High-Affinity Binding of a FYVE Domain to Phosphatidylinositol 3-Phosphate Requires Intact Phospholipid but Not FYVE Domain Oligomerization†

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ABSTRACT: FYVE domains are small zinc-finger-like domains found in many proteins that are involved in regulating membrane traffic and have been shown to bind specifically to phosphatidylinositol 3-phosphate (PtdIns-3-P). FYVE domains are thought to recruit PtdIns-3-P effectors to endosomal locations in vivo, where these effectors participate in controlling endosomal maturation and vacuolar protein sorting. We have compared the characteristics of PtdIns-3-P binding by the FYVE domain from Hrs-1 (the hepatocyte growth factor-regulated tyrosine kinase substrate) with those of specific phosphoinositide binding by Pleckstrin homology (PH) domains. Like certain PH domains (such as that from phospholipase C-δ_1), the Hrs-1 FYVE domain specifically recognizes a single phosphoinositide. However, while phosphoinositide binding by highly specific PH domains is driven almost exclusively by interactions with the lipid headgroup, this is not true for the Hrs-1 FYVE domain. The phospholipase C-δ_1 PH domain shows a 10-fold preference for binding isolated headgroup over its preferred lipid (phosphatidylinositol 4,5-bisphosphate) in a membrane, while the Hrs-1 FYVE domain greatly prefers (more than 50-fold) intact lipid in a bilayer over the isolated headgroup (inositol 1,3-bisphosphate). By contrast with reports for certain PH domains, we find that this preference for membrane binding over interaction with soluble lipid headgroups does not require FYVE domain oligomerization.

FYVE domains are small zinc-finger-like domains of around 70−80 amino acids that were first identified in several yeast and mammalian proteins implicated in membrane traffic (*1*−*3*). Studies of EEA1,[1] the early endosome antigen-1, indicated that its FYVE domain plays an important role in localizing the protein to endosomes (*2*) and that this localization depends on PI 3-kinase activity (*4*). Several groups have subsequently demonstrated that the FYVE domains from EEA1, Hrs-1, Vps27p, Vac1p, Fab1p, and Pib1p all bind specifically to PtdIns-3-P in vitro (*5*−*7*) and that, where studied, protein fragments containing FYVE domains are targeted to endosomal locations in vivo. PtdIns-3-P appears to be restricted in its location to the cytoplasmic face of early endosomes and internal vesicles of multivesicular bodies (*8*). By participating in the recruitment of specific FYVE domain-containing effector molecules to these locations, PtdIns-3-P is thought to play a key role in regulating steps in endosomal maturation and vacuolar protein sorting (*8*, *9*). The FYVE domain of Hrs-1 (hepatocyte growth factor-regulated tyrosine kinase substrate), for example, appears to be one of several determinants for the subcellular localization of this protein in mammalian cells (*10*). An intact FYVE domain, PI 3-kinase activity, and active receptor-mediated endocytosis are all required for epidermal growth factor (EGF) stimulated phosphorylation of Hrs-1, presumably at an endosomal compartment that is "marked" by PtdIns-3-P and can recruit FYVE domain-containing effectors (*11*).

X-ray crystal structures have been determined for the FYVE domains from Vps27p (*12*) and Hrs-1 (*13*). As anticipated from the FYVE domain sequences, the structure is stabilized by two Zn^{2+} ions that are coordinated by the eight cysteines (seven cysteines and one histidine in the case of Vps27p-FYVE) that define the finger. The third and fourth of the conserved cysteines are part of a positively charged "signature" motif that defines FYVE domains, with the sequence R(R/K)HHCRxCG (*1*). The basic side chains from residues in this motif dominate the physical character of the FYVE domain surface, forming a positively charged pocket that is likely to provide a large part of the PtdIns-3-P binding site (*12*). Precisely how the FYVE domains recognize PtdIns-3-P is not yet clear: attempts to model binding based on the crystal structures differ substantially (*12*, *13*). There is

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[1] Abbreviations: Btk, Bruton's tyrosine kinase; EEA1, early endosome antigen-1; EGF, epidermal growth factor; FGD1, faciogenital dysplasia protein-1; Grp1, general receptor for phosphoinositides; GST, glutathione *S*-transferase; Hrs-1, hepatocyte growth factor-regulated tyrosine kinase substrate-1; Ins(1,3)P_2, inositol (1,3)bisphosphate; Ins(1,4,5)P_3, inositol (1,4,5)trisphosphate; PH, Pleckstrin homology; PI 3-kinase, phosphatidylinositol 3-kinase; PLC, phospholipase C; PLC, δ-PHPH domain from PLC-δ_1; PMSF, phenylmethanesulfonyl fluoride; PtdIns, phosphatidylinositol; PtdIns-3-P, phosphatidylinositol 3-phosphate; PtdIns-4-P, phosphatidylinositol 4-phosphate; PtdIns-5-P, phosphatidylinositol 5-phosphate; PtdIns(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; PtdIns(3,4)P_2, phosphatidylinositol 3,4-bisphosphate; PtdIns(3,5)P_2, phosphatidylinositol 3,5-bisphosphate; PtdIns(3,4,5)P_3, phosphatidylinositol 3,4,5-trisphosphate; SUV, small unilamellar vesicle.

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agreement, however, that the basic residues in the FYVE domain signature motif are involved in binding, and analysis of NMR chemical shift differences upon PtdIns-3-P association with the EEA1 FYVE domain has confirmed this (*14*). The same NMR studies provided experimental evidence for the involvement of a set of residues in PtdIns-3-P binding that is not completely consistent with either hypothetical model, indicating that the actual binding configuration has yet to be elucidated.

