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HIP1 Functions in Clathrin-mediated Endocytosis through Binding to Clathrin and Adaptor Protein 2*

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Polyglutamine expansion in huntingtin is the underlying mutation leading to neurodegeneration in Huntington disease. This mutation influences the interaction of huntingtin with different proteins, including huntingtin-interacting protein 1 (HIP1), in which affinity to bind to mutant huntingtin is profoundly reduced. Here we demonstrate that HIP1 colocalizes with markers of clathrin-mediated endocytosis in neuronal cells and is highly enriched on clathrin-coated vesicles (CCVs) purified from brain homogenates. HIP1 binds to the clathrin adaptor protein 2 (AP2) and the terminal domain of the clathrin heavy chain, predominantly through a small fragment encompassing amino acids 276–335. This region, which contains consensus clathrin- and AP2-binding sites, functions in conjunction with the coiled-coil domain to target HIP1 to CCVs. Expression of various HIP1 fragments leads to a potent block of clathrin-mediated endocytosis. Our findings demonstrate that HIP1 is a novel component of the endocytic machinery.

Huntingtin-interacting protein 1 (HIP1)[1] was identified through its binding to the N-terminal domain of huntingtin, a region containing a polymorphic stretch of glutamine residues (1, 2). When the number of glutamines expands beyond 35, individuals who express the mutated protein develop Huntington disease, which is characterized by selective neuronal degeneration and is manifest clinically by chorea and intellectual decline. Binding of HIP1 to huntingtin is reduced dramatically following polyglutamine expansion (1), may contribute to the initiation and progression of the disease.

HIP1 belongs to a family of proteins consisting of HIP1 and HIP12/(HIP1R) (3). HIP12 was identified based on its homology with HIP1, but unlike HIP1, it does not bind to huntingtin (4). In addition to a central coiled-coil domain, HIP1 and HIP12 contain an ENTH (epsin N-terminal homology) domain. The ENTH domain is thought to function in clathrin-mediated endocytosis through binding to phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2)-containing membranes (5–7). ENTH domains are found in multiple endocytic proteins including epsin 1 and 2, adaptor protein 180 (AP180) and its homologue, clathrin assembly lymphoid myeloid leukemia protein (CALM). Interestingly, each of these proteins also bind to clathrin (8–11). In the case of epsin 1 and 2, clathrin binding is to the N-terminal domain of the clathrin heavy chain (CHC) and is mediated in part via a type-I clathrin-binding sequence, also called the clathrin box (12). Proteins that bind both PtdIns(4,5)P_2 and clathrin are thought to promote the linkage of clathrin triskelia to the lipid bilayer (7). Whether HIP1 or HIP12 binds to clathrin is currently unknown. However, HIP12 colocalizes with markers of receptor-mediated endocytosis in mammalian cells and cofractionates with CCVs (3). Moreover, mutations in the yeast HIP1/HIP12 homologue Sla2p result in a temperature-sensitive defect in clathrin-mediated endocytosis (13–17).

Here, we report that HIP1 colocalizes with endocytic markers, is enriched on CCVs, and binds directly to clathrin and AP2. Moreover, expression of various HIP1 fragments causes a potent block of clathrin-mediated endocytosis, directly implicating HIP1 in endocytic function. These data suggest that neurological defects seen in Huntington disease may be related to abnormalities in endocytosis.

EXPERIMENTAL PROCEDURES

Antibodies—Rabbit polyclonal anti-HIP1 (HIP1FP) antibody was described previously (4). The mouse monoclonal anti-HIP1 antibody (mAb HIP1-9) was generated by immunization of Balb/C mice with a GST fusion protein encoding the huntingtin-interacting domain of human HIP1 (1). Hybridomas were generated and cloned and HIP1 mAbs were selected by enzyme-linked immunosorbent assay with purified HIP1. Positive hybridomas were further characterized by Western blot, and mAb HIP1-9 was found to interact specifically with human and mouse HIP1. Mouse mAbs recognizing α-adaptin, CHC, or Rab5 were from Transduction Laboratories, mouse mAb directed against CHC was from Affinity Bioreagents Inc., and rabbit polyclonal anti-α-adaptin was from Santa Cruz. Mouse mAbs directed against the Flag- and tetra-His epitopes were from Sigma and Qiagen, respectively.

DNA Constructs—HIP1 mammalian expression constructs were generated with HIP1 splice variant 2 (4), which encodes the 1003-amino acid isoform of HIP1. HIP1 cDNA sequences were cloned into the mammalian expression vector pCI (Promega) and tagged at their respective C termini with the following cDNA sequence: 5′-GGAGGTGGAGACTACAAGGACGACGATGACAAGTAG-3′. This cDNA encodes a linker of three glycines, the Flag tag, and a stop codon. The following

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[1] The abbreviations used are: HIP1, huntingtin-interacting protein 1; CCVs, clathrin-coated vesicles; CHC, clathrin heavy chain; ENTH, epsin N-terminal homology; GST, glutathione *S*-transferase; TD, terminal domain; PtdIns(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; AP, adaptor protein; mAb, monoclonal antibody; PBS, phosphate-buffered saline; MES, 4-morpholineethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis.

