EHD1 and Eps15 Interact with Phosphatidylinositols via Their Eps15 Homology Domains*

Received for publication, October 10, 2006, and in revised form, March 1, 2007. Published, JBC Papers in Press, April 5, 2007, DOI 10.1074/jbc.M609493200

Naava Naslavsky, Juliati Rahajeng, Sylvie Chenavas, Paul L. Sorgen1, and Steve Caplan2

From the Department of Biochemistry and Molecular Biology and Eppley Cancer Center, University of Nebraska Medical Center, Omaha, Nebraska 68198-5870

The C-terminal Eps15 homology domain-containing protein, EHD1, regulates the recycling of receptors from the endocytic recycling compartment to the plasma membrane. In cells, EHD1 localizes to tubular and spherical recycling endosomes. To date, the mode by which EHD1 associates with endosomal membranes remains unknown, and it has not been determined whether this interaction is direct or via interacting proteins. Here, we provide evidence demonstrating that EHD1 has the ability to bind directly and preferentially to an array of phospholipids, preferring phosphatidylinositols with a phosphate at position 3. Previous studies have demonstrated that EH domains coordinate calcium binding and interact with proteins containing the tripeptide asparagine-proline-phenylalanine (NPF). Using two-dimensional nuclear magnetic resonance analysis, we now describe a new function for the Eps15 homology (EH) domain of EHD1 and show that it is capable of directly binding phosphatidylinositol moieties. Moreover, we have expanded our studies to include the C-terminal EH domain of EHD4 and the second of the three N-terminal EH domains of Eps15 and demonstrated that phosphatidylinositol binding may be a more general property shared by certain other EH domains. Further studies identified a positively charged lysine residue (Lys-483) localized within the third helix of the EH domain, on the opposite face of the NPF-binding pocket, as being critical for the interaction with the phosphatidylinositols.

The internalization of receptors is a critical process for eukaryotic cells. Receptors can be internalized from the plasma membrane by a variety of well described mechanisms, including via clathrin-coated pits, independently of clathrin, and through caveolae (1). Once internalized, the small vesicles containing the internalized cargo fuse with early endosomes (also known as sorting endosomes), and the receptors are then either sent to late endosomes and on to the lysosomal pathway for degradation or recycled back to the plasma membrane, where they may participate in additional rounds of internalization (2). Receptor recycling occurs either directly from the sorting endosomes in a process known as “fast recycling” or indirectly in a process termed “slow” or “regulated” recycling (3). Slow recycling has been better characterized and traverses a complex series of tubular and vesicular membrane structures that emerge from the microtubule-organizing center and is collectively known as the endocytic recycling compartment (3, 4). Despite advances in recent years, the process of recycling is not as well understood as internalization.

Among the key regulatory proteins that control endocytic transport and recycling are the Rab family of GTP-binding proteins (5–7). Over 60 different mammalian Rab proteins have been identified thus far, and their highly regulated GTP binding and subsequent hydrolysis lead to recruitment of a wide array of effector proteins that are important for vesicular transport and fusion processes within the endocytic pathways. For example, Rab5 has a well defined role in controlling transport through and fusion of early endosomes (8), whereas Rab4 (9, 10), Rab11 (11, 12), Rab25 (13), Rab22 (14, 15), and Rab21 (16) have been implicated at varying stages of sorting from the early endosome and recycling from the endocytic recycling compartment. In addition to Rab proteins and their direct effectors, SNARE3 proteins and other non-Rab proteins have also been implicated in the regulation of various stages of endocytosis.

The Eps15 homology (EH) domain-containing family of proteins also has a well documented role in the regulation of endocytic transport and recycling. These proteins contain at least one copy of the EH domain, a highly conserved ~100-amino acid stretch. The EH domain was originally identified in three copies at the N terminus of the protein Eps15 (17, 18). Structurally, each EH domain is composed of two sets of helix-loop-helix motifs (known as EF-hands) predicted to bind calcium and linked by a short antiparallel β-sheet (reviewed in Ref. 19). In addition to binding calcium, it was discovered that EH domains bind to proteins containing the tripeptide asparagine-proline-phenylalanine (NPF) (20, 21). Despite the wide variation of other functional domains found in EH domain-containing proteins, most of these proteins have a defined regulatory role in endocytic membrane transport (reviewed in Refs. 22 and

---

*This work was supported by National Institutes of Health Grants GM072631 (to P. L. S.) and GM074876 (to S. C.) and American Heart Association Grants 0560050z (to P. L. S.) and 0460012z (to S. C.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1To whom correspondence may be addressed. Tel.: 402-559-7557; Fax: 402-559-6650; E-mail: psorgen@unmc.edu.

2To whom correspondence may be addressed. Tel.: 402-559-7556; E-mail: scaplan@unmc.edu.

