HIP1 and HIP1r Stabilize Receptor Tyrosine Kinases and Bind 3-Phosphoinositides via Epsin N-terminal Homology Domains

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Huntingtin-interacting protein 1-related (HIP1r) is the only known mammalian relative of huntingtin-interacting protein 1 (HIP1), a protein that transforms fibroblasts via undefined mechanisms. Here we demonstrate that both HIP1r and HIP1 bind inositol lipids via their epsin N-terminal homology (ENTH) domains. In contrast to other ENTH domain-containing proteins, lipid binding is preferential to the 3-phosphate-containing inositol lipids, phosphatidylinositol 3,4-bisphosphate and phosphatidylinositol 3,5-bisphosphate. Furthermore, the HIP1r ENTH domain, like that of HIP1, is necessary for lipid binding, and expression of an ENTH domain-deletion mutant, HIP1rΔE, induces apoptosis. Consistent with the ability of HIP1r and HIP1 to affect cell survival, full-length HIP1 and HIP1r stabilize pools of growth factor receptors by prolonging their half-life following ligand-induced endocytosis. Although HIP1r and HIP1 display only a partially overlapping pattern of protein interactions, these data suggest that both proteins share a functional homology by binding 3-phosphorylated inositol lipids and stabilizing receptor tyrosine kinases in a fashion that may contribute to their ability to alter cell growth and survival.

Huntingtin-interacting protein 1-related (HIP1r) is a clathrin-binding protein that is the only known mammalian relative of huntingtin-interacting protein 1 (HIP1) (1), a protein that transforms fibroblasts via undefined mechanisms (2). HIP1 is also part of a t(5;7) chromosomal translocation that results in transformation of fibroblasts via undefined mechanisms (2). HIP1 is a huntingtin-interacting protein-1-related; HIP1, huntingtin-interacting protein-1; PDGFR, platelet-derived growth factor-β receptor; ENTH, epsin N-terminal homology; PtdIns, phosphatidylinositol; EGFR, epidermal growth factor; EGFR, EGF receptor; GFP, green fluorescent protein; GST, glutathione S-transferase; HA, hemagglutinin; FITC, fluorescein isothiocyanate; DAPI, 4,6-diamidino-2-phenylindole; PIP, phosphatidylinositol phosphate; IRES, internal ribosome entry site.

cancer (4). The transformation of fibroblasts by HIP1 is associated with altered levels of growth factor receptors (2).

The mechanism by which HIP1 overexpression alters growth factor receptor levels may be a result of its role in trafficking of growth factor receptors. HIP1 and HIP1r each contain a clathrin light chain-binding coiled-coil region (5), a leucine zipper, and a C-terminal TALIN homology domain. TALIN is a protein that binds actin and is involved in cell-substratum interactions (6). In addition, HIP1 and HIP1r contain epsin N-terminal homology (ENTH) domains. This domain in epsin and AP180 predominantly binds to phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P2) and is important in regulating clathrin-mediated endocytosis (7, 8). HIP1 and HIP1r both have a punctate immunolocalization and co-localize partially with clathrin, AP-2, and endocytosed transferrin (9–13).

Thus, structural and functional data suggest that HIP1 and HIP1r are involved in vesicle trafficking either at the plasma membrane, during sorting of vesicles, or at the trans-Golgi network. Unlike HIP1, HIP1r has lower affinity for clathrin, does not bind α-adaptin (5), does not bind huntingtin directly, and does bind actin via its TALIN homology domain (9). HIP1 homologues have been found in yeast (14) and Caenorhabditis elegans (15). Sla2p, the yeast homologue of HIP1 and HIP1r, is required for endocytosis, polarization of the cortical actin cytoskeleton, and growth (14).

We are interested in gaining a better understanding of what activities HIP1 and HIP1r might perform to affect tumorigenesis. Although the weight of evidence suggests that HIP proteins function in endocytosis, it is unclear how HIP1 and HIP1r might use clathrin-mediated trafficking to alter survival or growth of cells. One hypothesis suggested by our studies of HIP1 and transformation is that altered HIP1 expression may be a way to regulate growth factor receptor density and signaling and, as a result, affect cellular growth, death, and differentiation of cells (2). Along this line, one mechanism by which transformed cells escape the requirement for growth factors is to increase the activation of receptor tyrosine kinase signaling pathways (16, 17). Cells may compensate for decreased amounts of growth factors in the environment by elevating growth factor receptor levels. Another hypothesis derived from previous work studying ENTH domains (7, 8) is that the ENTH domains of HIP1 and HIP1r may be involved in regulating their roles in growth factor receptor endocytosis and thereby could be critical in their ability to promote cell growth or survival.

To test if there is a link between HIP expression, growth factor receptor signaling, and the role of the ENTH domains, we describe the generation of HIP1r constructs analogous to those generated previously for HIP1 (4). These mutants lack each of the HIP1/HIP1r shared domains, and their effects on...
cell survival and growth factor receptor signaling were tested. Full-length HIP1r had no transient effects on cell survival, but expression of a mutant lacking the ENTH domain, HIP1rΔE, led to apoptosis when transfected into cells. In addition, the lipid binding characteristics mediated by the ENTH domains of both HIP1 and HIP1r were analyzed. To our surprise we found that, in contrast to other ENTH domain-containing proteins which bind PtdIns(4,5)P₂, HIP1 and HIP1r bound preferentially to 3-phosphoinositides. We also found that overexpression of HIP1 and HIP1r inhibited the degradation of ligand-stimulated growth factor receptors. Consistent with this, we obtained using stable HIP1-transformed fibroblasts (2), transient transfection of either HIP1 or HIP1r into 293T cells stabilized pools of either EGFR or PDGFRβ following ligand-induced endocytosis. In contrast, transfection of the HIP1 and HIP1r mutants lacking the ENTH domains did not stabilize growth factor receptors. By using a dominant negative mutant, rate-limiting activities of HIP1 and HIP1r were placed downstream of dynamin.

In light of these data, we propose that both HIP1r and HIP1 may stabilize growth factor receptor levels via altered intracellular trafficking. The finding that HIP proteins can function downstream of dynamin together with the fact that the inositol lipids that bind the HIP ENTH domains are concentrated on intracellular vesicular membranes suggest that the HIP proteins modulate the intracellular trafficking of receptors. This is in addition to a role described previously in clathrin vesicle formation at the plasma membrane. Our data suggest that the HIP proteins are either not rate-limiting in the kinetics of the internalization phase of endocytosis or under certain cellular conditions do not participate in the receptor uptake phase of the clathrin trafficking pathway.

**MATERIALS AND METHODS**

**HIP1r Constructs**—Full-length human HIP1r (NCBI accession number KIAA0655, amino acids 1–1069) was retrieved from a fetal cDNA library by PCR and cloned into the mammalian expression vectors pcDNA3 (pcDNA3-HIP1r) or pcDNA3.1-mycHis (pcDNA3.1-HIP1r) (Invitrogen). Deletion constructs were made with a combination of PCR and restriction digestions using standard methods. For pcDNA3.1-HIP1r/TH, the TALIN homology region of HIP1r was generated by PCR and cloned into pcDNA3.1-mycHis by PCR. Primers 5’-ACC AGC AGG GAA TTC GGA ACA TGG AGG CCA GCC-3’ (underlined sequence denotes engineered EcoRI site) and 5’-GCT GGA CCC CTG GGG GAA GCT TTA GTT CAC GA-3’ (underlined sequence denotes engineered HindIII site) were used. PCR was performed with full-length HIP1r as template using the Expand High Fidelity PCR system (Roche Applied Science). PCR products and the pcDNA3.1 vector were digested with EcoRI and HindIII, ligated together, and confirmed by automated sequencing.