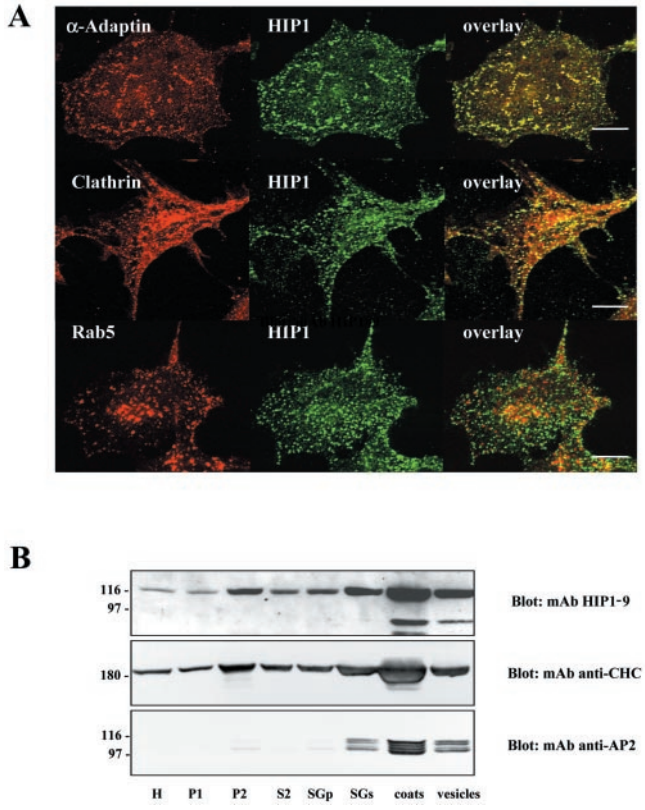

FIG. 1. **HIP1 colocalizes with markers of endocytosis and is enriched in CCVs.** *A*, localization of HIP1 (*green*) with α-adaptin, clathrin, and Rab5 (*red*) was analyzed in neuronal NT2 cells. Overlays of the images are shown in the *right panels*. *Bars*, 10 μm. *B*, purification of CCVs was performed by subcellular fractionation of E18 rat brain homogenates. Aliquots of 100 μg of homogenate (fraction *H*), pellet 1 (*P1*), pellet 2 (*P2*), supernatant 2 (*S2*), pellet obtained after sucrose gradient centrifugation (*SGp*), supernatant obtained after sucrose gradient centrifugation (*SGs*), coats, and vesicles were analyzed on SDS-PAGE and immunoblotted with anti-HIP1-9, anti-CHC, and anti-α-adaptin.

HIP1-GST fusion proteins were created by polymerase chain reaction amplification from full-length HIP1 cDNA with subsequent cloning into the appropriate pGEX vectors (Amersham Pharmacia Biotech): GST-HIP1-(219–616), GST-HIP1-(276–335). For the His_6-tagged terminal domain (TD) of clathrin, a GST construct encoding the 579 amino acids of the CHC in pGEX-2T (generous gift of James Keen, Thomas Jefferson University, Philadelphia) was digested with *Bam*HI and *Eco*RI, and the resulting insert was subcloned into the pTrcHisA (Invitrogen) at the same sites. All expression constructs were verified by DNA sequencing.

Immunofluorescence—Cells were washed in phosphate-buffered saline (PBS; 20 mM NaH_2PO_4, 0.9% NaCl, pH 7.4), fixed in 4% paraformaldehyde in PBS, and permeabilized in 0.3% Triton X-100, 1% paraformaldehyde. Nonspecific binding sites were blocked by incubation with 3% normal goat serum in PBS. For double immunofluorescence, HIP1 polyclonal antibody was incubated in 2% normal goat serum in PBS at 4 °C overnight followed by incubation with mAbs directed against endocytic markers for 2 h at room temperature. Cells were washed extensively in PBS between each incubation. Cells were then incubated with secondary antibodies, washed, mounted, and observed by confocal microscopy.

Transferrin Uptake Assays—COS-7 cells plated on poly-L-lysine-coated coverslips were transfected, cultured for 24 h, and serum-starved overnight. The cells were then incubated with Cy3-conjugated human transferrin (25 μg/ml) for 20 min at 37 °C, washed three times with PBS, and fixed with 3.5% paraformaldehyde in PBS. Following fixation, coverslips were processed for immunostaining with a monoclonal anti-Flag antibody as above.

Purification of CCVs—CCVs from rat brain were purified as described (18). A short version of the purification was adapted for transfected cells. Specifically, cells were homogenized in buffer A (0.1 M MES, pH 6.5, 1 mM EGTA, 0.5 mM $MgCl_2$, 0.83 mM benzamidine, 0.23 mM phenylmethylsulfonyl fluoride, 0.5 μg/ml aprotinin, and 0.5 μg/ml leupeptin) using a glass-Teflon homogenizer (10 strokes, 1500 rpm). The homogenate (H) was centrifuged at 17,800 × g_{max} for 20 min, and the supernatant (S1) was collected and centrifuged at 56,100 × g_{max} for 1 h. The pellet (P2) was resuspended in buffer A using a glass-Teflon homogenizer (3 strokes, 1500 rpm) followed by dispersion through a 25-gauge needle. The resuspended pellet was loaded on top of a solution containing 8% sucrose in buffer A made in D_2O and centrifuged for 2 h at 115,800 × g_{max}. The pellet containing the CCVs was collected.