We were interested to compare the mode of PtdIns-3-P binding by FYVE domains with that of specific phosphoinositide recognition by Pleckstrin homology (PH) domains. The PH domains from phospholipase C-δ_1 (PLC-δ_1), the general receptor for phosphoinositides-1 (Grp1), and Bruton's tyrosine kinase (Btk) each recognize a specific polyphosphoinositide, almost exclusively through interactions with the inositol phosphate headgroup (*15*, *16*). Although the degree of ligand-binding specificity shown by the Hrs-1 and FGD1 FYVE domains is similar to that seen for these PH domains, we find that the two types of domain differ dramatically in their reliance on lipid headgroup for phosphoinositide recognition. Whereas headgroup interactions dominate PH domain binding (*17*), this is not true for the FYVE domains. For high-affinity binding of FYVE domains to PtdIns-3-P, the intact lipid and its presence within a membrane structure appear to be critical. These findings illustrate a difference in the mode of phosphoinositide recognition by the two membrane targeting modules, possible origins of which are discussed. Finally, our lipid-binding affinity measurements, in light of previous literature, provide insights into the strength of binding required for recruitment of effector molecules to PtdIns-3-P-containing membranes.

EXPERIMENTAL PROCEDURES

Materials. PtdIns-3-P, PtdIns(3,4)P_2, and PtdIns(3,4,5)P_3 were purchased from Matreya Inc. (Pleasant Gap, PA). PtdIns, PtdSer, PtdIns-4-P, and PtdIns(4,5)P_2 were from Sigma-Aldrich (St. Louis, MO). PtdIns-5-P, PtdIns(3,5)P_2, Ins(1,3)P_2, and di-C_4-PtdIns-3-P were from Echelon Inc. (Salt Lake City, UT). PtdCho and di(dibromostearoyl)-PtdCho were from Avanti Polar Lipids (Birmingham, AL).

Production of FYVE Domains. The polymerase chain reaction (PCR) was used to amplify a DNA fragment encoding residues 138−226 of human Hrs-1 (*18*) from a HeLa cell cDNA library. Unique *Bam*HI and *Bgl*II restriction sites were introduced by PCR at the termini of the fragment. Similarly, DNA encoding the FYVE domain (residues 714−795) from FGD1, the Cdc42 guanine nucleotide exchange factor responsible for faciogenital dysplasia (*19*), was amplified by PCR from a WI-38 human lung fibroblast cDNA library. In this case, a unique *Bam*HI site was introduced at the end encoding the N-terminus of the FYVE domain, and an *Eco*RI site was introduced at the opposite end. The FYVE domain fragments were ligated into the *Bam*HI site (Hrs-1) or between the *Bam*HI and *Eco*RI sites (FGD1) of pGEX-2TK (Amersham-Pharmacia), and the sequences of inserts were confirmed by automated dideoxynucleotide sequencing. For expression of His-tagged GST-fusion proteins, fragments encoding residues 715−807 of FGD1 and residues 138−221 of Hrs-1, followed in each case by six histidines, were subcloned into *Bam*HI and *Bam*HI/*Eco*RI digested pGEX-2T, respectively.

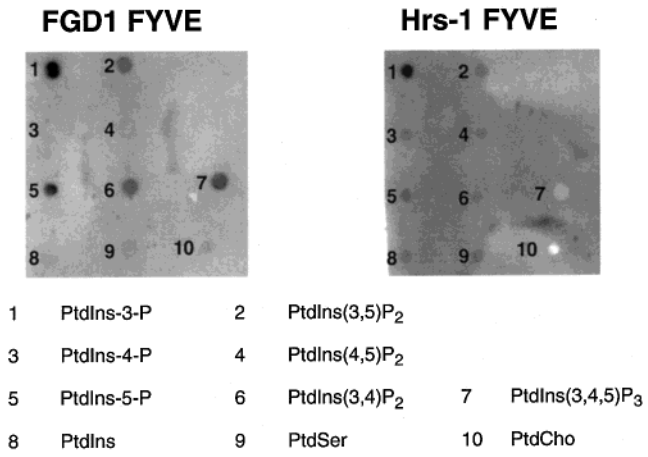

FIGURE 1: Visualization of FYVE domain phosphoinositide-binding specificity using a dot-blot procedure. Ten 1 μL spots of phospholipid were spotted onto nitrocellulose in the pattern listed in the key (see Experimental Procedures), and filters were probed with ^{32}P-labeled GST-fusion proteins of the FGD1 or Hrs-1 FYVE domains. Bound radioactivity was visualized using a Phosphorimager.

For GST-fusion protein purification, cells were lysed by sonication in 50 mM Tris-HCl, pH 7.5, and 100 mM NaCl, containing 10% glycerol, 1 mM PMSF, and 2.5 mM β-mercaptoethanol. Protein was bound to glutathione−agarose (Sigma) for 20 min at 4 °C. Beads were washed four times with lysis buffer, and protein was eluted with 15 mM reduced glutathione in lysis buffer. Glutathione was removed by size-exclusion chromatography or dialysis. For preparation of monomeric FYVE domains, GST-fusion proteins were washed three times while bound to glutathione agarose with 20 mM Tris-HCl, pH 8.0, and 150 mM NaCl, containing 20% (w/v) glycerol and 2.5 mM $CaCl_2$, and were subsequently eluted in this buffer containing 15 mM reduced glutathione. Two units of thrombin was then added per milligram of protein, and digestion was allowed to proceed at room temperature for 5 h. The cleaved protein was dialyzed overnight at 4 °C against 25 mM HEPES, pH 8.0, containing 100 mM NaCl and 1 mM β-mercaptoethanol (HBS) with 3500 MWCO tubing. Protein was then loaded onto a Ni−NTA column (Qiagen), washed with HBS containing 75 mM imidazole, and eluted with 150 mM imidazole and 300 mM imidazole in HBS. Finally, the FYVE domain was gel filtered using a Superose 12 column (Amersham-Pharmacia) in HBS containing no β-mercaptoethanol.