Human HIP1r contains a BamHI restriction site at base pair position 2513 (Fig. 1A). pcDNA3-HIP1r was digested with EcoRI and BamHI to release the 5’ portion of HIP1r and inserted into EcoRI and BamHI digested pcDNA3.1-HIP1r/TH to generate pcDNA3.1-HIP1r. pcDNA3-HIP1r has an EcoRI site in the multicloning site and an internal BamHI in the HIP1r insert. The EcoRI- and BamHI-digested fragment was inserted into the EcoRI and BamHI sites in the pcDNA3.1 vector to generate pcDNA3.1-HIP1r/ΔTH. To generate pcDNA3.1-HIP1r/ΔE, pcDNA3.1-HIP1r/ΔE (see below) was digested with EcoRI and BamHI. This fragment was ligated into the EcoRI- and BamHI-digested portion of pcDNA3.1-HIP1r (containing the vector and the 3’ end of HIP1r). To generate pcDNA3.1-HIP1r/ΔE, the EcoRI-BamHI fragment from pcDNA3.1-HIP1r/ΔE was ligated into EcoRI- and BamHI-digested pcDNA3.1. Human HIP1r contains three internal KspI restriction sites (Fig. 1A). To generate pcDNA3.1-HIP1r/Δ1535, pcDNA3.1-HIP1r was digested with KspI and the 5’ and 3’ ends ligated together to produce a construct whose product lacks amino acids 153–632 of HIP1r. pcDNA3.1-HIP1r/Δ1535 was prepared by ligating fragment from the pcDNA3.1-HIP1r construct, containing human FL-HIP1r, into EcoRI- and Xhol-digested pGEX-4T3 (Amersham Biosciences). To create pGEX-HIP1r/ΔE, pGEX-4T-3 was digested first with BamHI and then digested with XhoI, filled in with BamH1, and then digested with Sall. The vector sequences plus the 5’ HIP1r sequences starting at bp 610 (up to the Sall site) were ligated with a 1.1-kb Sall-SspI fragment (HIP1r bp 2500–3514) also derived from pKIAA0655. The resultant construct pGEX-HIP1r/ΔE was confirmed at its junctions by automated sequencing.

To create pGEX-HIP1r/ENTH, a PCR product encompassing the first 450 bp of human HIP1r with engineered XhoI and NotI restriction sites at the 5’ and 3’ ends, respectively, was generated. The PCR product and the pGEX-4T3 vector were digested with XhoI and NotI and ligated using T4 DNA ligase. Constructs were confirmed by automated sequencing. For construction of pGEX-HIP1r/ΔLZ, a 233-bp PCR product encompassing bp 1437–1760 of human HIP1r was generated with engineered EcoRI and XhoI restriction sites at the 5’ and 3’ ends, respectively. The digested PCR product was cloned into EcoRI- and XhoI-digested pGEX-4T3 and confirmed by sequencing. pGEX-HIP1r constructs were transfected into BL21 bacteria, and proteins were isolated by bacterial GST fusion protein purification using a glutathione-Sepharose 4B column (Amersham Biosciences) according to the manufacturer’s instructions. Following purification, the protein samples were dialyzed against phosphate-buffered saline and concentrated. When used to make antibodies, the GST portion was cleaved off with thrombin.

pcDNA3.1-HA was the kind gift of Dr. Sandra Schmid (University of California, San Diego). pcDNA3.1(+) vIRES-GFP was the kind gift of Dr. Kathleen Collins (University of Michigan). Mutant dynamin 1 was subcloned into the vIRES-GFP vector utilizing BamHI sites flanking the entire insert. Full-length HIP1 and HIP1r were subcloned into the pcDNA3.1(+) vIRES-GFP vector from the pcDNA3 constructs described previously (4).

**HIP1r Antibodies**—The polyclonal anti-HIP1r antibody U2359 was generated using pGEX-HIP1r/ΔE as the antigen (Cocalico Biologicals, Inc., Reamstown, PA). pGEX-HIP1r/TH was used as the antigen for polyclonal antibody U374. The monoclonal antibodies IE1, IE5, and IC5 were made by the University of Michigan Hybridoma Core Facility using pGEX-HIP1r/TH or pGEX-HIP1r/ΔLZ as the antigen. All antibodies were screened by enzyme-linked immunosorbent assay, immunofluorescence, and Western blot.

**Immunoprecipitation and Western Blotting**—293T cells in 100-mm dishes were transfected with 2.5 µg of the various HIP1 or HIP1r constructs using 90 µl of Superfect transfection reagent (Invitrogen) according to the manufacturer’s directions. Cells were harvested 48 h later and resuspended in lysis buffer containing 1% Triton X-100 and protease inhibitors. Protein concentrations were determined using theBradford reagent (Bio-Rad). For immunoprecipitations, 2–3.5 mg of protein were mixed with 20 µl of the polyclonal HIP1r antibody U359 and incubated at 4 °C overnight. Fifty microliters of protein G-Sepharose (Amersham Biosciences) were added and the mixture was incubated at 4 °C for 30 min. Beads were pelleted, washed 3–6 times in lysis buffer, and resuspended in SDS-PAGE loading buffer. One-tenth of the supernatants and the entire pellet from the immunoprecipitations were run on SDS-PAGE gels. Samples were run on 6–8% SDS-PAGE gels and transferred to nitrocellulose. Membranes were blocked in 5% milk/TBST and incubated at 4 °C overnight with anti-clathrin (TD, 1:200), anti-adaptin γ (BD Transduction Laboratories, clone 88, 1:1000), or anti-adaptin α (Sigma, clone 1002, 1:500), and signals were detected using ECL. Membranes were then overblotted with the HIP1r antibodies IE1 + IE5 (1:20 dilution of tissue culture supernatant) or UM359 (1:2000 dilution of serum).

**Lipid Binding**—FIP arrays were obtained from Echelon. Lipid binding was done following the manufacturer’s protocol by using 12.5 µg of isolated protein in TBST at 4 °C overnight. HIP1r mutants were detected using a mixture of the monoclonal IE1 and IC5 antibodies at 1:5000 in TBST. Anti-mouse secondary antibodies (Amersham Biosciences) were used at 1:5000 in TBST, 3% fatty acid-free bovine serum albumin, and 0.5% liposomes containing 40% phosphatidylinositol, 10% phosphatidylcholine, 10% phosphatidylethanolamine, and 10% of the following: phosphatidylserine (PtdIns), phosphatidic acid, PtdIns(3P), PtdIns(4P), PtdIns(5P), PtdIns(3,4,5P₃), PtdIns(3,5P₂), and PtdIns(4,5P₂). The mixture was resuspended in 1:1 chloroform/methanol containing 0.1% HCl, dried under nitrogen, and resuspended to a final concentration of 0.5 µg/µl. Lipid mixtures (10 µl of liposomes in 50 µl of 50 mM Hepes, 125 mM NaCl, 0.5 mM EDTA) were sonicated in a bath sonicator (45 °C) until a clear suspension was formed. Liposomes were collected by centrifugation at 16,000 × g for 10 min and resuspended in ice-cold cytosol buffer (0.2 M sucrose, 25 mM Hepes (pH 7.0), 125 mM
potassium acetate, 2.5 mM magnesium acetate, 1 mM dithiothreitol, protease inhibitors, and 0.1 mg/ml bovine serum albumin) at 2 mg/ml of total lipids. 100 µg of liposomes were mixed with isolated protein at 4 °C for 1 h and precipitated for 5 min at 16,000 × g. Pellets and one-fifth of the supernatants were run on 7% SDS-PAGE gels. Gels were transferred to nitrocellulose and analyzed by Western blotting for HIP1 or HIP1r.

The Course of HIP1r Construct Expression—293T cells in 100-mm plates were transfected with 2.5 µg of the various pcDNA3.1-HIP1r constructs using Superfect transfection reagent. Cells were collected into lysis buffer at 1, 2, 4, and 7 days post-transfection. Fifty µg of protein was run on 10 or 15% SDS-PAGE gels and transferred to nitrocellulose. Membranes were blotted with anti-HIP1r polyclonal antibody (BD Biosciences, 1:10000) and signals detected by ECL. Apoptosis Assays—COS 7 cells were transected with pcDNA3.1-HIP1r, pcDNA3.1-HIP1r/ΔE, or pcDNA3.1-HIP1r/ΔE and fixed with 3% formaldehyde at 24 and 48 h post-transfection. Cells were permeabilized with 0.1% Triton X-100 and blocked with 1% milk for 20 min, followed by staining with DAPI and anti-HIP1 monoclonal antibody 4B10 or anti-HIP1r monoclonal antibody IC5. Bound antibodies were visualized with anti-mouse IgG-FITC (Vector Laboratories). Cells expressing the HIP constructs were scored for apoptosis by nuclear morphology. At least 100 cells were counted for each sample, and transfections were performed in triplicate.