3The abbreviations used are: SNARE, soluble N-ethylmaleimide-sensitive factor attachment protein receptors; PtdIns, phosphatidylinositol; PtdIns3P, phosphatidylinositol 3-phosphate; PtdIns(3,5)P2, phosphatidylinositol 3,5-biphosphate; PtdIns(3,4,5)P3, phosphatidylinositol 3,4,5-trisphosphate; LPPG, 1-palmitoyl-2-hydroxy-sn-glycerol-3-(phospho-RAC-(1-glycerol)); BOG, n-octyl-β-D-glucopyranoside; EH, Eps15 homology; HSCC, heteronuclear single quantum correlation; GST, glutathione S-transferase; HRP, horseradish peroxidase; CMC, critical micelle concentration; PI3K, phosphatidylinositol 3-kinase; MHC, major histocompatibility complex; BSA, bovine serum albumin.
Among the best characterized EH domain-containing proteins is Eps15 itself (18). Eps15 and the related Eps15R are both found within assembly sites for clathrin-coated pits, serving as molecular scaffolds by associating with the AP-2 adaptor protein complex (24) and the NPF-containing protein, epsin 1 (25).

Recently, focus has turned to understanding the regulatory role of an atypical family of EH domain-containing proteins, the C-terminal EHD family (reviewed in Ref. 26). Unlike other mammalian EH domain-containing proteins, this subgroup of four homologous proteins has a single EH domain at its C terminus (27–30), a central coiled-coil region involved in oligomerization (31–33), and an N-terminal regulatory region that binds to nucleotides (32, 33). Emerging evidence has implicated all four C-terminal EHD proteins in the process of endocytosis (26). EHD1, the best characterized C-terminal EHD protein thus far, has a well documented role in controlling recycling from the endocytic recycling compartment to the plasma membrane, a function similar to that attributed to Rab11 (31, 33–43). Although no direct interaction has been demonstrated between EHD proteins and Rab proteins, recent studies have shown that EHD1 interacts directly with certain Rab effectors. For example, the divergent Rab4/Rab5 effector, Rabenosyn-5, interacts with the EH domain of EHD1 via several of its five NPF motifs (37). Similarly, the Rab11 effector Rab11-FIP2, which contains three NPF motifs, interacts with EHD1 through NPF-EH domain interactions and plays a role in recycling from the endocytic recycling compartment (33).

One of the interesting features of EHD1 is that despite not having a hydrophobic transmembrane-spanning region, it localizes to an array of vesicular and tubular membrane structures. Membrane localization depends upon the ability of the EHD1 P-loop to bind nucleotides (35, 36). In addition, EHD1 mutants incapable of oligomerizing become cytosolic in their subcellular localization, suggesting that oligomerization is a requirement for membrane association (32, 33) and EHD1 function is severely compromised when these cytosolic mutants are expressed (34–36). Thus far, however, the mode by which EHD1 and other C-terminal EHD proteins associate with membranes has remained unknown. Indeed, until now, the question as to whether EHD1 associates indirectly with lipids through its various interaction partners or whether it directly binds to membranes has not been determined.

In the study herein, we demonstrate that EHD1 is capable of directly interacting with membranes, by preferentially binding to phosphatidylinositols with a phosphate at position 3 via its EH domain. Our data lead to the conclusion that EH domains from other C-terminal EHD proteins, such as EHD4, as well as an N-terminal EH domain-containing protein (Eps15) are also capable of lipid binding. Moreover, we have identified an EHD1 residue (Lys-483) localized to the third helix of the EH domain as being critical for the interaction with the phosphatidylinositols. Overall, we describe phosphoinositide-binding as a new function for EH domains, which may in part explain the association of EHD proteins with membranes.

**Experimental Procedures**

**Recombinant DNA Constructs**—Full-length EHD1 and EHD3, the second EH domain of Eps15, and the EH domains of EHD4 and EHD1 were subcloned into the GST fusion bacterial expression vector pGEX-6P-2 (GE Life Sciences). Wild-type Myc-EHD1 and Myc-EHD1 ΔEH have been previously described (36). Myc-EHD1 K483E was prepared by site-directed mutagenesis with the QuikChange kit (Stratagene, La Jolla, CA).

**Phosphatidylinositols and Other Lipids**—18:1 Phosphatidylinositol (PtdIns, 8:0, 1,2-dioctanoyl-sn-glycero-3-(phosphoinositol 3,4,5-trisphosphate) (PtdIns(3,4,5)P3), 8:0, 1,2-dioctanoyl-sn-glycero-3-(phosphoinositol 3,5-bisphosphate) (PtdIns(3,5)P2), 8:0 phosphatidic acid, and 16:0 1-palmitoyl-2-hydroxy-sn-glycero-3-(phospho-RAC-(1-glycerol)) (LPPG) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). 80 octyl-β-D-glucopyranoside (BOG) was obtained from Calbiochem. Each lipid was used as a lyophilized powder and all were completely soluble in phosphate-buffered saline (pH 7.4) buffer.

Critical micelle concentration (CMC) measurements are not available for eight-carbon phosphatidylinositol phosphates, and LPPG and would be cost-prohibitive to determine (44). However, the CMC for PtdIns is 60 μM, and the addition of phosphates to the inositol ring is expected to increase the head group size, charge, and therefore the CMC (44). For example, the CMC of di-C8-phosphatidylerine has been measured at 2.28 mM (45). Accordingly, the 0.75 mM concentration utilized of PtdIns(3,4,5)P3, PtdIns(3,5)P2, and BOG (CMC 20–25 mM) is expected to render them monodispersed and unable to form micelles or membranes.