Dot-Blot Assay. Purified GST-fusion proteins (10 μg) produced using pGEX-2TK clones were labeled with ^{32}P exactly as described (*20*−*22*), while bound to glutathione−agarose, and were then eluted with glutathione for use in dot-blot assays.

As described (*15*, *22*), phospholipids at 2 mg/mL in 1:1 chloroform:methanol solution (containing 0.1% HCl) were spotted (1 μL) onto nitrocellulose sheets in the pattern shown in Figure 1. After being dried, the nitrocellulose was blocked overnight at 4 °C in Tris-buffered saline (TBS) plus 3% BSA (without detergent). ^{32}P-Labeled fusion protein at 0.7 μg/mL in TBS/3% BSA was then used to probe the phosphoinositide-containing nitrocellulose for 30 min at room temperature. Filters were washed five times with TBS (without detergent) and air-dried, and bound radioactivity was visual-

ized using a Phosphorimager (Molecular Dynamics).

Preparation of Small Unilamellar Vesicles (SUV's). Limit-sonicated small unilamellar vesicles (SUV's) were generated exactly as described (*15*, *17*, *22*). Di(dibromostearoyl)-phosphatidylcholine (PtdCho) was codissolved with the phosphoinositide of interest in 1:1 chloroform:methanol containing 0.1% HCl, to give a molar ratio of 97:3 (PtdCho: $PtdInsP_n$). The lipid mixture was then dried under a stream of nitrogen, followed by exposure to high vacuum. Dried lipids were hydrated by addition of assay buffer (25 mM HEPES, pH 7.2, 100 mM NaCl) to generate a suspension containing 25 mM total phospholipid, and the pH of this mixture was checked (and corrected if necessary). SUV's were then generated by multiple cycles (more than 20) of freezing (in liquid nitrogen) and thawing, with extensive bath sonication at 45 °C, until the suspensions became optically clear, indicating that small vesicles had been formed. Using $PtdIns(4,5)P_2$-containing vesicles generated with this approach, we previously showed that 57% of the $PtdIns(4,5)P_2$ is accessible on the outer surface of the vesicles for PH domain binding (*17*).

Centrifugation Assays for Lipid Vesicle Binding. Binding of proteins to lipid vesicles was assessed (in assay buffer as defined above) by their ability to pellet with vesicles of defined composition upon ultracentrifugation. Since SUV's will ordinarily not pellet in an ultracentrifuge, we used PtdCho that contains dibrominated acyl chains as our "background" lipid, to increase the vesicle density and allow efficient pelleting (*23*). A centrifugation assay for binding of the FYVE domain to a given lipid involved preparation of 80 μL samples in seven tubes, each containing a different concentration of phospholipid but a constant concentration (10 μM) of FYVE domain. The lipid concentrations chosen ranged from 0 to 2.5 mM total available lipid (estimated as 50% of the total lipid), corresponding to 0−75 μM phosphoinositide (present at only 3 mole percent). Following mixing of vesicles and protein, the samples were centrifuged for 1 h at 25 °C at 85 000 rpm in a Beckman Optima TLX ultracentrifuge, using a TLA-120.1 rotor. Sixty microliters of the supernatant was then taken and assayed for protein content. After the remaining supernatant was discarded, the vesicle pellet was resuspended in 80 μL of assay buffer by bath sonication, and 60 μL was taken for protein assay. Protein assays employed the Pierce BCA assay, as directed by the manufacturers. For protein assays using resuspended vesicles, SDS (1%) was added after incubation with the detection reagent in order to remove scattering artifacts when measuring absorbance with a spectrophotometer. A standard curve for each protein was generated in tandem, using the Pierce BCA assay, and the percentage of protein added in the assay that bound to the lipid vesicles (and therefore pelleted) was determined as the average suggested by assay of pellet and supernatant. Molar partition coefficients (*K*), as defined (*24*), were then estimated by fitting the data (in ORIGIN) to the equation:

$$\%\ \text{protein bound} = 100\left(\frac{K[\text{lipid}]}{1 + K[\text{lipid}]}\right)$$

where [lipid] is the concentration of available lipid ($\gg$[protein]$_{bound}$), approximated by one-half the total lipid concentration (assuming 50% is available on the SUV outer leaflet), and *K* is a partition coefficient that corresponds to the proportionality constant between the concentration of protein bound to the outer SUV leaflet and its concentration in bulk solution. Determination of *K* makes no assumptions of stoichiometry, although K_D for phosphoinositide binding can be estimated as (mole ratio)/*K* if 1:1 binding of phosphoinositides is assumed.

Competition for Vesicle Binding by Lipid Headgroups. To compare binding of the headgroups of PtdIns-3-P ($Ins(1,3)P_2$) and $PtdIns(4,5)P_2$ ($Ins(1,4,5)P_3$) to FYVE and PH domains with the binding of the corresponding intact lipids in a PtdCho vesicle background, competition experiments were employed. Competition experiments for the PLC-δ_1 PH domain were performed with 5 μM protein and with a constant (effective) $PtdIns(4,5)P_2$ concentration of 15 μM ([total lipid]$_{available}$ = 0.5 mM), while FYVE domain experiments employed 10 μM FYVE domain and an effective PtdIns-3-P concentration of 28 μM ([total lipid]$_{available}$ = 0.93 mM). Lipid vesicles were preincubated with increasing concentrations of free headgroup or di-C_4-PtdIns-3-P (up to 1 mM), and protein was added last. The sample was then mixed, and residual vesicle binding was assayed as described above.