Pull-down and Binding Assays—Adult rat brains were homogenized in buffer B (10 mM HEPES, pH 7.4, 0.83 mM benzamidine, 0.23 mM phenylmethylsulfonyl fluoride, 0.5 μg/ml aprotinin, and 0.5 μg/ml leupeptin), and a cytosolic fraction was generated by centrifugation at 205,000 × g_{max} for 30 min at 4 °C. Triton X-100 was added to the cytosolic fraction (2 mg) to a 1% final concentration, and the samples were incubated overnight at 4 °C with GST fusion proteins precoupled to glutathione-Sepharose beads. After incubation, samples were washed three times in buffer B containing 1% Triton X-100. In other cases, a His_6-tagged fusion protein encoding the clathrin TD (25 μg) was incubated overnight at 4 °C in buffer B with the various GST fusion proteins precoupled to glutathione-Sepharose beads. After incubation, samples were washed three times in buffer B. For all assays, specifically bound proteins were eluted in SDS-PAGE sample buffer and processed for Western blot analysis.

Immunoprecipitation Analyses—Forty-eight hours following transfection, 293T cells were washed in PBS, scraped in buffer B, and sonicated, and Triton X-100 was added to a 1% final concentration. The lysates were rocked for 30 min at 4 °C and then centrifuged at 245,000 × g_{max} for 15 min. Supernatants were incubated overnight at 4 °C with anti-Flag antibody precoupled to protein G-Sepharose. After incubation, samples were washed three times in buffer B containing 1% Triton X-100. Bound proteins were eluted in SDS-PAGE sample buffer and processed for Western blot analysis.

RESULTS AND DISCUSSION

HIP1 Is Localized to Endocytic Vesicles—Several lines of evidence suggest a potential role for HIP1 in clathrin-mediated endocytosis. We thus examined whether endogenous HIP1 colocalizes with endocytic markers. As HIP1 is most abundant in brain (1, 2), these studies were performed in the neuronal precursor cell line NT2. HIP1 demonstrates punctate staining that is closely colocalized with AP2 (Fig. 1*A*). Moreover, HIP1 colocalizes with the CHC but shows only partial overlap with the early endosomal marker Rab5. Similar results were obtained in primary striatal neurons (data not shown). These data suggest that HIP1 is closely associated with endocytic vesicles. To explore this possibility further, we purified CCVs from rat brain extracts. The coats were then stripped from the vesicles using 0.5 M Tris, pH 9.0. HIP1 was enriched in both the coat fraction and the stripped vesicle fraction when compared with homogenates, with the highest degree of enrichment detected in the coats (Fig. 1*B*). The enrichment of HIP1 in the clathrin coats was comparable with that of clathrin and AP2 (Fig. 1*B*). In contrast, other accessory proteins like amphiphysin, epsin, and Eps15 are far less enriched on CCVs (19, 20). Thus, HIP1 is a major component of CCVs.

Two Regions in HIP1 Are Necessary for Endocytic Targeting—To define the domains necessary for the localization of HIP1, we prepared CCVs from mammalian cells transfected with various HIP1 expression constructs (Fig. 2*A*). All constructs were verified by Western blot following transfection to demonstrate that the expressed fragments were of the correct size (Fig. 2*B*). We used a modified version of the CCV purification (see "Experimental Procedures"). However, this procedure gives a comparable enrichment of clathrin as does the classical protocol (Fig. 2*C*). Full-length HIP1 was enriched in the CCV fraction (Fig. 2*C*). The relative enrichment of HIP1 in the CCV fraction compared with the homogenate was not as great as that seen for the endogenous protein (Fig. 1*B*), probably because HIP1 is overexpressed in the cells. In contrast to full-length HIP1, HIP1-Δcc, lacking the coiled-coil region, corre-