**Antibodies**—Peptide-specific affinity-purified polyclonal anti-EHD1 antibodies have been previously described (33), goat anti-GST-horseradish peroxidase (HRP) was purchased from GE Life Sciences, and anti-PtdIns(3,4,5)P3 (46) and Alexa fluorochromes conjugated to secondary antibodies were obtained from Molecular Probes, Inc. (Carlsbad, CA).

**Protein Purification**—GST fusion proteins were generated by standard methods. For NMR studies, 15N labeling was done as previously described (47), and the GST was cleaved from the EH domains with PreScission Protease (GE Life Sciences) and concentrated as previously described (47).

**Protein-Lipid Binding Assay**—P-lipid (phosphatidylinositol) strips, P-lipid (phosphatidylinositol) arrays, and SphingoStrips (Echelon Biosciences, Salt Lake City, UT) were blocked in TBST-BSA (10 mM Tris, pH 8, 150 mM NaCl, 0.1% Tween 20 (w/v)) supplemented with 3% fatty acid-free BSA (Sigma) for 1 h at room temperature and then incubated with 1 μg/ml GST fusion protein diluted in TBST-BSA overnight at 4 °C. After washes with TBST-BSA, the membranes were blotted with goat anti-GST conjugated to HRP and visualized by enhanced chemiluminescence. Densitometric analysis was applied to determine the relative affinity of EHD1 binding to the various phosphatidylinositols. Numerical densitometric values were attributed to each of the five concentrations measured after subtracting backgrounds, and the total additive value for all five measurements was used as an indication of affinity. The highest value, for EHD1 binding to PtdIns(3,5)P2, was arbitrarily assigned “100% binding,” and all other phosphatidylinositols were normalized compared with PtdIns(3,5)P2.

**Protein-Liposome Binding Assay**—PolyPinosomes (Echelon) contain phosphatidylcholine (65 mol %), PE (29 mol %), bio-
EH Domains Bind Phosphatidylinositols

To determine whether EHD1 is capable of directly interacting with lipids, we first utilized an assay in which a wide variety of lipids, including phosphatidylserine, phosphatidylinositol, sphingosine, cholesterol, and others (Fig. 1, A and B), GST-EHD1 bound to a series of phosphatidylinositol moieties, including phosphatidic acid, phosphatidylinositol-3-phosphate (PtdIns3P), phosphatidylinositol-4-phosphate, phosphatidylinositol-5-phosphate, PtdIns(3,5)P2, and PtdIns(3,4,5)P3, but showed no binding to various other lipids, including phosphatidylethanolamine, phosphatidylcholine, sphingosine, cholesterol, ceramide, and others (Fig. 1, A and B). To quantitatively assess the binding specificity of these lipids for EHD1, GST-EHD1 was used to probe an array of phosphatidylinositol moieties.

RESULTS

EHD1 Associates Directly with Phosphatidylinositol Lipids—To determine whether EHD1 is capable of directly interacting with lipids, we first utilized an assay in which a wide variety of lipids, immobilized on nitrocellulose, were probed with GST as a control (data not shown) or with a GST-EHD1 fusion protein. As demonstrated in Fig. 1, A and B, GST-EHD1 bound to a series of phosphatidylinositol moieties, including phosphatidic acid, phosphatidylinositol-3-phosphate (PtdIns3P), phosphatidylinositol-4-phosphate, phosphatidylinositol-5-phosphate, PtdIns(3,5)P2, and PtdIns(3,4,5)P3, but showed no binding to various other lipids, including phosphatidylethanolamine, phosphatidylcholine, sphingosine, cholesterol, ceramide, and others (Fig. 1, A and B). To quantitatively assess the binding specificity of these lipids for EHD1, GST-EHD1 was used to probe an array of phosphatidylinositol moieties.

To determine whether EHD1 is capable of directly interacting with lipids, we first utilized an assay in which a wide variety of lipids, including phosphatidylserine, phosphatidylinositol, sphingosine, cholesterol, and others (Fig. 1, A and B), GST-EHD1 bound to a series of phosphatidylinositol moieties, including phosphatidic acid, phosphatidylinositol-3-phosphate (PtdIns3P), phosphatidylinositol-4-phosphate, phosphatidylinositol-5-phosphate, PtdIns(3,5)P2, and PtdIns(3,4,5)P3, but showed no binding to various other lipids, including phosphatidylethanolamine, phosphatidylcholine, sphingosine, cholesterol, ceramide, and others (Fig. 1, A and B). To quantitatively assess the binding specificity of these lipids for EHD1, GST-EHD1 was used to probe an array of phosphatidylinositol moieties.
EH Domains Bind Phosphatidylinositols

EH Domains that was incubated with the liposomes precipitated with liposomes containing PtdIns(3,4,5)P3 and PtdIns(3,5)P2, whereas less than 3% precipitated with PtdIns. The GST-only control did not bind to any of the lipid compositions. Additional control experiments with streptavidin beads but without liposomes demonstrated that the efficient pull-down of GST-EHD1 and GST-EHD3 was entirely dependent on the presence of the liposomes. Moreover, the preferential binding to PtdIns(3,5)P2 and PtdIns(3,4,5)P3 as compared with PtdIns is consistent with our findings in Fig. 1. Taken together, our data support a direct interaction between EHD1 and phosphoinositoid lipids.