Analytical Ultracentrifugation. Sedimentation equilibrium experiments employed an XL-A analytical ultracentrifuge (Beckman). Samples were loaded into six-channel Epon charcoal-filled centerpieces, using quartz windows. Experiments were performed at 25 °C using two different speeds (18 000 and 23 500 rpm), detecting at 280 or 300 nm, with identical results. Solvent density was taken as 1.0037 g/mL, and the partial specific volume of the FYVE domain was estimated from its amino acid composition as 0.714 mL g^{-1}. Experiments were performed with the FYVE domain at 58 μM (pET-derived protein) and 63 μM (cleaved from GST fusion). Data were fit using the Optima XL-A data analysis software (Beckman/MicroCal).

RESULTS

To investigate specific PtdIns-3-P recognition by mammalian FYVE domains, we began by using a "dot-blot" lipid-interaction assay (*15*) to compare binding of the isolated FYVE domains from Hrs-1 and FGD1 (as ^{32}P-labeled GST-fusion proteins) to a series of phosphoinositides and other cellular phospholipids. As expected (*5*−*7*), both the Hrs-1 and FGD1 FYVE domains gave a significant signal with PtdIns-3-P in this assay (Figure 1). For the Hrs-1 FYVE domain, no significant signal was detected for any other lipid tested, indicating remarkable specificity for PtdIns-3-P that matches the highest degree of specificity seen with PH domains using this and similar assays (*15*, *25*). The FGD1 FYVE domain appeared to be slightly less selective and also showed significant binding to PtdIns-5-P, as well as weak signals with $PtdIns(3,4)P_2$, $PtdIns(3,5)P_2$, and $PtdIns(3,4,5)P_3$.

To compare the PtdIns-3-P binding affinity of a FYVE domain with values measured for PH domains that bind phosphoinositides specifically, we employed a centrifugation-based vesicle-binding assay that we have used for our previous PH domain studies (*15*, *22*) (see Experimental Procedures). Purified GST-FYVE fusion proteins bound strongly to vesicles containing PtdIns-3-P but showed a

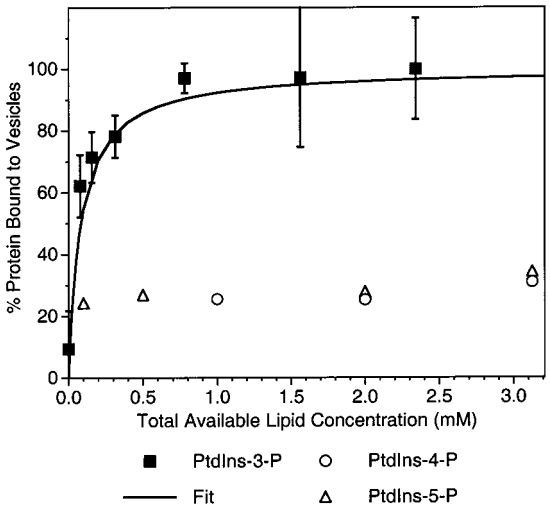

FIGURE 2: Vesicle binding by the Hrs-1 FYVE domain. Binding of the monomeric Hrs-1 FYVE domain (present at 10 μM in assay samples) to vesicles containing di(dibromostearoyl)phosphatidylcholine with 3 mole percent PtdIns-3-P (■), PtdIns-4-P (○), or PtdIns-5-P (△), measured using the centrifugation assay described in Experimental Procedures. Data are plotted as the percent protein bound to pelleted vesicles against the total *available* lipid concentration, estimated as one-half of the total lipid concentration. Effective phosphoinositide concentrations are 3% of the millimolar value plotted on the horizontal axis. Data for PtdIns-3-P binding represent the mean of three experiments (±standard deviation). The fit of the PtdIns-3-P data to the binding function described in Experimental Procedures yields a partition coefficient (*K*) of 12 020 M^{-1}, which would correspond to a K_D of 2.5 μM if 1:1 PtdIns-3-P binding by the FYVE domain were assumed.

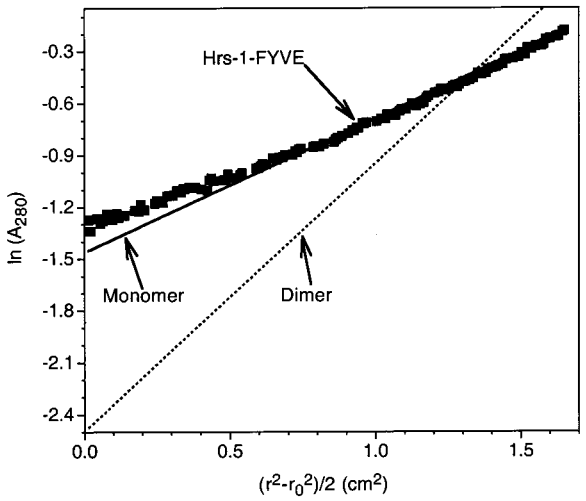

FIGURE 3: Hrs-1 FYVE domain is monomeric. Sedimentation equilibrium analytical ultracentrifugation studies show that the Hrs-1 FYVE domain produced by cleavage from a GST-fusion protein sediments as a monomeric species. A plot is shown of the logarithm of the absorbance at 280 nM [ln(A_{280})] against the square of the radius $(r^2 - r_0^2)/2$, where r is the radial position in the sample and r_0 is the radial position of the meniscus. For an ideal single species, this plot is linear and the gradient of the line is proportional to the molecular mass of the species. Idealized results are plotted for the monomeric FYVE domain (solid line) and a dimeric FYVE domain (broken line). The data collected for a 23 500 rpm spin at 25 °C of the Hrs-1 FYVE domain produced by cleavage from a GST-fusion protein coincide very closely with the expected data for a monomer and give a monomeric molecular mass in data fitting (see Results).

tendency to precipitate that prevented quantitative interpretation (data not shown). By contrast, isolated FYVE domains expressed from pET vectors without fusion to GST did not bind PtdIns-3-P-containing vesicles. Since fusion to GST induces dimerization (*26*), we hypothesized on the basis of these initial observations that FYVE domains require dimerization in order to bind PtdIns-3-P in lipid vesicles. We previously found that the dynamin PH domain only binds phosphoinositides when dimeric (*22*). Moreover, Kutateladze et al. have reported that the EEA1 FYVE domain binds much more strongly to PtdIns-3-P-containing vesicles when fused to GST than when not tagged (*14*).