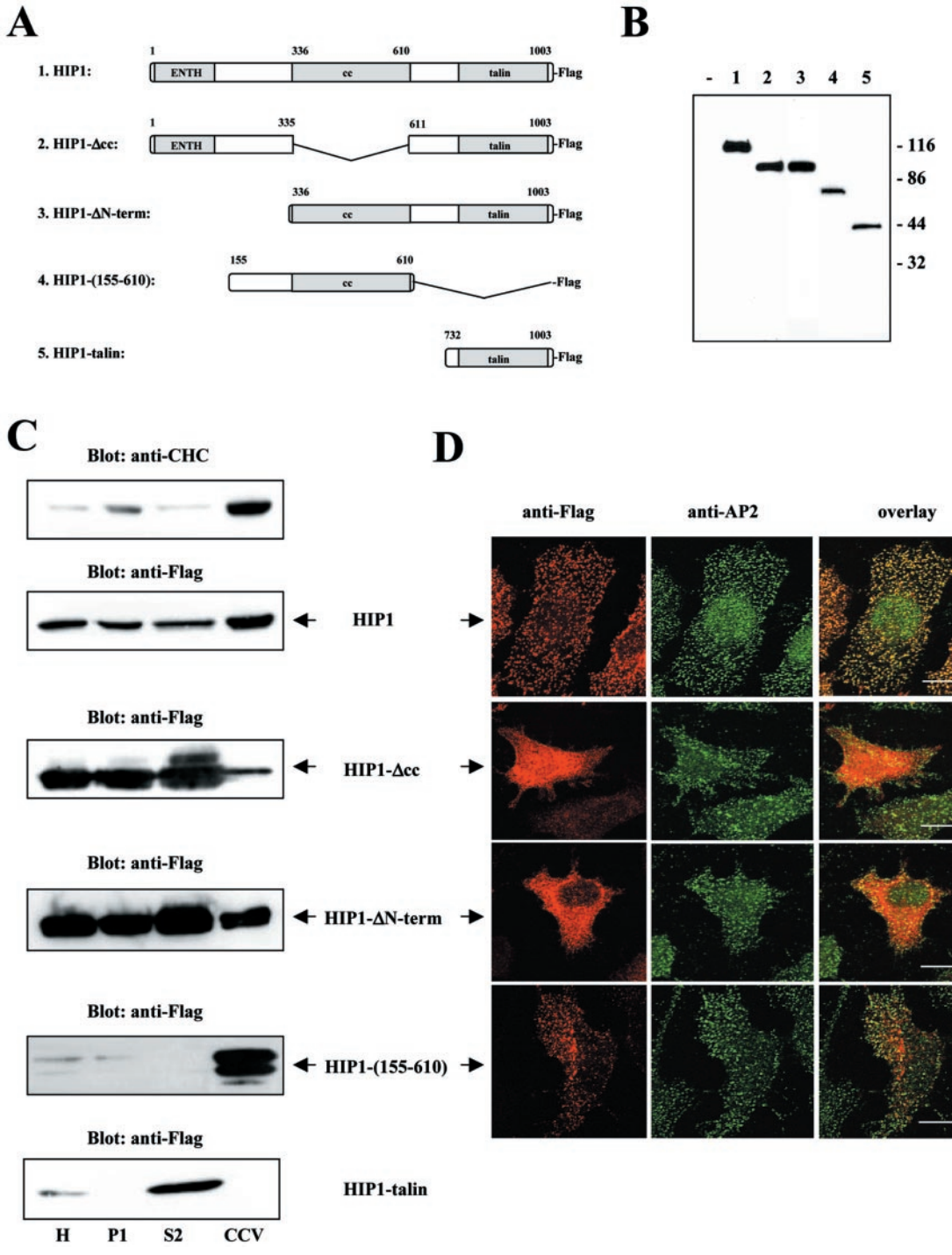

FIG. 2. **HIP1 targeting to CCVs.** *A*, domain model of full-length HIP1 and HIP1 mutants (amino acid *position numbers* are indicated *above* the constructs). *B*, expression of each construct (*1–5*) was analyzed by Western blot and compared with non-transfected cells (−) using an anti-Flag antibody. *C*, 293T cells were transfected with various constructs of HIP1 and CCVs were purified as described under "Experimental Procedures." Aliquots of various fractions from the purification (100 μg) were analyzed by Western blot with anti-CHC antibody or anti-Flag antibody. *D*, expression of transfected HIP1 and various HIP1 mutants (*red*) was visualized using an anti-Flag antibody following transfection in HeLa cells. Expression of AP2 is shown in *green*. Overlays of the images are shown on the *right panels*. *Bars*, 10 μm.

sponding to amino acids 336–610, and HIP1-ΔN-term, lacking the N-terminal portion, corresponding to amino acids 1–335, are not enriched in the CCV fraction compared with the homogenate (Fig. 2*C*). These results suggest that the coiled-coil region and the N-terminal portion of HIP1 contain information necessary for the targeting of HIP1 to CCVs. These domains also appear sufficient for targeting as the construct HIP1-(155–610) is enriched in the CCV fraction (Fig. 2*C*). As a negative control, we used a construct encoding the talin domain of HIP1 (Fig. 2*A*). This protein was not present in the CCVs (Fig. 2*C*).

We next examined the constructs for colocalization with AP2. Transfected full-length HIP1 showed extensive colocalization with AP2 (Fig. 2*D, top row*). In contrast, deletion of either the N-terminal domain or the coiled-coil domain abolished AP2 colocalization (Fig. 2*D, two middle rows*). Expression of HIP1-(155–610) containing the coiled-coil region and upstream sequences resulted in extensive colocalization of HIP1 with AP2 (Fig. 2*D, bottom row*). These data indicate that sequences important for colocalization of HIP1 and AP2 are contained between amino acids 155–610.