Inhibition of Phosphatidylinositol 3-Kinase (PI3K) Activity Affects the Subcellular Distribution of EHD1—Within cells, EHD1 resides on tubular and vesicular membrane-bound organelles. Although efficient/specific antibodies are not available for many of the phospholipids, commercial antibodies suitable for immune fluorescence are available for PtdIns(3,4,5)P3 (46), one of the phospholipids to which EHD1 displayed relatively high affinity (Figs. 1 and 2). To determine whether EHD1 localizes in vivo to PtdIns(3,4,5)P3-containing membranes, we fixed and incubated HeLa cells with antibodies directed against endogenous EHD1 and PtdIns(3,4,5)P3 (Fig. 3). As shown, EHD1 localizes to an array of vesicular and tubular endosomal structures, consistent with its function in the recycling pathway (Fig. 3A). PtdIns(3,4,5)P3 localized primarily to vesicular membranes (Fig. 3B). As indicated in the merged micrograph (Fig. 3C) and insets, a subset of the PtdIns(3,4,5)P3-containing membrane structures was also positive for EHD1. Co-immunostaining of endogenous EHD1 with phosphatidylinositol 4,5-bisphosphate showed little to no overlap with EHD1 (data not shown), consistent with our in vitro data. These experiments indicate that in vivo EHD1 and PtdIns(3,4,5)P3 partially co-localize, suggesting that this may result from direct interactions of EHD1 with PtdIns(3,4,5)P3 and/or other phospholipids.

To provide additional in vivo evidence for the influence of inositol phosphotides on the localization of EHD1, we treated EHD1-transfected cells with three different PI3K inhibitors (Fig. 3, D–P). As previously shown (52), PI3K inhibitors LY294002 (Fig. 3E) and wortmannin (Fig. 3F) effectively altered the localization of internalized major histocompatibility complex class I (MHC-I) proteins compared with untreated cells (Fig. 3D). An additional highly selective PI3K inhibitor, ZSTK474 (53), similarly induced the localization of internalized liposomies immobilized on nitrocellulose membranes in decreasing concentrations from 100 to 6.2 pmol (Fig. 1C) and quantified by densitometry (Fig. 1D). As demonstrated, phosphatidylinositol moieties with a phosphate at position 3 of the inositol ring, including PtdIns(3,5)P2 and PtdIns(3,4,5)P3, showed binding even at 25 and 12.5 pmol, respectively. These data indicate that EHD1 can directly bind to phosphatidylinositols with phosphates on the inositol ring.

To further validate our findings that EHD1 can interact directly with phospholipids, we used a pull-down assay, whereby streptavidin beads coated with either PtdIns(3,4,5)P3 or PtdIns were then incubated with GST-EHD1, GST-EHD3 (the closest mammalian homolog to EHD1 at 85% identity), or GST alone. By this in vitro assay, we again demonstrated that EHD1 as well as EHD3 bind to PtdIns(3,4,5)P3 and PtdIns (Fig. 2A).

To determine whether EHD1 interacts directly with phospholipids in the context of a monolayer more closely resembling physiological membrane composition, we utilized liposomes composed of 1% biotinylated PE, 29% PE, and 65% phosphatidylcholine, with either 5% PtdIns(3,4,5)P3, PtdIns(3,5)P2, or PtdIns (PolyPIPosomes; Echelon). The liposomes were incubated with GST-EHD1, GST-EHD3, or GST alone as a control. Isolated complexes of biotinylated liposomes bound to GST proteins were further purified by precipitation with streptavidin-coated Sepharose beads (Fig. 2B). Approximately 10–20% of both GST-EHD1 and GST-

**FIGURE 2.** EHD1 interacts with phosphatidylinositol-containing beads and liposomes. A, streptavidin beads coated with either biotinylated PtdIns(3,4,5)P3, or PtdIns were incubated with either GST, GST-EHD1, or GST-EHD3 for 2 h at 4 °C and then precipitated. Recombinant proteins were eluted, separated by SDS-PAGE, transferred to nitrocellulose filters, and immunoblotted with anti-GST-HRP. The right panel (Input) depicts 20% of each recombinant protein that was incubated with the beads. B, 10 μg of purified recombinant GST-EHD1, GST-EHD3, and GST were incubated with 15 μmol of biotinylated liposomes containing 5 mol % of either PtdIns(3,4,5)P3, PtdIns(3,5)P2, or PtdIns for 15 min at room temperature. Liposome-protein complexes were then centrifuged, incubated with streptavidin beads, and recovered with a second centrifugation. Bound GST fusion proteins were then eluted, separated by SDS-PAGE, transferred to nitrocellulose filters, and immunoblotted with anti-GST-HRP. The bottom panel (Input) depicts 20% of each recombinant protein that was incubated with the liposomes. As a control, streptavidin beads were incubated with GST-EHD1, GST-EHD3, and GST in the absence of liposomes (right). 20% of each recombinant protein that was incubated with the liposomes is depicted as *Input* (right).
EH Domains Bind Phosphatidylinositols