Growth Factor Transfection and Stimulation—For the EGFR stabilization experiments, 293T cells were grown to 50–60% confluency in 100-mm dishes and transfected with 5 µg of pRK5-EGFR (kindly provided by the molecular signaling group at the Ludwig Institute for Cancer Research) and 5 µg of either the various pcDNA3.1-HIP1r constructs or pcDNA3-HIP1 using Superfect transfection reagent. One day later, cells were starved for 20–24 h, treated with cycloheximide (100 µg/ml) for 30 min, and stimulated with EGF (100 ng/ml) in the presence of cycloheximide. Samples were collected at 0, 1, 2, and 4 h after stimulation. Fifteen micrograms of protein were run on 6% SDS-PAGE gels and transferred to nitrocellulose. Membranes were blotted with anti-phospho-EGFR (Cell Signaling, Tyr-845, 1:5000), anti-EGFR (Cell Signaling, 1:2000), anti-HIP1r/1C5 or IC5 (or UM374, 1:2000), or anti-HIP1r (4B10) polyclonal antibodies and signals detected by SuperSignal West Pico Chemiluminescent Substrate (Pierce). Experiments with dominant negative caspase 9 also included 5 µg of pcDNA3-DCN9 (gift of Dr. Gabriel Nunez) or vector in the transfection. Dominant negative caspase 9 was detected using anti-caspase 9 antibody (Cayman Chemical Co., 1:1000). For PDGFβR experiments, 5 µg of Sema-PDGFRβ (11) were transfected with either vector, pcDNA3.1-HIP1r, pcDNA3.1-HIP1r/ΔE, pcDNA3-HIP1, or pcDNA3-HIP1/ΔE. Cells were starved, stimulated, and harvested as described. PDGFβR expression was detected using polyclonal anti-PDGFβR antibody (BD Pharmingen, 1:1000).

Immunofluorescence—COS 7 cells were plated onto coverslips and transfected with pRK5-EGFR and either vector, pcDNA3.1-mycHis/ HIP1r, pcDNA3.1-HA/HIP1r, or pcDNA3.1-HA/Dynamin444A using Superfect. The cells were starved the next day for 20 h, treated for 30 min with cycloheximide (Sigma; 100 µg/ml) at 37 °C, and stimulated with EGF (100 ng/ml) for 0 or 30 min. For each time point, cells were fixed with 3% formaldehyde, permeabilized with Triton X-100, and blocked with 5% milk/PBST. The primary antibodies used were anti-EGFR polyclonal antibody (Cell Signaling), anti-Myc monoclonal antibody (Cell Signaling), anti-HA monoclonal antibody (Babco), and anti-EEA1 antibody (Cell Signaling). FITC anti-rabbit and Texas Red anti-mouse (Vector Laboratories) were the secondary antibodies used. Images were obtained with a Zeiss confocal microscope. For HIP1 and HIP1r, COS 7 cells were plated onto coverslips, transfected with pcDNA3-HIP1 and pcDNA3.1-HIP1r, and fixed with 3% formaldehyde at 24 h post-transfection. Cells were permeabilized with 0.1% Triton X-100 and blocked with 1% milk for 20 min, followed by staining with anti-HIP1 monoclonal antibody 4B10 and anti-HIP1r polycloncal antibody UM359. Bound antibodies were visualized with anti-mouse IgG-FITC or Texas Red anti-rabbit ( Fitzgerald). (Vector Laboratories). Cells were analyzed with a Zeiss confocal microscope and the images processed using Adobe Photoshop software.

Flow Cytometry—For analysis of endocytic uptake of endogenous EGF, HeLa cells were plated 2 days prior to transfection into 6-well dishes at an approximate density of 2 × 105 cells/well. By the time of transfection, cells were 60–80% confluent. The next day, pcDNA3.1/tiraIRES-GFP, pcDNA3.1/tiraIRES-GFP/HIP1, pcDNA3.1/tiraIRES-GFP/HIP1/ΔE, or pcDNA3.1/tiraIRES-GFP/dynamin1-K444A were transfected into HeLa cells (2 µg/well of a 6-well dish). Following transfection, cells were grown in medium containing 10% fetal bovine serum for 24 h and then starved in serum-free medium for an additional 20 h. Cells were then stimulated with EGF for the times indicated, trypsinized, washed twice with ice-cold phosphate-buffered saline containing 1% fetal bovine serum, and incubated with anti-EGFR antibody conjugated to phycoerythrin (Pharmingen) for 60 min with gentle rocking at 4 °C. Following staining, cells were washed three times with ice-cold phosphate-buffered saline and were subjected to flow cytometric analysis on a BD Biosciences FACs Elite within 4 h after staining, with measurement of fluorescence intensity in the green and red wavelengths (for GFP positivity and phycoerythrin-conjugated anti-EGFR, respectively). Distinct populations of GFP-positive and negative cells were analyzed for mean fluorescence intensity. For graphical analysis, the fraction of fluorescence remaining after stimulation was plotted as a percentage of initial fluorescence against time. For analysis of transferrin uptake, 293T cells were transfected with the same constructs as above, and following starvation, cells were incubated with Alexa-Flour 633-labeled transferrin as described previously (2).

RESULTS

HIP1r Mutants and Antibody Characterization—In order to define the relevance of the various protein domains in the activity of HIP1r, deletion mutants lacking these domains were generated (Fig. 1A). In addition, monoclonal and polyclonal antibodies were raised against HIP1r using various HIP1r-GST fusion proteins as antigens (see "Materials and Methods"). The monoclonal antibodies HIP1r/1C5 and HIP1r/1E1 resulted from use of the HIP1r TALIN homology region as the antigen, and both antibodies recognized all of the deletion mutants (Fig. 1A) except those that did not contain the TALIN homology region (HIP1r/ΔT and HIP1r/ΔEΔT) (Fig. 1B). The polyclonal anti-HIP1r antibody UM359, which was generated against a ΔE-HIP1r-GST fusion protein, immunoprecipitated all of the HIP1r mutants (see the Supplemental Material Fig. 1a, lower panel). The monoclonal antibody HIP1r/1E5 was generated from a region of HIP1r that contained the coiled-coil domain and, as expected, did recognize HIP1r/ΔT. This is shown by the fact that all of the HIP1r mutants were recognized by Western blot using a mix of the monoclonal antibodies 1E1 and 1E5 (Supplemental Material Fig. 1a, bottom panel).

Prior to using the HIP1r constructs (Fig. 1A), they were tested for expression levels and for association with endocytic proteins that had been published previously (5, 9–13) to interact with HIP1 and HIP1r. 293T cells were transfected with the various HIP1 (4) and HIP1r constructs, immunoprecipitated with the polyclonal anti-HIP1 antibody UM323 or the polyclonal anti-HIP1r antibody UM359, and immunoblotted for various endogenous endocytic proteins. As has been reported previously, we found that HIP1r (Supplemental Material Fig. 1A, lane 2) and HIP1 (Supplemental Material Fig. 1b, lane 2) associated with clathrin. The HIP1 mutants HIP1/ΔT and HIP1r/ΔE and the HIP1r mutants HIP1r/ΔTH, HIP1r/ΔE, and HIP1r/ΔEΔT were able to associate with clathrin equally well compared with a full-length HIP1 and HIP1r (Supplemental Material Fig. 1b, lanes 3 and 5; Fig. 1a, lanes 5–7). This provides evidence that the mutants we have created are folding properly in the cell. In addition, these data confirm the previous work showing that the LMD motif and coiled-coil domains are necessary for association with clathrin (Supplemental Material Fig. 1b, lanes 4 and 6) (11).