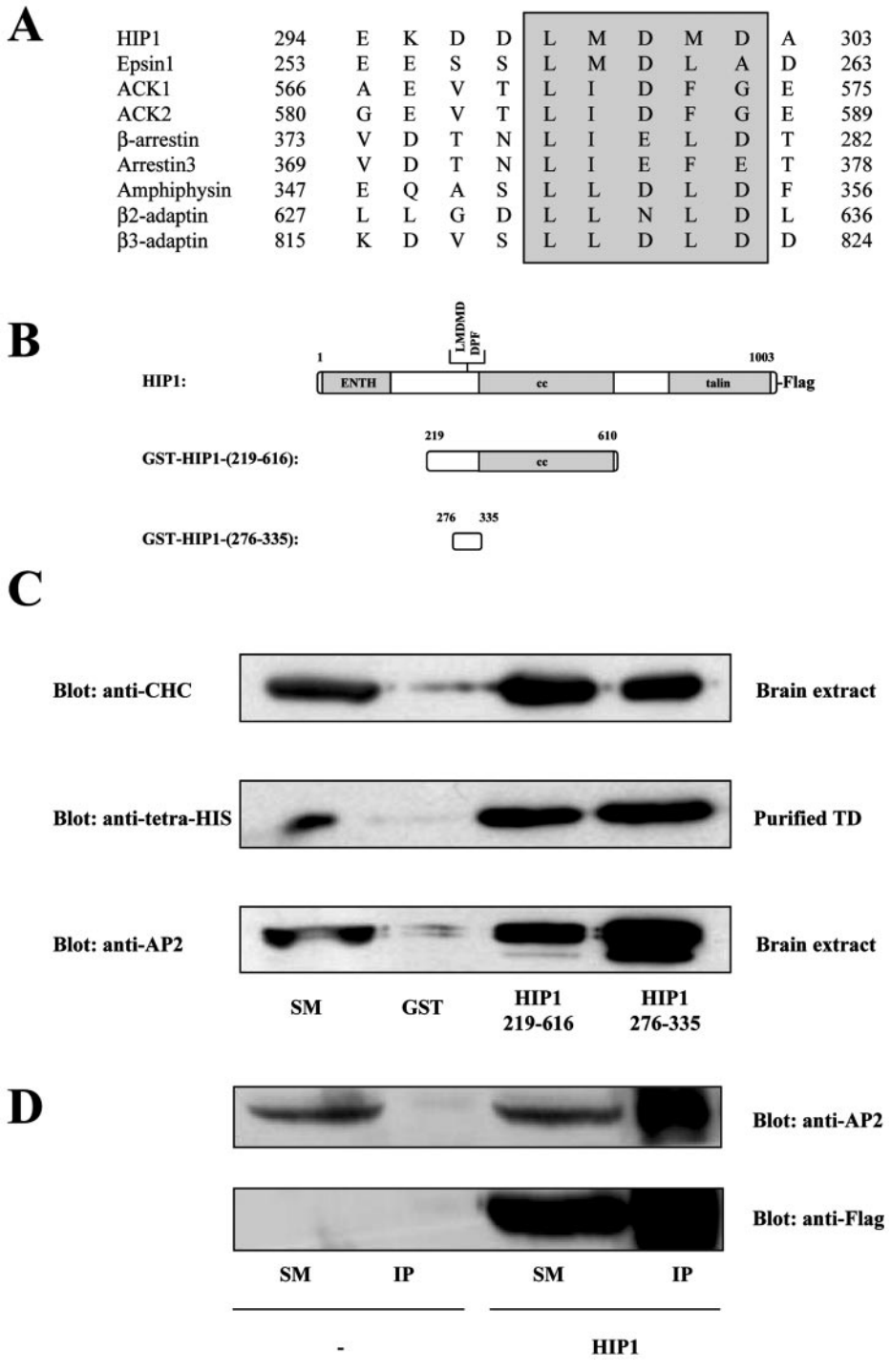

FIG. 3. **HIP1 binds clathrin and AP2.** *A*, sequence alignment of HIP1 with various clathrin box-containing proteins. *B*, schematic presentation of Flag-tagged full-length HIP1 and sequences present in different HIP1-GST fusion proteins. *C*, soluble proteins from brain extracts or purified His_6-tagged clathrin TD fusion protein were affinity-purified with equal amounts of GST fusion proteins encoding domains of HIP1 bound to glutathione-Sepharose beads. Proteins specifically bound to the beads were analyzed by Western blotting with anti-CHC, anti-tetra-His, and anti-α-adaptin antibodies as indicated. *D*, 293T cells were transfected with Flag-tagged full-length HIP1 and processed in parallel with nontransfected cells (−). Proteins were extracted and immunoprecipitated with anti-Flag antibody. Immunoprecipitated proteins were analyzed by Western blotting with anti-α-adaptin and anti-Flag antibodies.