MHC-I molecules to enlarged endocytic structures (Fig. 3G). The same concentrations of these PI3K inhibitors also affected the localization of EHD1. Upon transfection in untreated cells, EHD1 showed a typical tubulo-vesicular distribution pattern, with some cytosolic EHD1 observed as well (Fig. 3, H and L). However, treatment of the cells with LY294002 (Fig. 3, I, M, and N), wortmannin (Fig. 3, J and O), or ZSTK474 (Fig. 3, K and P) led to a mild “hypertubulation” of EHD1, where vesiculation could be observed all along the tubular membranes. These experiments show that perturbation of a subset of phosphorylated phosphatidylinositol moieties alters the localization of EHD1 in vivo, underlining the potential importance of EHD1/phospholipid binding.

The EHD1 EH Domain (EH-1) Binds to Phosphorylated Phosphatidylinositols in a Ca2+ -independent Manner—We next aimed to determine whether the isolated EH domain of EHD1 (EH-1) is capable of directly interacting with phospholipids. Previous studies have shown that EH domains contain either “canonical” or “pseudo-” EF-hands that bind Ca2+ (54), and we first tested the ability of EH-1 to bind to Ca2+. To this end, we purified 15N-labeled EH-1 in phosphate-buffered saline buffer (pH 7.4) and subjected the 12-kDa protein domain to NMR spectroscopy (Fig. 4A). The primary NMR experiment used to characterize the EH-1 domain was the 15N HMQC experiment. The 15N HMQC is a two-dimensional NMR experiment where each amino acid (except proline) gives one signal (or chemical shift) that corresponds to the N-H amide group. These chemical shifts are sensitive to the chemical environment, and even small changes in structure and/or dynamics can change the chemical shift of an amino acid.

To determine experimentally if EH-1 binds Ca2+, we first characterized the spectral pattern in the absence of Ca2+ and superimposed it with titrations of 0.1, 0.5, 2, and 5 mM Ca2+ (Fig. 4A). From the shifts in amino acid peaks (marked by dashed ovals and rectangles), it was apparent that EH-1 binds Ca2+. Interestingly, the 15N HMQC data indicate that in the absence of Ca2+, EH-1 exists in two conformations (Fig. 4A, Conformation A and Conformation B; dashed purple ovals provide examples of various peaks that are split into two). The titration of any concentration of Ca2+ induces a shift in these peaks from both conformations to that of only Conformation B. However, an additional two peaks were shifted, inducing a third conformation (Fig. 4A, Conformation C; red dashed rectangles at the bottom) only upon titration of the highest concentration of Ca2+ (5 mM), demonstrating the coordination of Ca2+ by a second, lower affinity Ca2+-binding region.

To determine if EH-1 binds to PtdIns(3,4,5)P3, we first studied the spectral pattern of this domain in the absence of lipid and Ca2+ (Fig. 4B; black peaks) and superimposed it with the spectral distribution observed upon titration of a 1:3 molar ratio of EH-1 to PtdIns(3,4,5)P3. As demonstrated, a number of the amino acid peaks showed significant shifts upon the addition of the PtdIns(3,4,5)P3 (Fig. 4B; red peaks and green dashed ovals), indicating that the lipid binds to EH-1 in the absence of Ca2+. The addition of 2 mM Ca2+ to the same EH-1/lipid mix-
tured (Fig. 4C; red peaks and green dashed ovals) or the subsequent removal of Ca\(^{2+}\) by the addition of 10 mM EDTA (Fig. 4D; red peaks and green dashed ovals) did not alter EH-1/PtdIns(3,4,5)\(_3\) binding, suggesting that Ca\(^{2+}\) binding does not play a major role in the regulation of EH-1/lipid binding.

We then tested the ability of other lipids to interact with EH-1 by examining their \(^{15}\)N HSQC spectra after titration of the lipids in the presence of Ca\(^{2+}\). PtdIns(3,5)\(_2\) showed a very similar pattern of binding to EH-1, with shifts detected in the same peaks observed for PtdIns(3,4,5)\(_3\) (Fig. 5A; red peaks and dashed green ovals). Phosphatidic acid, another lipid that displayed binding in the lipid array, also induced a similar pattern of peak shifts when titrated with EH-1 (Fig. 5B). On the other hand, PtdIns, which showed no binding to EHD1 when immobilized on nitrocellulose but was capable of interacting by the liposome and bead assays, showed subtle but measurable \(^{15}\)N HSQC shifts for only a few residues, indicative of weak binding (Fig. 5C). BOG, a lipid containing a glucose rather than inositol head group, also displayed minor shifts similar to PtdIns (data not shown). LPPG, a 16-carbon lipid with a negatively charged glycerol head group rather than inositol, showed no shift whatsoever in its \(^{15}\)N HSQC pattern (Fig. 5D), confirming that EH-1 binds specifically to certain phosphatidylinositol moieties.