Next, we tested whether HIP1 and HIP1r associate with AP-1, an endocytic protein complex localized primarily to the trans-Golgi network and late endosomes. Immunoprecipitation and Western blotting for γ-adaptin, the large subunit of the AP-1 tetrameric complex, showed that neither HIP1 nor HIP1r associated with AP-1 (data not shown). We also confirmed that although HIP1 associates with α-adaptin, the large subunit of the adaptor protein AP-2, HIP1r did not (data not shown) (5, 9–13). This is consistent with the differences in domain structure between HIP1, which contains a consensus binding se-
quence for AP-2 (the DPF motif), and HIP1r, which lacks this motif (Fig. 1A).

Most interesting, the HIP1r/H9004E mutant that lacks the putative inositol lipid-binding domain did not reproducibly associate with AP-2 (data not shown) but did associate with clathrin (Supplemental Material Fig. 1B, lane 3). We have found previously that expression of this mutant, but not the full-length HIP1r, induces cell death in 293T (4) and BT549 breast cancer cells (2). One reason that HIP1r/H9004E may not have associated with AP-2 is that it was concentrated in a relatively AP-2-deficient perinuclear area of the cell by confocal immunofluorescence (Supplemental Material Fig. 2B). In comparison, when full-length HIP1 was overexpressed in the same cells, it was more widely distributed in the cytoplasm in a punctate pattern (Supplemental Material Fig. 2a). The HIP1r/AE mutant also showed a similar and more perinuclear subcellular localization compared with full-length HIP1r (compare Supplemental Material Fig. 2, c and d). Although HIP1r co-localized partially with HIP1, it consistently showed an additional localization to the more peripheral ruffled plasma membrane (Supplemental Material Fig. 2c, white arrow, and g). This HIP1r location to the actin containing membrane ruffles confirms the HIP1r subcellular localization described previously (15).

HIP1 and HIP1r Bind Preferentially to 3-Phosphate Containing Inositol Lipids via Their ENTH Domains—The ENTH domain is found in several proteins implicated in endocytosis including Epsin 1 and AP180/CALM (7, 8), CLINT ((19) also known as enthoprotin (20) or EpsinR (21)), as well as the HIP family. The Epsin 1 and AP180/CALM ENTH domains are documented lipid-binding motifs that have been shown to bind predominantly to PtdIns(4,5)P2 and PtdIns(3,4,5)P3 (7, 8). Recently, the ENTH domains from several proteins including those of the HIP family have also been found to bind tubulin (22). In contrast, the lipid binding specificity of the HIP family of ENTH domains has not been studied. It should be noted that Mishra et al. (13) did find that phosphoinositides were a necessary component of the liposomes for HIP1 binding. However, the preparation of inositol lipids was not purified, and therefore conclusions about lipid specificity were not made in that study.

One obvious hypothesis derived from the different subcellular localization of the ΔE mutants (Supplemental Material Fig. 2, b and d) is that important protein or lipid binding activities of the ENTH domain are not present in the deletion mutants. These binding activities would anchor HIP proteins to the particular subcellular location of those proteins or lipids. In addition, it is well established that inositol lipids are used by the cell to generate diversity in the destinations of protein cargo during trafficking (23). To begin to explore if a lipid binding activity of the HIP family is important in their cellular function/location, we assayed the lipid binding specificity of the HIP1 and HIP1r ENTH domains by using a variety of techniques. First, myc-His tagged HIP1 and HIP1r/AE were overex-

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**Fig. 1. HIP1r mutants and antibody characterization.** A, the human HIP1r protein has an epsin N-terminal homology (ENTH) domain, a central coiled-coil domain containing a leucine zipper, and a C-terminal TALIN homology domain. These domains were deleted to produce the constructs pcDNA3.1-HIP1r/TH, pcDNA3.1-HIP1r/Δ153, pcDNA3.1-HIP1r/ΔTH, pcDNA3.1-HIP1r/ΔENTH, and pcDNA3.1-HIP1r/ΔET. Restriction sites for KspI (K) and BamHI (B) are marked. B, polyclonal and monoclonal antibodies were raised against purified HIP1r fragments. Western blots (WB) using monoclonal antibodies 1C5 and 1E1, which recognize the TALIN homology region, are shown. Asterisks denote the location of HIP1r mutants.
pressed and purified from 293T extracts by nickel column chromatography. PIP arrays were used to assess initially the binding of these purified proteins to PtdIns lipids. Most surprising, the highest affinity of binding for HIP1 was to PtdIns(3,4)P2 and PtdIns(3,5)P2 (Fig. 2A, left panel). As expected, HIP1/H9004E did not bind these lipids (Fig. 2A, right panel). We also looked at the lipid binding of 35S-labeled proteins that were synthesized using reticulocyte lysates. The in vitro translated proteins were incubated with liposomes containing a variety of phosphatidylinositol lipids. Pelleted liposomes and supernatants were run on SDS-PAGE gels and analyzed by autoradiography. HIP1 bound to liposomes containing PtdIns(3)P, PtdIns(3,4)P2, and PtdIns(3,5)P2 but did not bind to PtdIns(4,5)P2 (Fig. 2B, top panel). As predicted, the HIP1/ΔE protein was unable to bind any of the inositol lipid-containing liposomes (Fig. 2B, bottom panel).

Because we unexpectedly found that the myc-His tag was not recognized by anti-His or anti-Myc antibodies or the nickel column when attached to the HIP1r C terminus (despite repeated sequence confirmation of the constructs), it was necessary to generate a series of HIP1r deletion mutants fused to GST to characterize the lipid binding properties of HIP1r (see “Materials and Methods”). First, purified FL-HIP1r-GST and ΔE-HIP1r-GST fusion proteins were used to test binding to PIP arrays. Like HIP1, full-length HIP1r bound preferentially to PtdIns(3,4)P2 and PtdIns(3,5)P2 using the PIP arrays (Fig. 2C, left panel), and significant binding was abolished in the mutant lacking the ENTH domain (Fig. 2C, right panel). HIP1r-GST also exhibited some lower affinity binding to PtdIns(4,5)P2 and PtdIns(3,4,5)P3 (Fig. 2C, left panel). HIP1r-GST fusion proteins were also subjected to liposome pelleting assays. Consistent with the PIP array results, full-length HIP1r bound preferentially to PtdIns(3,4)P2 and PtdIns(3,5)P2 using the PIP arrays (Fig. 2C, left panel), and significant binding was abolished in the mutant lacking the ENTH domain (Fig. 2C, right panel). HIP1r-GST also exhibited some lower affinity binding to PtdIns(3,4)P2 and PtdIns(3,4,5)P3 (Fig. 2C, left panel). HIP1r-GST fusion proteins were also subjected to liposome pelleting assays. Consistent with the PIP array results, full-length HIP1r bound preferentially to PtdIns(3,4)P2 and PtdIns(3,5)P2-containing liposomes (Fig. 2D). We also observed less reproducible binding in some cases to other 3-phosphorylated inositol lipids, namely PtdIns(3)P and PtdIns(3,4,5)P3 (Fig. 2D, E, and data not shown). The ENTH domain was necessary for binding, as the ΔE-HIP1r and LZ-HIP1r mutants failed to bind any of the tested lipids (Fig. 2E). The HIP1r-GST mutant containing only the ENTH domain (ENTH-HIP1r) also pelleted with liposomes but was less specific and bound to PtdIns(3)P, PtdIns(4)P, PtdIns(3,4)P2, PtdIns(4,5)P2, and PtdIns(3,4,5)P3, as well as phosphatidic acid (Fig. 2E). The latter data suggest that sequences not present in the ENTH mutant are necessary for conferring preferential binding to 3-phosphorylated inositol lipids.
The differences in lipid binding reported here compared with previous mammalian ENTH domain-containing proteins suggest that HIP1 and HIP1r may participate in a distinct part of the pathway of clathrin trafficking different from the other ENTH domain-containing proteins. Another possibility is that the binding of the HIP proteins to inositol lipids has a completely different function that remains to be determined. However, we think this interaction is significant as several proteins known to co-localize with endosomal membranes also bind to 3-phosphate-containing bisphosphoinositides (24, 25). For example, the yeast protein Ent3p has been shown recently to have a PtdIns(3,5)P_2-binding ENTH domain that mediates its role in intracellular multivesicular body protein sorting (26).