Hip1 Binds to AP2 and the Clathrin Terminal Domain—Analysis of HIP1 revealed the amino acid sequence ^{298}LMDMD (Fig. 3*A*). Related sequences are found in a variety of endocytic proteins and are responsible for mediating interactions with the clathrin TD. HIP1 also contains a DPF motif (amino acids 324–326), a signature for AP2 binding (Fig. 3*B*). To address the potential clathrin and AP2 binding properties of HIP1, we designed two GST fusion proteins encompassing amino acids 219–616 and 276–335 (Fig. 3*B*). Both fusion proteins bound strongly to clathrin and AP2 from soluble rat brain extracts (Fig. 3*C*). Clathrin binding was significantly greater to HIP1-(219–616) than to HIP1-(276–335) (binding ratio = 1.22 ± 0.06; mean ± S.E., $n = 6$). In contrast, AP2 demonstrated consistently less binding to HIP1-(219–616) than to HIP1-(276–335) (binding ratio = 0.66 ± 0.09; mean ± S.E., $n = 7$). The significantly different ratios for clathrin and AP2 binding ($p < 0.001$; t test) suggest that the binding of AP2 is direct and does not result from an indirect pull-down mediated through clathrin. Moreover, the increased binding of clathrin to HIP1-(219–616) *versus* HIP1-(276–335) suggests that a second binding site for clathrin exists in HIP1, likely in the coiled-coil region (amino acids 336–610). The HIP1-GST fusion proteins also bind to purified His_6-tagged clathrin TD demonstrating that clathrin/HIP1 interaction is direct (Fig. 3*C, middle panel*). Binding of the TD to HIP1-(219–616) and HIP1-(276–335) was equivalent, suggesting that the second clathrin-binding site located in the coiled-coil region does not bind clathrin through the TD. In addition, we were able to immunoprecipitate the clathrin adaptor AP2 with full-length HIP1 in transfected cells (Fig. 3*D*).

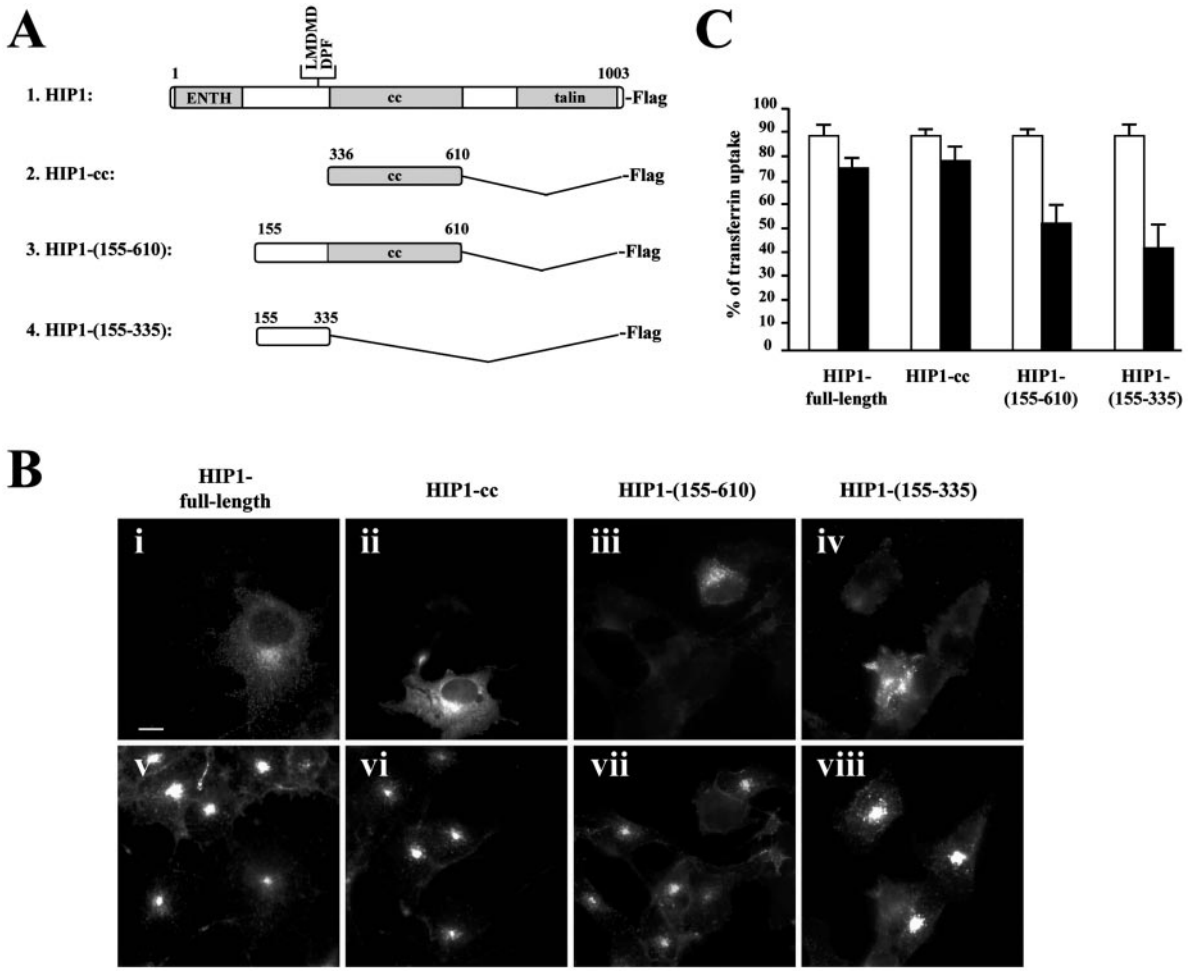

FIG. 4. **Transferrin uptake in COS-7 cells.** *A*, schematic of full-length HIP1 and HIP1 mutants. *B*, the uptake of Cy3-labeled transferrin into COS-7 cells (*panels v–viii*) was analyzed in the presence of various constructs of HIP1. Transfected cells were revealed by immunofluorescence with an anti-Flag antibody (*panels i–iv*). *Bars*, 10 μm. *C*, the graph represents the mean ± S.E. from six separate experiments of the percentage of transferrin uptake for the different constructs. The *white* and *black bars* represent non-transfected and transfected cells, respectively.