The EH Domain of EHD4 and the Second of the Three Eps15 EH Domains Are Also Capable of Interacting with PtdIns(3,5)\(_2\)—To the best of our knowledge, direct interactions between EH domains and phospholipids have not previously been reported. To determine whether lipid binding is a specific property limited to the EHD1 EH-1 domain or a more general characteristic shared by other EH domain-containing proteins, we purified two additional EH domains for binding analysis by \(^{15}\)N HSQC. We first chose EH-4, the EH domain of EHD4, one of the four highly homologous C-terminal EHD proteins (Fig. 6A; 15N HSQC peaks in black). Similar to EH-1 and EH-4, the addition of LPPG to Eps15 EH-2 had no effect upon the distribution of the \(^{15}\)N HSQC peaks (Fig. 6B; green peaks). However, titration

---

**FIGURE 4.** The EHD1 EH domain (EH-1) interacts with PtdIns(3,4,5)\(_3\) in a Ca\(^{2+}\)-independent manner. A–D. \(^{15}\)N-labeled EH-1 was purified and subjected to \(^{15}\)N HSQC analysis following titration with varying concentrations of Ca\(^{2+}\) (see color-coded key) (A), in the presence of PtdIns(3,4,5)\(_3\) without Ca\(^{2+}\) (B), in the presence of PtdIns(3,4,5)\(_3\) with 2 mM Ca\(^{2+}\) (C), or in the presence of PtdIns(3,4,5)\(_3\) with 2 mM Ca\(^{2+}\) and 10 mM EDTA (D). 0.25 mM EH-1 and 0.75 mM lipid were mixed in a 1:3 molar ratio. The purple dashed ovals illustrate residues that shift upon the addition of any concentration of Ca\(^{2+}\), whereas the red dashed rectangles point out amino acid peaks that shift only upon the addition of 5 mM Ca\(^{2+}\). Green dashed ovals indicate amino acid shifts resulting from the addition of phosphoinositides.
of PtdIns(3,5)P$_2$ again caused a significant number of peaks to redistribute (Fig. 6B; dashed blue ovals and amino acids noted from the previously solved structure of Eps15 EH-2 (55)), indicating that this N-terminal positioned EH domain is also capable of binding to phospholipids, such as PtdIns(3,5)P$_2$. Taken together, these data support the likelihood that phospholipid binding is a more general property of certain EH domains.

**DISCUSSION**

Despite the lack of a hydrophobic transmembrane region, the C-terminal EHD proteins localize to tubular and/or vesicular membrane structures (31, 34–36). Moreover, a number of studies have demonstrated that the proper membrane localization of EHD1 is crucial for its function (32, 33, 35, 36). However, the mechanisms by which these EHD proteins associate with membranes have remained unclear.

Recent studies provide evidence that a regulatory p-loop motif at the N-terminal region of EHD1 and EHD3 is capable of binding ATP and that nucleotide binding is required for their membrane localization (32, 33). Mutations in a conserved glycine (G65R) in the nucleotide-binding p-loop cause mislocalization of EHD1 and EHD3 to the cytosol (34–36). Moreover, it has recently been demonstrated that oligomerization of EHD proteins through a central coiled-coil is necessary for their association with endocytic membranes (31–33, 36), suggesting...
that regulation of EHD localization to membranes is complex, entailing more than a single domain or region of the protein. In addition, studies have shown that either truncation of the entire C-terminal EH domain or mutations within key conserved amino acids of the domain alter the localization of EHD1 and EHD3 in cells, causing a shift from primarily tubular to entirely vesicular membrane structures (31, 36). This suggests either a change in protein-protein interactions or a change in the mode of lipid binding. However, the specific regions of EHD proteins that potentially mediate associations with membranes have yet to be determined. Indeed, even the question as to whether EHD proteins interact directly with lipids or bind indirectly via other lipid-binding proteins has remained enigmatic to date.

Several EHD interaction partners have been shown to interact directly with lipids. For example, EHD1 and EHD3 interact with several of the five NPF motifs within Rabenosyn-5, via their EH domains (37). Since Rabenosyn-5 contains a FYVE domain (56), a domain with direct affinity for PtdIns3P (57–59), this raises the possibility that Rabenosyn-5 might mediate indirect interactions between EHD proteins and phospholipids. However, we have previously shown that efficient RNA interference knockdown of Rabenosyn-5 does not alter the subcellular localization pattern of EHD1 (37), suggesting that this is not the only mechanism for association of EHD1 with membranes. Another EHD1 and EHD3 interaction partner, Rab11-FIP2 (33), also directly interacts with lipids (60), but the significance of this interaction for EHD localization has yet to be determined. EHD proteins also interact with Syndapins, but the EHD proteins appear to recruit Syndapins to membranes (41, 61), suggesting that this interaction does not regulate EHD-membrane associations.

A recent report suggests that EHD proteins interact directly with phospholipids (27). To the best of our knowledge, we now provide the first evidence that EH domains are capable of directly interacting with phosphatidylinositols. By an in vitro immobilized lipid binding assay, we have demonstrated that full-length EHD1 binds directly and specifically to a subset of phosphatidylinositols, particularly those with a phosphate in the 3-position. Additional methods were used to confirm these interactions, including binding to lipid-coated beads and a liposome binding assay. Although PtdIns showed significant binding in the bead assay, possibly due to enhanced presentation of the inositol ring, the latter method represents a more stringent assay to examine lipid binding in a manner that might be more reflective of physiological membranes.