To test directly the hypothesis that dimerization of FYVE domains is required for their phosphoinositide binding, we analyzed a form of the Hrs-1 FYVE domain that was initially produced as a GST-fusion protein but from which GST had been removed proteolytically. A GST/Hrs-1 FYVE fusion protein with a C-terminal hexahistidine tag was purified as described in Experimental Procedures. Following cleavage with thrombin, the isolated FYVE domain was separated from GST by metal-affinity chromatography and size-exclusion chromatography and was analyzed for PtdIns-3-P binding using the centrifugation-based vesicle-binding assay. As shown in Figure 2, this protein pelleted efficiently with phosphatidylcholine (PtdCho) vesicles containing 3 mole percent PtdIns-3-P but not with similar vesicles containing PtdIns-4-P or PtdIns-5-P. Thus, fusion to GST is not required for strong binding of the Hrs-1 FYVE domain to PtdIns-3-P.

As mentioned above, when expressed from pET11a without fusion to GST, the Hrs-1 FYVE domain failed to bind any of phospholipids tested, despite differing from the GST-fusion-derived FYVE domain only in lacking the C-terminal hexahistidine tag (which does not influence PtdIns-3-P binding by the GST fusion). One possible explanation for this difference in binding was that the FYVE domain cleaved from the GST-fusion protein actually exists as a dimer—perhaps in a metastable folded state. This phenomenon has been observed with the N-terminal domain of CD2, which occurs as a dimer only when produced by cleavage from a GST-fusion protein (*27*). To test this possibility and to rule out possible aggregation of the cleaved Hrs-1 FYVE domain, we used analytical ultracentrifugation sedimentation equilibrium to measure the molecular mass of the protein. When loaded in the centrifuge cell at 63 μM, the cleaved FYVE domain sedimented as a single 10 464 Da species, while the pET11a-derived Hrs-1 FYVE domain (loaded at 58 μM) behaved as a 9213 Da species (this protein lacks the hexahistidine tag). As shown in Figure 3, the Hrs-1 FYVE domain produced by cleavage from a GST-fusion protein clearly sediments as a monomeric (expected molecular mass = 10 980 Da), and not a dimeric, species. Dimerization of the Hrs-1 FYVE domain in solution is therefore not a prerequisite for its binding to PtdIns-3-P in vesicles.

From these findings it can be argued that a monomeric form of the Hrs-1 FYVE domain binds PtdIns-3-P-containing vesicles with substantial affinity. The failure of protein produced from pET11a constructs to bind PtdIns-3-P may result from misfolding or incomplete charging with Zn^{2+}, but we have not investigated this issue any further. For binding of the GST-fusion-derived monomeric Hrs-1 FYVE domain to PtdIns-3-P in small unilamellar vesicles (SUV's), the molar partition coefficient (see Experimental Procedures) estimated by curve fitting (Figure 2) is 12 020 M^{-1}. If a

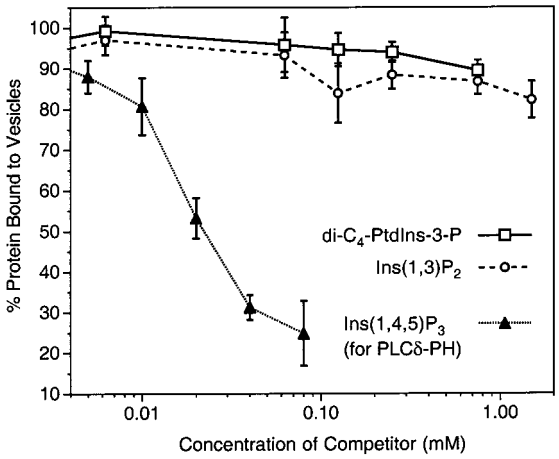

FIGURE 4: Headgroup competition assays. Binding of the Hrs-1 FYVE domain (present at 10 μM) to vesicles containing PtdIns-3-P (with $[PtdIns\text{-}3\text{-}P]_{effective}$ of 28 μM) is completely unaffected by addition of Ins(1,3)P_2 to concentrations of up to 1.5 mM or by addition of soluble dibutanoyl-PtdIns-3-P to concentrations of 800 μM. By contrast, when binding of the PLC-δ_1 PH domain (at 5 μM) is studied to vesicles containing PtdIns(4,5)P_2 ($[PtdIns(4,5)P_2]_{effective} = 15$ μM), the headgroup of the lipid [Ins(1,4,5)P_3] is a very effective competitor for vesicle binding. Addition of Ins(1,4,5)P_3 to just 75 μM is sufficient for almost complete disruption of PLCδ-PH binding to PtdIns(4,5)P_2-containing membranes. Data for the PLCδ-PH competition are from Kavran et al. (*15*).

stoichiometry of 1 PtdIns-3-P per FYVE domain molecule is assumed, this corresponds to an apparent K_D of approximately 2.5 μM for PtdIns-3-P binding. This K_D value is comparable with the K_D measured by isothermal titration calorimetry for PtdIns(4,5)P_2 binding by the PH domain from PLC-δ_1 ($\approx$2 μM) (*17*) but is actually around 4-fold stronger than the PLCδ-PH/PtdIns(4,5)P_2 interaction studied using precisely the same centrifugation-based vesicle binding assay (*15*). Thus, binding of the monomeric FYVE domain from Hrs-1 to lipid vesicles containing PtdIns-3-P is similar in strength, if not stronger, than binding of monomeric PLCδ-PH to vesicles containing PtdIns(4,5)P_2.