In addition, we have found that PtdIns 3-kinase was activated in cells that overexpressed HIP1 but that phospholipase C-γ, in contrast, was not activated (2). This together with our 3-phosphoinositide binding data suggested the hypothesis that HIP1 binding to 3-phosphorylated inositol lipids may contribute to Akt activation by these lipids. If this hypothesis were true, a dominant negative HIP1 that lacked lipid binding might lead to Akt inhibition and subsequent apoptosis as Akt activation is a cell survival signal. On the other hand, constitutively activated Akt would be expected to bypass this dominant negative HIP1-induced apoptosis as it would be expected to be downstream of HIP1. Indeed, we have found that constitutively active Akt (Myr-Akt), but not oncogenic Ras-V12, is able to rescue dominant negative HIP1ΔΔE-mediated apoptosis (Supplemental Material Fig. 3). The latter data, albeit indirect, suggests that 3-phosphoinositides may indeed be important in the fundamental cellular functions of the HIP family.

The HIP1r Mutant Lacking the ENTH Domain Induces Cell Death—We have shown previously (4) that HIP1 is overexpressed in several cancers, especially prostate and colon cancer. In addition, it has been shown that specific antibodies against HIP1r are produced in patients with colon cancer, suggesting that HIP1r is also overexpressed in colon cancers (27). Furthermore, expression of the ENTH deletion mutant of HIP1, but not full-length HIP1, induces cell death in a dominant interfering manner (4). The discrepancies between our data that suggest HIP1r has a cellular survival function and the data of Hackam et al. (44) and Gervais et al. (30) that suggest HIP1 is primarily pro-apoptotic are not yet understood. We speculate that the cellular environment may influence whether or not the full-length HIP proteins have pro-apoptotic versus survival functions. By removal of the ENTH domain, potential pro-apoptotic domains (such as the pseudo-DED domain described by Gervais et al. (30)) may be unmasked.

To begin to test which domains of HIP1r were important to its function, we expressed the HIP1r deletion mutants in 293T cells. A time course of protein expression after transient transfection of HIP1r deletion mutants showed that HIP1rΔΔE and HIP1rΔΔEΔT were not as stable as other HIP1r mutants (Fig. 3A). This result parallels our previous finding that the HIP1rΔΔE mutant was not as stable as other HIP1r mutants and induced apoptosis. In contrast, expression of full-length HIP1r and most of the deletion mutants remained stable for at least 7 days post-transfection (Fig. 3A). We have also overexpressed the ENTH domain of HIP1 in cells, and it did not induce apoptosis (Ref. 4 and data not shown).

In contrast to full-length HIP1 and HIP1r (Supplemental Material Fig. 2, a and c), many cells transfected with HIP1rΔΔE or HIP1ΔΔE and visualized by immunofluorescence 48 h post-transfection were condensed and blebbing, characteristic of apoptotic cells. In order to quantitate the apoptosis caused by the HIP1rΔΔE mutant and to compare this mutant with the HIP1ΔΔE mutant, cells were transfected in triplicate with HIP1rΔΔE, full-length HIP1r, or HIP1ΔΔE, stained with anti-HIP1r or anti-HIP1 antibodies, and then assayed for apoptosis by scoring apoptotic nuclear morphology. DAPI-stained nuclei of transfected cells were scored as apoptotic if they possessed condensed or fragmented nuclei. Whereas full-length HIP1r-transfected cells had a minimal percentage of apoptosis, cells transfected with HIP1ΔΔE and HIP1rΔΔE consistently exhibited increased incidence of cellular apoptosis 48 h post-transfection (Fig. 3B). Most interesting, compared with HIP1ΔΔE, HIP1rΔΔE did not show significant apoptosis at 24 h post-transfection. This was also confirmed with terminal dUTP nick-end labeling analysis in an independent experiment (data not shown). These results, together with our previous data documenting a dominant interfering function of HIP1ΔΔE (4), imply that HIP1r, like HIP1, may have a role in cellular survival. The difference in time course also suggests that HIP1 and HIP1r may have functions in the cell that are distinct from each other in addition to those that are overlapping.

The HIP Family Stabilizes Growth Factor Receptors—The activities of HIP1 and HIP1r that might lead to the survival of cells and contribute to tumorigenesis remain to be defined. Recently, we have found that HIP1 is overexpressed in multiple epithelial tumors, and stable overexpression of HIP1 in NIH3T3 fibroblasts causes transformation. In HIP1-overexpressing cells, this process may be mediated in part by elevated levels of epidermal growth factor receptor (EGFR) and the resultant activation of mitogenic signaling pathways (2).
order to determine whether the elevated levels of EGFR in the transformed cells was a direct result of HIP1 overexpression or a more secondary effect of transformation, we have begun to evaluate the effect of transient HIP1 and HIP1r overexpression on growth factor receptor stability after ligand stimulation. Briefly, to allow for sufficient sensitivity of detection and semi-quantitation of growth factor receptor half-life, 293T cells were co-transfected with EGFR or PDGF/H9252R and the various full-length or mutant HIP constructs. The transfected cells were then stimulated with ligand and analyzed for the rate of receptor tyrosine kinase degradation. In order to validate our assay, we utilized the well characterized GTPase-deficient dynamin1 dominant negative mutant dynamin1-K44A (28). Dynamin is critical for release of invaginated clathrin-coated pits from the plasma membrane to form clathrin-coated vesicles (29). The dynamin1-K44A construct exerts a dominant interfering effect on endogenous dynamin, resulting in the loss of normal dynamin function. The net effect of expression of this mutant in cells is to prevent ligand-induced endocytosis and degradation of growth factor receptors and thereby increase surface levels of the receptor (28).

Following transfection of the constructs and starvation in serum-free medium, transfected cells were stimulated with EGF for various lengths of time. It is noteworthy that we found it necessary to include cycloheximide in the assay to inhibit the confounding rapid translation of new EGFR. If cycloheximide was not included, there were no reproducible differences detected in EGFR half-life in any of the cells transfected with EGFR, dynamin1-K44A (Dyna-K44A), and HIP1/ΔE as indicated and stimulated with EGF. 20 μg of protein were loaded on SDS-PAGE gels and analyzed by Western blot.

![Image](image-url)

**Fig. 4.** HIP1 expression stabilizes receptor tyrosine kinases during post-stimulation endocytosis and degradation. A, levels of EGFR are stabilized by the overexpression of HIP1 and the dynamin1-K44A mutant. 20 μg of protein were loaded on SDS-PAGE gels and analyzed for EGFR expression following stimulation with 100 ng/ml EGF. Actin levels were used as a loading control. B, comparison of EGFR levels and EGFR phosphorylation after stimulation with various amounts of EGF for 60 min. Gels were loaded with 20 μg of protein extracts from cells transfected with EGFR and the indicated construct. C, comparison of EGFR levels and EGFR phosphorylation in cells transfected with EGFR and full-length HIP1 or HIP1/ΔE, starved for 48 h, and stimulated with 100 ng/ml EGF for 0 or 2 h. 50 μg of protein was loaded on SDS-PAGE gels and analyzed. D, the dynamin1-K44A mutant overcomes EGFR destabilization caused by HIP1/ΔE. 293T cells were co-transfected with EGFR, dynamin1-K44A, and HIP1/ΔE as indicated and stimulated with EGF. 20 μg of protein were loaded on SDS-PAGE gels and analyzed by Western blot.
HIP1r- and stimulated with EGF. HIP1r/H9262 can be partially overcome by dynamin1-K44A. Cells were co-transfected with EGFR, dynamin1-K44A (H9004 E, or Dyna K44A), and either HIP1r, HIP1r/H9004E, or HIP1r/H9004E (lanes 1 – 9), or HIP1 (lanes 10 – 12). Samples were taken 0, 1, and 2 h after starvation and stimulation with human EGF and subject to Western blot analysis. B, comparison of PDGFβR levels following stimulation with PDGF-βR. 293T cells were transfected with vector, HIP1r or HIP1r/ΔE, along with 5 μg of pSRα-PDGFR. Cells were harvested 0, 1, and 3 – 4 h after starvation and stimulation with PDGF-βR and subjected to Western blot analysis. C, same experiment as B except that HIP1 and HIP1ΔE were assayed. D, down-regulation of EGFR by HIP1r/ΔE can be partially overcome by dynamin1-K44A. Cells were co-transfected with EGFR, dynamin1-K44A (Dyna K44A), and either HIP1r, HIP1r/ΔE, or HIP1 and stimulated with EGF. 15 μg of protein were loaded on SDS-PAGE gels and analyzed by Western blot.