The sequence that represents the clathrin box in HIP1, ^{298}LMDMD, corresponds well with the previously described consensus sequence (21–23). The clathrin box and the DPF are arranged in tandem in HIP1 just upstream of the coiled-coil domain. Several other proteins contain a similar arrangement including amphiphysin I (24), epsins (12), and arrestins (25). A tandem arrangement of clathrin- and AP2-binding sites is predicted to stabilize clathrin/adaptor interactions because the interaction of the clathrin TD with the AP2 complex is rather weak (12).

HIP1 Functions in Endocytosis—To directly assess whether HIP1 functions in endocytosis, we performed transferrin uptake in COS-7 cells transfected with the various constructs described in Fig. 4*A*. We observed efficient transferrin uptake in cells transfected with either full-length HIP1 (Fig. 4*B, panels i* and *v*) or the isolated coiled-coil region (*panels ii* and *vi*). Interestingly, expression of the construct HIP1-(155–610) or HIP1-(155–335) strongly reduced transferrin uptake (Fig. 4*B, panels iii, vii, iv,* and *viii*). Fig. 4*C* is a graph of the quantified results from six separate experiments. We conclude that the region upstream of the coiled-coil domain of HIP1 containing the AP2- and the clathrin TD-binding sites is a potent inhibitor of endocytosis.

Many proteins involved in clathrin-mediated endocytosis contain specialized domains mediating protein-protein or protein-lipid interactions (26, 27). We have now identified HIP1 as a novel component of clathrin-coated pits and vesicles. HIP1 binds directly to clathrin and AP2, and expression of a short fragment of HIP1, encompassing binding sites for both proteins, causes a potent block of transferrin uptake. The binding of HIP1 to clathrin and AP2 occurs through a clathrin box and a DPF consensus AP2-binding site, respectively. In addition, HIP1 contains an ENTH domain, described in other proteins to bind to PtdIns(4,5)P_2-containing membranes (5–7). It is thus tempting to speculate that HIP1 functions to link components of the clathrin coat to PtdIns(4,5)P_2-containing endocytic vesicles.

HIP1 is enriched on CCVs to the same extent as clathrin, AP2, and AP180. AP180 cooperatively assembles clathrin with AP2 (28). AP180 binds to the TD of clathrin and the α-appendage of AP2 through its clathrin assembly domain. This domain is characterized by multiple copies of a conserved motif, DLL (29). One such motif is found in the coiled-coil region of HIP1 at amino acid position 450. However, it remains to be determined whether HIP1 can confer clathrin-assembly activity.

The identification of the neuronal protein HIP1 as a novel component of the endocytic machinery leads us to suggest that HIP1 may function in synaptic vesicle endocytosis. This process is a specialized form of clathrin-mediated endocytosis that ensures fast and specific retrieval of synaptic vesicle membranes and is regulated by a multitude of accessory factors (30). HIP1 was originally identified based on its interaction with huntingtin (1, 2). Huntingtin is concentrated in presynaptic termini (31) and interacts with another accessory protein involved in

endocytosis in neurons, endophilin A3 (SH3GL3) (32). Moreover, huntingtin associates with CCVs (33). The altered interaction between mutant huntingtin and HIP1, together with the direct evidence of the role of HIP1 in endocytosis, leads us to suggest that mutant huntingtin can significantly influence HIP1 function and its role in clathrin-mediated endocytosis in neurons.