Membrane binding by PLCδ-PH and other phosphoinositide-specific PH domains is dominated by high-affinity recognition of the inositol phosphate headgroup (*16*). For example, PLCδ-PH binds nearly 10-fold more strongly to the isolated headgroup Ins(1,4,5)P_3 than to PtdIns(4,5)P_2 (*17*), and the PH domains from Grp1 and DAPP1 show similar characteristics (*15*). Indeed, since the headgroup is the only region in which different phosphoinositides are distinct, it is expected to be the primary site of interaction with domains that recognize specific phosphoinositides. However, for the Hrs-1 and FGD1 FYVE domains, we were unable in preliminary studies to detect significant binding to ^{3}H-labeled Ins(1,3)P_2 (not shown). Competition binding studies presented in Figure 4 highlight a critical difference between the mode of phosphoinositide recognition by the Hrs-1 FYVE domain and the PLC-δ_1 PH domain. Ins(1,4,5)P_3 is a very effective inhibitor of PLCδ-PH binding to vesicles containing 3% PtdIns(4,5)P_2. When present at a concentration of 75 μM, Ins(1,4,5)P_3 completely prevents binding of PLCδ-PH (at 5 μM) to PtdIns(4,5)P_2-containing vesicles (with an IC_{50} of approximately 20 μM). By contrast, even at a concentration of 1500 μM, Ins(1,3)P_2 has no detectable effect on binding of the monomeric Hrs-1 FYVE domain (at 10 μM) to PtdIns-3-P-containing vesicles. Since the Hrs-1 FYVE domain used here does not dimerize at concentrations 10-fold higher than used for this experiment, the explanation for the failure of Ins(1,3)P_2 to compete for vesicle binding cannot arise from a simple avidity effect (see Discussion). One possible explanation for the failure of this competition is that interactions of the FYVE domain with the glyceryl ester backbone of PtdIns-3-P are required. This simple explanation was excluded by the inability of freely soluble dibutanoyl-PtdIns-3-P (with short acyl chains) at concentrations up to 800 μM to compete with the FYVE domain from vesicles containing dihexadecanoyl-PtdIns-3-P (Figure 4).

DISCUSSION

The experiments presented here allow direct comparison of specific phosphoinositide binding by a FYVE domain and a well-studied PH domain (from PLC-δ_1). Dot blots and vesicle-binding studies indicate that the selectivity of the Hrs-1 FYVE domain for PtdIns-3-P over other phosphoinositides is as great as, if not greater than, that shown by PLCδ-PH for its target, PtdIns(4,5)P_2. While binding of Hrs-1 FYVE to PtdCho vesicles containing 3% PtdIns-4-P or PtdIns-5-P is virtually undetectable compared with PtdIns-3-P binding (Figure 2), PLCδ-PH shows only an approximately 5−6-fold preference for PtdIns(4,5)P_2 over PtdIns-4-P (*17*, *28*) or PtdIns(3,4)P_2 (*28*) in PtdCho vesicles. While these observations indicate that the Hrs-1 FYVE domain is more selective in its phosphoinositide recognition than PLCδ-PH, we find surprisingly that PtdIns-3-P binding by the FYVE domain is recapitulated far less well by the isolated lipid headgroup than is the case for PLCδ-PH binding to PtdIns(4,5)P_2 and for other specific PH domain interactions. There are several possible explanations for this difference, as outlined below.

Our initial hypothesis was that Hrs-1 FYVE domain binding to PtdIns-3-P in vesicles is multivalent. Univalent Ins(1,3)P_2 or dibutanoyl-PtdIns-3-P (di-C_4-PtdIns-3-P) would compete very inefficiently with PtdIns-3-P in vesicles for binding to a species with multiple binding sites for the lipid headgroup, since the energies of individual binding sites would add to create a high-avidity interaction with the vesicle surface. If this hypothesis is correct, the individual FYVE domains could contain multiple PtdIns-3-P binding sites, which seems unlikely for such a small protein module, or they could oligomerize to generate the avidity effect. Analytical ultracentrifugation studies show that the Hrs-1 FYVE domain remains monomeric at concentrations at least 6-fold higher than those used for the vesicle binding studies reported here (Figure 3). Thus, by contrast with the case for the PH domains from dynamin-1 and dynamin-2 (*22*), there is no requirement for strong FYVE domain oligomerization in order for the Hrs-1 FYVE domain to bind phosphoinositide-containing vesicles. From NMR analysis of translational diffusion coefficients, Kutateladze et al. (*14*) observed that the FYVE domain from EEA1 begins to oligomerize at concentrations above 0.2 mM. A weak tendency to oligomerize when free in solution would translate into a much stronger tendency to self-associate when FYVE domains are concentrated and restricted to two dimensions at a membrane surface (*29*). Under these circumstances, weak monovalent PtdIns-3-P binding and weak self-association could cooperate to generate an effective high-avidity interaction, with which univalent Ins(1,3)P_2 or di-C_4-PtdIns-3-P

would not compete efficiently. Furthermore, it is possible that conformational changes, indicated by the NMR chemical shift perturbations seen upon binding of di-C_4-PtdIns-3-P binding to the EEA1 FYVE domain (*14*), lead to stabilization of an oligomeric form of the FYVE domain only at the membrane surface.