Fig. 5. HIP1r-transfected cells have increased levels of growth factor receptors. A, HIP1r stabilizes total and Tyr-845-phosphorylated EGFR. 293T cells were transfected with EGF and either vector (lanes 1 – 3), HIP1r (lanes 4 – 6), HIP1r/ΔE (lanes 7 – 9), or HIP1 (lanes 10 – 12). Samples were taken 0, 1, and 2 h after starvation and stimulation with human EGF and subject to Western blot analysis. B, comparison of PDGFβR levels following stimulation with PDGF-βR. 293T cells were transfected with vector, HIP1r or HIP1r/ΔE, along with 5 μg of pSRα-PDGFR. Cells were harvested 0, 1, and 3 – 4 h after starvation and stimulation with PDGF-βR and subjected to Western blot analysis. C, same experiment as B except that HIP1 and HIP1ΔE were assayed. D, down-regulation of EGFR by HIP1r/ΔE can be partially overcome by dynamin1-K44A. Cells were co-transfected with EGFR, dynamin1-K44A (Dyna K44A), and either HIP1r, HIP1r/ΔE, or HIP1 and stimulated with EGF. 15 μg of protein were loaded on SDS-PAGE gels and analyzed by Western blot.

In contrast, there was a dramatic prolongation of the EGFR half-life to greater than 2 h in HIP1-transfected cells following stimulation. This is shown by steady levels of the EGFR at all time points in the presence of HIP1 (Fig. 4A, lanes 1 – 4). A similar effect where the EGFR half-life was prolonged beyond the last time point in our assay was seen in dynamin1-K44A-transfected cells (Fig. 4A, lanes 9 – 12), whereas transfection of a wild type dynamin construct caused higher levels of EGFR degradation (data not shown).

It should also be noted that the Western blots presented are a portion of those actually analyzed. It was necessary for us to evaluate different exposures or to run varying amounts of each of our extracts to achieve blots where the semi-quantitation of EGFR in the extracts from the various transfection conditions were in a range of ECL signal that was linear. This allowed us to conclude that the half-life of the EGFR varied during the time course of each experiment. This allowed us to quantitate the growth factor half-lives without having to quantitate ratios of growth factor levels and modifier proteins. It also allowed us to conclude that the half-lives of the HIPs and dynamin mutant were much longer than 4 h, as the presence of cycloheximide did not lead to detectable changes in their levels in the presence or absence of growth factor receptor stimulation.

Co-expression of full-length HIP1r produced analogous results, with stabilization of the EGFR to a half-life of greater than 2 h and higher levels of EGFR phosphorylation after stimulation compared with vector-transfected controls (Fig. 5A, compare lanes 4 – 6 to lanes 1 – 3). It is also significant that the starting levels of EGFR prior to EGF stimulation were frequently increased in the HIP or dynamin dominant negative transfected cells. This is likely due to a continual effect of HIPs and the dynamin1-K44A mutant on the turnover of EGFR. As expected, this difference in starting amounts of EGFR was not observed in the absence of cycloheximide.

To determine whether HIP1r and HIP1 were able to alter the stability of other receptors whose endocytosis is mediated by clathrin and its cofactors, we assayed the effect of HIP1r and HIP1 on the degradation of the PDGFβR after stimulation with its ligand, PDGF-βR. Following stimulation, overexpression of HIP1r or HIP1 led to diminished degradation of the PDGFβR, albeit to a lesser extent than that seen for the EGFR (Fig. 5B, compare lanes 4 – 6 to lanes 1 – 3; Fig. 5C, compare lanes 4 – 6 to lanes 1 – 3). The approximate half-life of the PDGFβR was pro-
longed from 1 to 3 h in the presence of full-length HIPs. The similar but distinct effects seen with the EGFR and PDGFβR when either HIP1 or HIP1r was overexpressed indicate not only a general role for the HIP1 family in post-stimulation regulation of receptor tyrosine degradation but also a possible selectivity of the HIP family in its activity of receptor stabilization.

Mutants Lacking the ENTH Domain Do Not Stabilize EGFRs—We and others (4, 10, 30) have previously gathered data that indicate HIP1 has an effect on cellular survival. It is possible that altered HIP1r expression also affects the survival of cells. We hypothesize that the pro-survival functions of HIP1 and HIP1r may be dependent upon their ability to stabilize and thereby up-regulate growth factor receptor tyrosine kinases through their role in clathrin trafficking. Conversely, the withdrawal of growth factor receptor stimulation has been noted to be sufficient to cause apoptosis (31). These facts led to the hypothesis that the pro-apoptotic effects of interfering with HIP1 or HIP1r function might result from the disruption of receptor tyrosine kinase trafficking.

To address this hypothesis, we co-transfected the HIP1/ΔE construct along with EGFR and analyzed its effect on EGFR half-life. We have provided evidence previously (4) that the HIP1/ΔE protein functions as a dominant interfering mutant. HIP1/ΔE did not have the same stabilizing effect as full-length HIP1; indeed, EGFR degradation following stimulation appeared to be accelerated in cells expressing HIP1/ΔE (Fig. 4D, lanes 4–6 compared with lanes 1–3). Phosphorylation of the EGFR was also diminished after ligand stimulation compared with vector-transfected control (Fig. 4C, lanes 5 and 6 compared with lanes 1 and 2).

Similar but distinct results were observed upon transfection of HIP1r/ΔE. Western blots of total EGFR show at least two species of EGFR of slightly different molecular weights. Levels of the upper phosphorylated or monoubiquitinated EGFR band were diminished at every time point in the HIP1r/ΔE-transfected samples compared with vector-transfected cells (Fig. 5A, compare lanes 7–9 to lanes 1–3). This activated EGFR isoform was not stable in the HIP1r/ΔE-expressing cells (Fig. 5A, lanes 7–9) but was in the HIP1r-transfected cells (Fig. 5A, lanes 4–6). Immunoblot analysis for phosphorylated EGFR confirmed that activated EGFR levels were, like in the HIP1/ΔE-expressing cells, reduced in the HIP1r/ΔE-expressing cells (Fig. 5A, lanes 7–9 compared with lanes 1–3).

Next, we analyzed the effects of the other HIP1r mutants on EGFR expression and ligand-stimulated activation. Like full-length HIP1r, the HIP1r/ΔTH mutant stabilized total EGFR levels and also showed increased tyrosine phosphorylation of the EGFR compared with vector-transfected cells (data not shown). Like HIP1r/ΔE, HIP1r/ΔEAT-transfected cells lacked the higher molecular weight EGFR species seen in vector- or HIP1r-transfected cells and showed reduced tyrosine phosphorylation of the receptor (data not shown). These results suggest that the ENTH domain is necessary for effective maintenance of EGFR signal duration, likely via stabilization of the receptor in early pH neutral endocytic vesicles. It is interesting to note again that the HIP1/ΔE-containing cells contain predominantly the slower migrating, phosphorylated EGFR band (Fig. 4D, lanes 4–6), whereas the HIP1r/ΔE-containing cells contain more of the faster migrating EGFR band (Fig. 5A, lanes 7–9). This suggests that the different patterns of EGFR banding, as seen in the HIP1/ΔE compared with the HIP1r/ΔE-transfected cells, may reflect subtle differences in the ways HIP1 and HIP1r affect total levels of EGFR versus total levels of tyrosine-phosphorylated EGFR.

Previously, we have shown that HIP1/ΔE-mediated apoptosis could be rescued by a dominant negative caspase 9 but not a dominant negative caspase 8, suggesting that HIP1/ΔE induced caspase 9-dependent apoptosis (4). To determine whether the same effect could be seen in terms of receptor stabilization, EGFR stimulation assays were performed with co-transfection of EGFR, HIP1/ΔE, and either vector or dominant negative caspase 9. Dominant negative caspase 9 did not alter the effect of HIP1/ΔE on EGFR, although this construct does correct the cell death induced by HIP1/ΔE (data not shown and see Ref. 4). These results indicate that the effect of HIP1/ΔE or HIP1r/ΔE on EGFR levels in our assays is not simply due to increased apoptosis of cells that express transfected EGFR and the ΔE mutants. Moreover, it suggests that caspase 9-dependent apoptosis mediated by the ΔE mutants may be a downstream effect of receptor down-regulation and that the apoptosis can be corrected without a correction of the receptor abnormality.