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REFERENCES

1. Kalchman, M. A., Koide, H. B., McCutcheon K., Graham, R. K., Nichol, K., Nishiyama, K., Kazemi-Esfarjani, P., Lynn, F. C., Wellington, C., Metzler, M., Goldberg, Y. P., Kanazawa, I., Gietz, R. D., and Hayden, M. R. (1997) *Nat. Genet.* **16,** 44–53
2. Wanker, E. E., Rovira, C., Scherzinger, E., Hasenbank, R., Walter, S., Tait, D., Colicelli, J., and Lehrach, H. (1997) *Hum. Mol. Genet.* **6,** 487–495
3. Engqvist-Goldstein, A. E., Kessels, M. M., Chopra, V. S., Hayden, M. R., and Drubin, D. G. (1999) *J. Cell Biol.* **147,** 1503–1518
4. Chopra, V. S., Metzler, M., Rasper, D. M., Engqvist-Goldstein, A. E., Singaraja, R., Gan, L., Fichter, K. M., McCutcheon, K., Drubin, D., Nicholson, D. W., and Hayden, M. R. (2000) *Mamm. Genome* **11,** 1006–1015
5. Kay, B. K., Yamabhai, M., Wendland, B., and Emr, S. D. (1999) *Protein Sci.* **8,** 435–438
6. Ford, M. G., Pearse, B. M., Higgins, M. K., Vallis, Y., Owen, D. J., Gibson, A., Hopkins, C. R., Evans, P. R., and McMahon, H. T. (2001) *Science* **291,** 1051–1055
7. Itoh, T., Koshiba, S., Kigawa, T., Kikuchi, A., Yokoyama, S., and Takenawa, T. (2001) *Science* **291,** 1047–1051
8. Hussain, N. K., Yamabhai, M., Ramjaun, A. R., Guy, A. M., Baranes, D., O'Bryan, J. P., Der, C. J., Kay, B. K., and McPherson, P. S. (1999) *J. Biol. Chem.* **274,** 15671–15677
9. Rosenthal, J. A., Chen, H., Slepnev, V. I., Pellegrini, L., Salcini, A. E., Di Fiore, P. P., and De Camilli, P. (1999) *J. Biol. Chem.* **274,** 33959–33965
10. Ye, W., and Lafer, E. M. (1995) *J. Biol. Chem.* **270,** 10933–10939
11. Tebar, F., Bohlander, S. K., and Sorkin, A. (1999) *Mol. Biol. Cell* **10,** 2687–2702
12. Drake, M. T., Downs, M. A., and Traub, L. M. (2000) *J. Biol. Chem.* **275,** 6479–6489
13. Holtzman, D. A., Yang, S., and Drubin, D. G. (1993) *J. Cell Biol.* **122,** 635–644
14. Raths, S., Rohrer, J., Crausaz, F., and Riezman, H. (1993) *J. Cell Biol.* **120,** 55–65
15. Moore, R. H., Sadovnikoff, N., Hoffenberg, S., Liu, S., Woodford, P., Angelides, K., Trial, J. A., Carsrud, N. D., Dickey, B. F., and Knoll, B. J. (1995) *J. Cell Sci.* **108,** 2983–2991
16. Wesp, A., Hicke, L., Palecek, J., Lombardi, R., Aust, T., Munn, A. L., and Riezman, H. (1997) *Mol. Biol. Cell* **8,** 2291–2306
17. Yang, S., Cope, M. J., and. Drubin, D. G. (1999) *Mol. Biol. Cell* **10,** 2265–2283
18. Maycox, P. R., Link, E., Reetz, A., Morris, S. A., and Jahn, R. (1992) *J. Cell Biol.* **118,** 1379–1388
19. Chen, H., Fre, S., Slepnev, V. I., Capua, M. R., Takei, K., Butler, M. H., Di Fiore, P. P., and De Camilli, P. (1998) *Nature* **394,** 793–797
20. Traub, L. M, Downs M. A., Westrich J. L., and Fremont, D. H. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 1096–1100
21. Ramjaun, A. R., and McPherson, P. S. (1998) *J. Neurochem.* **70,** 2369–2376
22. Drake, M. T., and Traub, L. M. (2001) *J. Biol. Chem.* **276,** 28700–28709
23. ter Haar, E., Harrison, S. C., and Kirchhausen, T. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 1096–1100
24. Slepnev, V. I., Ochoa, G. C., Butler, M. H., and De Camilli, P. (2000) *J. Biol. Chem.* **275,** 17583–17589
25. Laporte, S. A., Oakley, R. H., Zhang, J., Holt, J. A., Ferguson, S. S., Caron, M. G., and Barak, L. S. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 3712–3717
26. McPherson, P. S. (1999) *Cell. Signal.* **11,** 229–238
27. Marsh, M., and McMahon, H. T. (1999) *Science* **285,** 215–220
28. Hao, W., Luo, Z., Zheng, L., Prasad, K., and Lafer, E. M. (1999) *J. Biol. Chem.* **274,** 22785–22794
29. Morgan, J. R.,. Prasad, K, Hao, W., Augustine, G. J., and Lafer, E. M. (2000) *J. Neurosci.* **20,** 8667–8676
30. Slepnev, V. I., and De Camilli, P. (2000) *Nat. Rev. Neurosci.* **1,** 161–172
31. Sharp, A. H., Loev, S. J., Schilling, G., Li, S. H., Li, X. J., Bao, J., Wagster, M. V., Kotzuk, J. A., Steiner, J. P., Lo, A., Hedreen, J., Sisodia, S., Snyder, S. H., Dawson, T. M., Ryugo, D. K., and Ross, C. A. (1995) *Neuron* **14,** 1065–1074
32. Sittler, A., Walter, S., Wedemeyer, N., Hasenbank, R., Scherzinger, E., Eickhoff, H., Bates, G. P., Lehrach, H., and Wanker, E. E. (1998) *Mol. Cell* **2,** 427–436
33. Velier, J., Kim, M., Schwarz, C., Kim, T. W., Sapp, E., Chase, K., Aronin, N., and DiFiglia, M. (1998) *Exp. Neurol.* **152,** 34–40