In addition to this possible contribution to membrane association, it has been suggested that the FYVE domain penetrates the hydrophobic region of the lipid bilayer and that this may add to the strength of PtdIns-3-P binding. Certainly, this contribution would only be relevant for binding to PtdIns-3-P in vesicles and not for association with di-C_4-PtdIns-3-P or Ins(1,3)P_2. As such, surface penetration could provide at least part of the explanation for the failure of the univalent, soluble, ligands to compete for vesicle binding. The X-ray crystal structures of the Vps27p and Hrs-1 FYVE domains revealed an exposed hydrophobic patch, immediately preceding the R(R/K)HHCRxCG signature motif in the FYVE domain sequence (*12*, *13*). This patch lies at the "tip" of the FYVE domain, and its hydrophobic nature is well conserved among FYVE domains. Resonances from residues in this region of the EEA1 FYVE domain disappeared in NMR spectra when micellar (but not soluble) PtdIns-3-P was added to the sample, supporting the suggestion that they are surface active (*14*). The structurally related C1b domain from PKCδ has a region similar to the hydrophobic protrusion of the FYVE domain (*30*), and monolayer studies of the PKCα C1 domain showed that it does penetrate the membrane (*31*), most likely using this hydrophobic protrusion. Finally, while this paper was in review, Kutateladze et al. reported an NMR determination of the EEA1 FYVE domain structure in complex with di-C_4-PtdIns-3-P, as well as observations of its interactions with PtdIns-3-P-containing mixed micelles (*32*). Evidence for conformational changes upon binding to di-C_4-PtdIns-3-P was seen but none that appeared to alter the oligomeric state of the domain. More importantly, the region immediately preceding the FYVE domain signature motif was shown to penetrate the surface upon binding of the EEA1 FYVE domain to mixed micelles containing PtdIns-3-P. This study therefore argues that polar interactions with the PtdIns-3-P headgroup and hydrophobic interactions with the membrane interior cooperate to drive high-affinity binding of the FYVE domain to PtdIns-3-P-containing membranes. According to this model of Kutateladze et al. (*32*), neither di-C_4-PtdIns-3-P nor PtdCho bilayers should be able to compete the FYVE domain away from PtdIns-3-P in PtdCho bilayers, although either component alone can bind weakly to the FYVE domain.

Thus, current knowledge suggests that the ability of FYVE domains to penetrate the membrane surface is likely to explain the difference between FYVE and PH domains in the extent to which isolated inositol phosphates can recapitulate specific phosphoinositide recognition. No evidence has been presented for membrane penetration by PH domains, and studies with the Grp1, PLC-δ_1, and TAPP1 PH domains (*15*) (data not shown) show that vesicle binding by these PH domains has no avidity advantage over their binding to univalent headgroups.

Requirements for PtdIns-3-P Binding in Vivo. A K_D for PtdIns(4,5)P_2 binding of approximately 1.7 μM is sufficient to drive substantial membrane targeting in vivo of the monomeric PH domain from PLC-δ_1 (*17*, *28*, *33*−*35*). However, a K_D for PtdIns-3-P binding of 2.5 μM (Figure 2) is not sufficient for significant membrane targeting of a monomeric FYVE domain. Gillooly et al. found that the isolated Hrs-1 FYVE domain is predominantly cytoplasmic when expressed in mammalian cells (*8*). Similarly, Lawe et al. found that fragments of EEA1 are targeted to endosomes in vivo only if they can bind to both PtdIns-3-P and Rab5 (*36*). The fact that a K_D of approximately 2 μM is sufficient for membrane targeting of the PLC-δ_1 PH domain, but not for FYVE domains, is likely to reflect the simple fact that cellular levels of PtdIns(4,5)P_2 exceed those of PtdIns-3-P by 20−25-fold (*37*). There is therefore no reason to expect that a K_D strong enough for PtdIns(4,5)P_2 targeting in vivo will be sufficient for PtdIns-3-P targeting.

Cellular PtdIns-3-P levels are roughly comparable with levels of PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 seen in agonist-treated cells (*37*). PH domains that can be recruited (as monomers) by these transiently occurring 3-phosphoinositides (the PH domains from Grp1, Btk, and DAPP1, for example) bind to them with K_D values between 30 and 50 nM (*16*). A K_D in this range should therefore also suffice for targeting of FYVE domains to endosomes. While the isolated Hrs-1 FYVE domain is predominantly cytosolic when expressed in BHK cells, Gillooly et al. showed that a "double FYVE" (2 × FYVE), in which two Hrs-1 FYVE domains are present in a single protein molecule, is targeted very efficiently to endosomes (*8*). The effective K_D for binding of a tandem pair of FYVE domains to a PtdIns-3-P-containing membrane is predicted to range from perhaps 0.01 nM to 10's of nanomolar, depending on how well the two FYVE domains cooperate (based on the results shown in Figure 2). Surface plasmon resonance (SPR) studies (*8*) suggested a K_D of 38 nM for binding of a GST-FYVE-fusion protein to membranes containing PtdIns-3-P [the use of a dimeric GST-fusion protein in these studies leads to an overestimate of the monomer affinity (*38*) and may represent a reasonable estimate of binding by 2 × FYVE]. With a K_D of 38 nM or less, 2 × FYVE is a very effective PtdIns-3-P probe (*8*), suggesting that Hrs-1 ordinarily achieves its endosomal localization through oligomerization or with the additional involvement of other, unrelated, interactions (*10*).