Although phosphatidylinositols are not exclusively localized to certain organelar membranes, PtdIns3P is highly enriched on early endosomal membranes (reviewed in Ref. 62), and PtdIns(3,5)P2 is concentrated on multivesicular bodies and

FIGURE 6. The EHD4 EH domain (EH-4) and the second Eps15 EH domain also interact directly with phosphatidylinositols. A and B, 15N-labeled EH-4 and second Eps15 EH domain were purified and subjected to 15N HSQC analysis by themselves (A and B; black peaks) or in the presence of PtdIns(3,5)P2 or LPPG (0.25 mM EH-4 or Eps15 EH domain and 0.75 mM lipid; 1:3 molar ratio). The blue dashed ovals depict residues that shift upon the addition of PtdIns(3,5)P2. Since the solution structure of the second Eps15 EH domain has been previously solved, and all of the amino acids have been assigned (55), some of the amino acids that shift upon PtdIns(3,5)P2 titration have been noted, with key residues shown in blue.

FIGURE 7. Mutations in Lys-483 cause EHD1 to shift from tubular to vesicular membranes. Wild-type Myc-EHD1 (A), Myc-EHD1 lacking the EH domain (Myc-EHD1 ΔEH) (B), Myc-EHD1 K475E (C), and Myc-EHD1 K483E (D) were transfected separately into HeLa cells on glass coverslips. After transfection (24 h), the cells were fixed, permeabilized, and immunostained with mouse anti-Myc antibodies and secondary anti-mouse Alexa-Fluor 568 antibodies. Representative images were obtained with a Zeiss LSM 5 Pascal confocal microscope. Bar, 10 μm.
**EH Domains Bind Phosphatidylinositols**

lysosomal membranes and required for cargo delivery to the former organelle (63). PtdIns(3,4,5)P$_3$ is synthesized both at the plasma membrane and in the cytoplasm and is associated with membrane ruffling and coatamer-coated vesicles important in Golgi trafficking (46). These patterns are consistent with the role attributed to EHD proteins in regulation of endocytic transport events.

The significance of EHD1-phospholipid interactions is highlighted by our in vivo studies, demonstrating that the localization of EHD1 is altered upon modulation of the cellular phosphatidylinositol levels by treatment with PI3K inhibitors. Although the complexity of pathways that regulate phosphatidylinositol levels renders it difficult to pinpoint precisely which lipids are important for EHD1 localization, these experiments demonstrate that phospholipids affect EHD1 localization and suggest that phospholipid binding is an important part of this regulation.

Given that the EH domain is the only recognizable domain in EHD1 (aside from a short p-loop and the central coiled-coil region; reviewed in Ref. 26), we hypothesized that it may be involved in phosphatidylinositol binding. To determine if the EH domain is capable of lipid-binding, we utilized two-dimensional $^{15}$N HSQC experiments. By these NMR-based strategies, we demonstrated that the isolated EH domain of EHD1 (EH-1) directly interacts with PtdIns(3,5)P$_2$ and PtdIns(3,4,5)P$_3$ in a Ca$^{2+}$-independent manner and induces clear shifts in a series of amino acid peaks in the $^{15}$N HSQC spectra. For control experiments, several lipids were titrated, including LPPG, BOG, and PtdIns. Whereas PtdIns(3,4,5)P$_3$ bound tightly to EH-1 in our studies, PtdIns and BOG (which have nonphosphorylated inositol and glucose head groups, respectively) exhibited only very weak binding. LPPG, which has a glycerol head group, displayed no measurable binding. Ca$^{2+}$ does not appear to play a major role in the in vitro binding of EH-1 to lipids, but the binding of EF-hands to Ca$^{2+}$ has been well documented (54).

Although the physiological role of Ca$^{2+}$ binding to EF-hands remains poorly understood, one might speculate that Ca$^{2+}$ plays a more significant role in vivo, possibly stabilizing the full-length protein and facilitating its interactions with lipids and/or NPF motifs. These results suggest that a phosphorylated inositol head group is preferable for Ca$^{2+}$-independent EH-1 binding.

Although we were able to detect lipid binding of the EH domain alone using the NMR-based approach, the other in vitro techniques were not successful in showing binding (data not shown). This was not surprising, since it has been documented that other lipid-binding domains often only exhibit lipid binding when they comprise part of a full-length protein or are expressed as tandem domains. The rationale for this has been attributed to the involvement of other regions of the protein in stabilization of the lipid association and/or poor affinity of the isolated domain to lipids. An example is the FYVE domain, which is found in Rabenosyn-5 and EEA1 and binds to PtdIns3P (58, 64). FYVE domain-containing proteins, such as EEA1, tend to oligomerize and generally display weak affinity for PtdIns3P when expressed as a single isolated FYVE domain (58).

Similarly, the EH-1 domain may require nucleotide binding through its p-loop and/or oligomerization via the coiled-coil region to facilitate lipid binding. Akin to the FYVE domains, EH-1 may display poor affinity for phosphatidylinositols that are attached to either a matrix or liposome, due to the fixed orientation of the lipids. Accordingly, without the stability provided by the rest of the protein and potentially by its oligomerization, binding cannot be detected readily by these methods. On the other hand, the binding assay by NMR is more sensitive and has the advantage that at the concentrations used, the phosphatidylinositol is monodispersed and probably has a better opportunity to result in stable association with EH-1.