Because previous studies have indicated that HIP1 and HIP1r have activities at the plasma membrane, it is possible that they are rate-limiting in these functions. Overexpression of HIP1 or HIP1r could inhibit the initial stages of clathrin-coated vesicle formation, similar to the dynamin1-K44A mutant (29). This mechanism would favor retention of growth factor receptors at the plasma membrane and allow continued activation of signaling pathways. To begin to test at which step of endocytosis the ΔE mutants might act predominantly, cells were transfected with EGFR and the ΔE mutants with or without dynamin1-K44A. Dynamin1-K44A was able to overcome the diminished levels of EGFR in cells expressing HIP1/ΔE and exhibited EGFR levels comparable with transfection with dynamin1-K44A alone (Fig. 4D, compare lanes 7–9 to lanes 10–12). In contrast, the ability of dynamin1-K44A to overcome the effects of HIP1r/ΔE was not nearly as robust. Co-transfection of dynamin1-K44A with HIP1r/ΔE did cause some increase in EGFR levels, but only in the EGFR species of lower molecular weight and not to the extent seen with HIP1/ΔE (Fig. 5D, compare lanes 16–18 with lanes 13–15). Based on this finding, HIP1 and HIP1r may have different but partially overlapping mechanisms of growth factor stabilization. These results suggest HIP1 acts in a rate-limiting way downstream of the initial stages of clathrin-vesicle formation to promote receptor tyrosine kinase stabilization. HIP1r may also act in the endocytic pathway after clathrin-vesicle formation, as the dynamin1-K44A was able to partially overcome HIP1r/ΔE-mediated down-regulation of EGFR. However, the data suggest that HIP1r may have some rate-limiting functions that are upstream of HIP1. This would be consistent with its localization to peripheral membrane ruffles (15).

Finally, we tested whether the ΔE mutants of HIP1 and HIP1r caused a more widespread down-regulation of receptor tyrosine kinase levels by assaying their effects on PDGFβR after starvation and stimulation with PDGF-ββ. Similar to EGFR, cells expressing HIP1r/ΔE and HIP1/ΔE exhibited diminished levels of PDGFβR at each time point after ligand stimulation compared with vector-transfected cells (Fig. 5B, compare lanes 7–9 to lanes 1–3; Fig. 5C, compare lanes 7–9 to lanes 1–3). However, the extent of receptor down-regulation was less pronounced in cells expressing HIP1/ΔE compared with HIP1r/ΔE-expressing cells.

Inhibition of Receptor Tyrosine Kinase Degradation by the HIP Family Occurs Post-endocytosis—Ligand-mediated degradation of receptor tyrosine kinases occurs via endocytosis of clathrin-coated pits to progressively form clathrin-coated vesicles, endosomes, and lysosomes via vesicle trafficking (32). Thus, there are numerous steps of trafficking at which HIP1 and HIP1r might act to inhibit the degradation of receptor
tyrosine kinases. In order to begin to understand at which stages of endocytosis HIP1 and HIP1r might act, immunofluorescent analysis of EGFR-containing vesicles in cells transfected with EGFR and either vector, HIP1, HIP1r, or dynamin1-K44A was performed. This assay was derived from a recently published assay of transforming growth factor-β receptor endocytosis (33). Vesicles containing EGFR were visualized after ligand stimulation by staining with anti-EGFR antibody. Prior to stimulation, EGFR was localized to the plasma membrane as denoted by the absence of vesicular structures in the cytoplasm (Fig. 6A, Ist three left-hand panels). After 30 min of stimulation, cells expressing EGFR alone demonstrated the formation of vesicular structures containing EGFR (Fig. 6A, three right-hand panels, top row). Similarly, HIP1- or HIP1r-overexpressing cells also had EGFR-positive vesicular structures after ligand stimulation, indicating endocytic uptake in the HIP-transfected cells (Fig. 6A, three right-hand panels, 2nd and 3rd rows). In cells transfected with EGFR and either vector or HIP1, EGFR-containing vesicular structures also co-localized with the early endosomal antigen EEA-1 as expected (Fig. 6A, three right-hand panels, top row). Similarly, HIP1- or HIP1r-overexpressing cells also had EGFR-positive vesicular structures after ligand stimulation, indicating endocytic uptake in the HIP-transfected cells (Fig. 6A, three right-hand panels, 2nd and 3rd rows). In cells transfected with EGFR and either vector or HIP1, EGFR-containing vesicular structures also co-localized with the early endosomal antigen EEA-1 as expected (Fig. 6B, top and middle rows; columns 3 and 6). In contrast, in cells transfected with the positive control dynamin1-K44A and EGFR, EGFR-containing vesicular structures did not form, and EGFR remained on the surface rather than being internalized (Fig. 6A, three right-hand panels, bottom row). There was also no co-localization of EGFR with EEA-1 (Fig. 6B, bottom row, column 6). This is consistent with prior results that indicated the dynamin1-K44A dominant negative mutant functions to inhibit clathrin-coated vesicle release from the plasma membrane (29). If HIPs had functioned in a rate-limiting fashion during clathrin vesicle formation, we would have expected the HIP-transfected cells to not form EGFR-positive vesicles in a manner similar to that observed with the dynamin1-K44A-positive control.

We also attempted to assay the effects of the ΔE mutants of HIP1 and HIP1r on EGFR endocytosis by using this assay. However, due to the enhanced degradation of the EGFR in the presence of the ΔE mutants, the sensitivity of this assay for the EGFR was not high enough to make solid observations. Because of this, we devised a more sensitive flow cytometry assay that quantitated the surface EGFR in transfected cells. In this assay we quantitated by flow cytometry the relative amounts of cell-surface EGFR following EGF stimulation in HIP1- or HIP1ΔE-transfected HeLa cells, which contain significant quantities of endogenous EGFR (28). In this assay HeLa cells were transfected with pcDNA3-IRES-GFP constructs containing nothing (i.e. vector alone), HIP1, dynamin1-K44A or HIP1ΔE cDNAs cloned in front of the IRES-GFP cassette. These constructs express GFP in addition to the protein of interest, allowing us to restrict flow cytometric analysis of EGFR to only those cells that were GFP-positive (transfected cells). Following stimulation with EGF, live, non-permeabilized cells were stained with an anti-EGFR antibody conjugated to phycoerythrin, and relative fluorescence of GFP-positive cells was measured by flow cytometry. Similar to the effect seen by the immunofluorescence assay of EGFR uptake, there was no effect of HIP1 or HIP1ΔE on endocytic clearance of surface EGFR (Fig. 6C). On the other hand, dynamin1-K44A promoted retention of the EGFR on the cell surface at all time points indicated. In addition, even though HIP1ΔE failed to have an effect on surface levels of the EGFR post-EGF stimulation, it did cause apoptosis in HeLa cells (Supplemental Material Fig. 4). Thus, this provides further evidence that a main effect of HIP1 and HIP1ΔE on EGFR degradation likely occurs subsequent to the uptake phase of ligand-stimulated receptor tyrosine kinase endocytosis. Because endocytosis of EGFR results primarily in degradation, whereas the endocytosis of transferrin receptor results primarily in recycling of the receptor, we examined the effect of HIP1 or HIP1ΔE on uptake of fluorescently labeled transferrin. It has been shown previously that full-length HIPs do not affect the uptake of transferrin (12, 15). The effect of the HIP1/ΔE mutant has not yet been tested in transferrin accumulation. To test this, the same IRES-GFP constructs were used as those used in the assay of endogenous EGFR uptake in HeLa cells. Similar to the effects seen for the EGFR, transferrin uptake was unaffected in HIP1 or HIP1/ΔE-transfected cells but was inhibited in dynamin1-K44A (positive control)-transfected cells (Fig. 6D). Thus, there was no effect of transient overexpression of HIP1 or its ENTH deletion mutant on the uptake phase of either degradative or recycling endocytic pathways.

