant actin projections formed following Hip1R reduction by transfection with GFP-LCB-(1–44). Cortactin was recently shown to be enriched in clathrin-coated pits and capable of binding Hip1R, dynamin, and F-actin (21, 37). Normally, cortactin localizes to the periphery of cells that are polarized or migrating, playing a role in regulating actin nucleation and stabilizing actin filament branching (38). In non-polarized cells, like 293T, cortactin is not usually abundant at the cell cortex, but upon reduction of Hip1R, we observe that its colocalization with actin at cell-substrate adhesions is increased. Thus it appears that Hip1R is involved in regulating actin dynamics through cortactin. Whether it sequesters cortactin for activation or inactivation near CCVs cannot yet be determined, although others have suggested that Hip1/R negatively regulates actin dynamics (21, 39).

**Proposed Mechanism for Hip1/R Action Linked to Clathrin—** Based on the *in vitro* binding data shown in Fig. 3, we propose a hierarchy of Hip1/R and clathrin interaction (Fig. 6). First, free LC has a preference for binding to hub over binding to Hip1/R (Fig. 6A), explaining how hub can “steal” LCs from Hip1R (Fig. 3A). Second, Hip1/R has a preference for LCs bound to hub over free LCs (Fig. 6B), explaining the fact that LCs are competed off Hip1R by hub-bound LCs (Fig. 3B). Third, the Hip1/R dimer binds more free LCs than hub-bound LCs so there must be a reduction in the stoichiometry because of steric hindrance from the presence of HCs (Fig. 6D), even though there is an increase in avidity for LCs when they are HC bound. This avidity increase is a likely result of the fact that LCs fold better when bound to HC (8). Thus, in the presence of hub, hub sequesters LCs away from Hip1/R. Hip1/R can rebind to folded hub-bound LCs but with a slower rate, because of the reduced LC accessibility on hub. Furthermore, in this state a Hip1/R dimer does not appear to be able to bind two hub-LC complexes. This binding paradigm suggests that in a cell, Hip1/R dimer binding to LCs on a clathrin triskelion is substoichiometric (Fig. 6C). Substoichiometric binding between Hip1/R and HC-bound LCs is additionally consistent with the partial effect of Hip1R on assembly of hub-LC complexes *in vitro* (Fig. 4).

Binding of the central domain of Hip1/R to LCs localizes part of Hip1/R to the outside surface of CCVs, where it could easily regulate CCV interaction with the actin cytoskeleton. Images of Hip1R as an extended “dumbbell” (10) reveal that it could also project into the interior of a CCV as far as the internal membrane vesicle where its ANTH domain could influence lipid rearrangement (Fig. 6D). Lipid interactions and actin polymerization, as well as LC binding by Hip1/R could all contribute to CCV formation and function. Our analysis of the binding preference between clathrin subunits and Hip1R indicates that the basic structure of the clathrin triskelion is likely to form before Hip1/R binds. Furthermore, Hip1/R binding is unlikely to disrupt a triskelion by removal of LCs. Binding of Hip1/R to a triskelion may then lower the threshold for clathrin assembly by neutralizing LC suppression. Assembly will be further promoted by cooperative interactions from HC distal leg domains (40) brought into proper alignment by various adaptor molecules (24). Rearrangement of polygonal lattices readily occurs with little energy cost and spontaneously contributes to the basket curvature and membrane deformation (32). However, this latter process is facilitated by various lipid-binding adaptors (41) and Hip1/R may consequently contribute. One unusual feature of Hip1/R is that it is the first such adaptor to interact with the externally oriented LC-hub portion of the clathrin coat rather than the internally oriented HC terminal domain. This may facilitate the multifunctional roles of Hip1/R and allow it to regulate the relationship between
CCVs and the actin cytoskeleton as well as influence CCV assembly by interaction with LCs and membrane lipids.

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Huntingtin-interacting Protein 1 (Hip1) and Hip1-related Protein (Hip1R) Bind the Conserved Sequence of Clathrin Light Chains and Thereby Influence Clathrin Assembly *in Vitro* and Actin Distribution *in Vivo*

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