EH domains have been described in over 50 different proteins (19), yet to date there has been no evidence presented that they are capable of interacting directly with lipids. Accordingly, we sought to understand whether lipid binding is a specific phenomenon restricted to the EH domain of EHD1 or whether it might represent a more general phenomenon exhibited by homologous EH domains of other proteins. To this aim, we tested the EH domain of another related C-terminal EH domain-containing protein, EHD4 (EH-4), as well as the second of the three EH domains found in Eps15 (Eps15 EH-2). When defined as a 95-residue entity, EH-4 shares 73.7% identity with EH-1. The Eps15 EH-2 domain is a representative of the N-terminal EH domain family but still shares 49.5% identity with EH-1, whereas other N-terminal EH domains, such as the first Intersectin EH domain, shares only 38.9% identity with EH-1. $^{15}$N HSQC spectra from both EH-4 and Eps15 EH-2 elicited patterns consistent with proper folding and titration of PtdIns(3,5)P$_2$ but not LPPG-induced shifts in amino acids indicative of binding to the former lipid. The results from these experiments demonstrate that direct EH-lipid binding is not restricted to EHD1 or EH domains of proteins belonging to the C-terminal EHD family. Whether or not all known EH domains interact directly with lipids remains to be determined.

Taking advantage of the fact that the second EH domain of Eps15 has been previously solved and all of its amino acids assigned (55), we were able to determine precisely which amino acids shift in our $^{15}$N HSQC experiments as a result of interaction with PtdIns(3,5)P$_2$ (Figs. 6B and 8A). The shifts were localized to a stretch of about 30 amino acids that span the second and third helices. Of interest, a subset of residues “strongly shifted” have been denoted by the blue color, whereas the remaining shifted residues are highlighted in red for the sequence alignment (Fig. 8A) and models (Fig. 8, B and C). The strongly shifted residues defined a key area of interaction that may exist between EH domains and phosphatidylinositol phosphates, involving the positively charged conserved lysine (Lys-39) and arginine (Arg-47) residues (side chains, green). Measuring the distance between these two positively charged lipid-binding side chains (Lys-39 and Arg-47) yielded a distance of 16 Å, sufficient to encompass a phosphorylated inositol head group. It is of interest that positively charged residues are maintained at homologous positions for EH-1, EH-4, and Eps15 EH-2, but in the first Intersectin EH domain, these residues are replaced by glycine and glutamate, respectively, suggesting that perhaps not all EH domains are suited for phosphatidylinositol binding.
The strongest evidence for the physiological significance of EH-1-lipid binding follows from our demonstration that mutation of the second conserved lysine residue in the EHD1 EH domain to glutamate (K483E) causes a dramatic change in the subcellular localization of EHD1, so that it associates entirely with vesicular rather than tubular membranes (Fig. 7D). The K475E mutation did not alter the subcellular localization of EHD1, suggesting that this positively charged residue is less important for the binding than Lys-483. Indeed, taking advantage of the solved structure for the Eps15 EH-2 domain and the degree of residue shifts observed upon the addition of PtdIns(3,5)P₂ (Fig. 6B), one might predict that Lys-483 plays a more significant role in lipid binding. This is because although both the Eps15 EH-2 Lys-39 and Arg-47 shift to a similar degree, the amino acid preceding Arg-47 (Gly-46) displays a much greater shift than that of the Ser-38, which precedes Lys-39. Our data demonstrate that a single point mutation mimics truncation of the entire EH domain (Fig. 7, B and D), highlighting the significance of Lys-483 in phospholipid binding and localization.

Based on the studies of De Beer and colleagues (55, 65), an NPF-binding pocket has been shown for the second Eps15 EH domain primarily between a key leucine residue at position 36 and a critical tryptophan at position 49, suggesting an overlap between the NPF- and lipid-binding regions (side chains, gray). However, despite the proximity of the NPF-binding amino acid backbone to that of the lipid-binding backbone (residues 39 and 47), when modeled it was apparent that the positively charged lipid-binding amino acid side chains project away from the defined NPF-binding pocket (Fig. 8, B and C). This is consistent with the notion that EH domains might simultaneously bind phospholipids and NPF-containing proteins.

In summary, our findings demonstrate for the first time that EHD1 and other EH domain-containing proteins can interact directly with phosphatidylinositols via their EH domains. Given the involvement of EH domain-containing proteins in the endocytic regulatory network, we propose that EH domain/lipid-binding may be part of the complex mechanisms that regulate endocytic transport and recycling.

Acknowledgment—We thank Greg Taylor for helpful discussions and critical reading of the manuscript.

REFERENCES
1. Conner, S. D., and Schmid, S. L. (2003) Nature 422, 37–44
2. Mellman, I. (1996) Annu. Rev. Cell Dev. Biol. 12, 575–625
3. Maxfield, F. R., and McGraw, T. E. (2004) Nat. Rev. Mol. Cell. Biol. 5,