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REFERENCES

1. Stenmark, H., and Aasland, R. (1999) *J. Cell Sci. 112*, 4175−4183.
2. Stenmark, H., Aasland, R., Toh, B. H., and D'Arrigo, A. (1996) *J. Biol. Chem. 271*, 24048−24054.
3. Hurley, J. H., and Misra, S. (2000) *Annu. Rev. Biophys. Biomol. Struct. 29*, 49−79.
4. Patki, V., Virbasius, J., Lane, W. S., Toh, B. H., Shpetner, H. S., and Corvera, S. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 7326−7330.
5. Burd, C. G., and Emr, S. D. (1998) *Mol. Cell 2*, 157−162.
6. Gaullier, J. M., Simonsen, A., D'Arrigo, A., Bremnes, B., Stenmark, H., and Aasland, R. (1998) *Nature 394*, 432−433.
7. Patki, V., Lawe, D. C., Corvera, S., Virbasius, J. V., and Chawla, A. (1998) *Nature 394*, 433−434.

8. Gillooly, D. J., Morrow, I. C., Lindsay, M., Gould, R., Bryant, N. J., Gaullier, J. M., Parton, R. G., and Stenmark, H. (2000) *EMBO J. 19*, 4577−4588.
9. Odorizzi, G., Babst, M., and Emr, S. D. (2000) *Trends Biochem. Sci. 25*, 229−235.
10. Komada, M., and Kitamura, N. (1997) *J. Biol. Chem. 272*, 20538−20544.
11. Urbé, S., Mills, I. G., Stenmark, H., Kitamura, N., and Clague, M. J. (2000) *Mol. Cell. Biol. 20*, 7685−7692.
12. Misra, S., and Hurley, J. H. (1999) *Cell 97*, 657−666.
13. Mao, Y., Nickitenko, A., Duan, X., Lloyd, T. E., Wu, M. N., Bellen, H., and Quiocho, F. A. (2000) *Cell 100*, 447−456.
14. Kutateladze, T. G., Ogburn, K. D., Watson, W. T., de Beer, T., Emr, S. D., Burd, C. G., and Overduin, M. (1999) *Mol. Cell 3*, 805−811.
15. Kavran, J. M., Klein, D. E., Lee, A., Falasca, M., Isakoff, S. J., Skolnik, E. Y., and Lemmon, M. A. (1998) *J. Biol. Chem. 273*, 30497−30508.
16. Lemmon, M. A., and Ferguson, K. M. (2000) *Biochem. J. 350*, 1−18.
17. Lemmon, M. A., Ferguson, K. M., O'Brien, R., Sigler, P. B., and Schlessinger, J. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 10472−10476.
18. Asao, H., Sasaki, Y., Arita, T., Tanaka, N., Endo, K., and Kasai, H. (1997) *J. Biol. Chem. 272*, 32785−32791.
19. Pasteris, G. N., Cadle, A., Logie, L. J., Porteous, M. E. M., Schwartz, C. E., Stevenson, R. E., Glover, T. W., Wilroy, R. S., and Gorski, J. L. (1994) *Cell 79*, 669−678.
20. Ron, D., and Dressler, H. (1992) *BioTechniques 13*, 866−869.
21. Margolis, B., and Young, R. A. (1995) in *DNA Cloning 2* (Glover, D. M., and Hames, B. D., Eds.) pp 1−14, IRL Press, Oxford, U.K.
22. Klein, D. E., Lee, A., Frank, D. W., Marks, M. S., and Lemmon, M. A. (1998) *J. Biol. Chem. 273*, 27725−27733.
23. Tortorella, D., and London, E. (1994) *Anal. Biochem. 217*, 176−180.
24. Peitzsch, R. M., and McLaughlin, S. (1993) *Biochemistry 32*, 10436−10443.
25. Dowler, S., Currie, R. A., Campbell, D. G., Deak, M., Kular, G., Downes, C. P., and Alessi, D. R. (2000) *Biochem. J. 351*, 19−31.
26. McTigue, M. A., Williams, D. R., and Tainer, J. A. (1995) *J. Mol. Biol. 246*, 21−27.
27. Murray, A. J., Lewis, S. J., Barclay, A. N., and Brady, R. L. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 7337−7341.
28. Garcia, P., Gupta, R., Shah, S., Morris, A. J., Rudge, S. A., Scarlata, S., Petrova, V., McLaughlin, S., and Rebecchi, M. J. (1995) *Biochemistry 34*, 16228−16234.
29. Grasberger, B., Minton, A. P., DeLisi, C., and Metzger, H. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 6258−6262.
30. Zhang, G., Kazanietz, M. G., Blumberg, P. M., and Hurley, J. H. (1995) *Cell 81*, 917−924.
31. Medkova, M., and Cho, W. (1999) *J. Biol. Chem. 274*, 19852−19861.
32. Kutateladze, T., and Overduin, M. (2001) *Science 291*, 1793−1796.
33. Hirose, K., Kadowaki, S., Tanabe, M., Takeshima, H., and Iino, M. (1999) *Science 284*, 1527−1530.
34. Stauffer, T. P., Ahn, S., and Meyer, T. (1998) *Curr. Biol. 8*, 343−346.
35. Varnai, P., and Balla, T. (1998) *J. Cell Biol. 143*, 501−510.
36. Lawe, D. C., Patki, V., Heller-Harrison, R., Lambright, D. G., and Corvera, S. (2000) *J. Biol. Chem. 275*, 3699−3705.
37. Stephens, L. R., Jackson, T. R., and Hawkins, P. T. (1993) *Biochim. Biophys. Acta 1179*, 27−75.
38. Ladbury, J. E., Lemmon, M. A., Zhou, M., Green, J., Botfield, M., and Schlessinger, J. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 3199−3203.

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