These results with the dynamin dominant negative, together with the lack of growth factor receptor stabilization in cells co-transfected with the ENTH domain deletion mutants, suggest that the HIP family does not function to inhibit growth factor receptor degradation, in a rate-limiting fashion, by inhibiting the internalization phase of endocytosis. Because the HIP-stabilized EGFR is also activated, we suggest that, in addition to a non-rate-limiting function at the cell surface, the HIP family also inhibits the conversion of the early endocytic vesicles to later inactivating acidic endosomes or lysosomes (Fig. 7). This is consistent with the fact that HIP1 and HIP1r localization in the cell is not only at the plasma membrane but also found in intracellular vesicles (10). The more membranous localization of HIP1r is consistent with the inability of dynamin1-K44A to fully overcome HIP1ΔE-mediated EGFR down-regulation. Finally, the 3-phosphorylated bisphosphoinositide binding properties of HIP1 and HIP1r (Fig. 2) are also consistent with HIP1 and HIP1r playing a role in intracellular trafficking as these lipids are concentrated in intracellular membranes (34).

**DISCUSSION**

Our findings demonstrate a unique binding preference of HIP1 and HIP1r via their ENTH domains for 3-phosphorylated bisphosphoinositides. This binding is likely necessary for proper functioning of HIP proteins. This is suggested by our observation that mutants lacking the ENTH domains are mis-localized in the cell and induce apoptosis. We reported previously that full-length HIP1 promotes growth and transformation of fibroblasts. A potential direct mechanism for this transformation is described here, where we show by using cycloheximide that both HIP1 and HIP1r stabilize activated receptor tyrosine kinases by inhibiting protein degradative pathways post-ligand stimulation. Consistent with this, the pro-apoptotic mutants, HIP1/ΔE and HIP1r/ΔE, do not stabilize growth factor receptors.

These results are of interest in light of data demonstrating a causative role for HIP1 in oncogenesis as well as the recent finding that HIP1 deficiency alters the levels of intracellular AMPA receptors in cultured central nervous system neurons (35). In addition, the HIP1 portion of the HIP1/PGDFβR fusion protein, independent of its dimerizing activity, is necessary for its transforming activity (18). We suggest that HIP1 and HIP1r act in a rate-limiting fashion downstream of receptor uptake to inhibit trafficking of receptor tyrosine kinases to the lysosome for subsequent degradation.

It is interesting to speculate that HIP1 and HIP1r, by binding inositol lipid determinants of clathrin-coated vesicles, early endosomes, and recycling endosomes via their ENTH domains, may cause the endocytic machinery to favor continued signaling either by stabilizing the early endosome or by stimulating growth...
FIG. 6. HIP1 and HIP1r and the HIP1/ΔE mutant do not alter EGFR uptake. A, COS 7 cells were transfected with EGFR and either vector (Vec), HIP1, HIP1r, or dynamin1-K44A. Cells were starved and stimulated with EGF for 30 min before fixation for confocal microscopic analysis. Staining for EGFR (green) shows the presence of EGFR in vesicles at 30 min after stimulation in vector-, HIP1-, or HIP1r-transfected cells (1st, 2nd, and 3rd row), but not in dynamin1-K44A-transfected cells (bottom row). B, the EGFR vesicles (green) seen at 30 min in vector- or HIP1-transfected cells co-localize (Coloc) with the endosomal marker, EEA1 (red). C, internalization of endogenous EGFR following stimulation with EGF in HIP1, HIP1/ΔE, and dynamin1-K44A-transfected HeLa cells. Following transfection, cells were starved and stimulated with 100 ng/ml EGF. Shown are the relative amounts of surface EGFR (i.e. EGFR fluorescence at indicated time/EGFR fluorescence prior to stimulation), expressed as a percentage, as measured by mean phycoerythrin fluorescence in 10^4 GFP-positive cells by flow cytometry. Mean EGFR fluorescence
factor receptor recycling to the cell surface (Fig. 7). This would be in lieu of growth factor receptor degradation by trafficking to the lysosome. We also speculate that this depends on the lipid components of the various intracellular compartments. Thus, HIP location could be regulated in part by the inositol lipid phosphorylation state, and may act in more than one step of trafficking to decrease the degradation of growth factor receptors and increase signal duration following receptor-ligand uptake. This in turn could lead to dysregulated growth of cells, with suppression of various cell cycle checkpoints, accumulation of mutations, and oncogenic transformation.

These results are also important because HIP1 and HIP1r are the first mammalian ENTH domain-containing proteins that bind preferentially to 3,4- and 3,5-phosphorylated inositol lipids. Previously identified ENTH domains, including those of AP180 and Epsin 1, have been shown to bind primarily PtdIns(4,5)P2 and PtdIns(3,4,5)P3 (7, 8). Both of the latter lipid moieties are enriched at the plasma membrane, whereas it has been suggested that PtdIns(3,4)P2 and PtdIns(3,5)P2 are localized to intracellular vesicles, predominantly those in the perinuclear sorting area (36). Localization of HIP1 and HIP1r via their ENTH lipid binding domains to intracellular vesicles would be consistent with their having an additional role in EGFR degradation post-internalization. Recent evidence points toward different functions for the ENTH domain of Epsin 1 versus that of AP180. The epsin ENTH domain has been shown to promote tubulation of lipid micelles, implying that the structure of the domain causes curvature of membranes (37). Thus, its function is consistent with a role in the internalization phase of endocytosis. The ENTH domain of AP180, also referred to as an ANTH domain, does not promote the curvature of the membrane. HIP1 and HIP1r ENTH domains appear to be more similar by sequence comparison to the AP180 ANTH domain than to the ENTH domain of epsin.

Another example of a putative endocytic protein that may have similar functions to HIP1 and HIP1r in endocytosis and trafficking remain to be elucidated. Although HIP1r and HIP1 have similar domain structures, their interacting partners and subcellular locales are somewhat different. Most important, they do have similar lipid binding specificities and similar inhibitory effects on ligand-stimulated receptor tyrosine kinase degradation, showing that there is overlap in their functions. Yet another example of a putative endocytic protein that may have similar functions to HIP1 and HIP1r is Eps15 and its relative Eps15R. Eps15 co-localizes with HIP1 (10), and its overexpression alters growth of NIH3T3 cells by enhancing their ability to grow at low density (38). Eps15 is monoubiquitinated in response to EGF and is a major substrate for the EGFR tyrosine kinase (39). However, like HIP1 and HIP1r, its activity in trafficking is not well defined. Testing whether Eps15 or mutants of Eps15 have activity in the EGFR stabilization assay described here will be of use.

in untransfected cells (i.e. GFP-negative cells in each well containing the different transfectants) was identical to the vector-transfected cells. This experiment was repeated twice with identical results. D, internalization of Alexa-fluor-633-transferrin in 293T cells transfected with HIP1, HIP1/ΔE, and dynamin1-K44A. Shown are the mean fluorescence intensities for 10^4 GFP-positive cells measured by flow cytometry, following the indicated times of transferrin incubation with the cells. Mean fluorescence intensity was not significantly different in untransfected cells versus vector-transfected cells.
There is growing evidence that intracellular sorting of endocytic vesicles plays a major role in regulating receptor stability. Other proteins that have an effect on growth factor receptor trafficking following the internalization phase of endocytosis include Hrs, Rab5, and Clil (40–43). The regulation of these proteins and their potential dysregulation in states of increased growth such as cancer warrants further study.

In summary, the results in this report provide evidence for a link between HIP1 or HIP1r expression, lipid binding, cellular survival, and growth factor receptor stability and signaling. The activities of the HIP family members are mediated in part by their ENTH domains. The evidence provided here that interfering with HIP1 or HIP1r function down-regulates growth factor receptor tyrosine kinase levels should be of great interest to those studying endocytosis, signaling, and cancer. Finally, the inhibition of HIP1 or HIP1r activity in tumors could concomitantly interfere with the function of multiple growth factor receptors, providing a multipronged approach to therapy.

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