Distinct Polyphosphoinositide Binding Selectivities for Pleckstrin Homology Domains of GRP1-like Proteins Based on Diglycine Versus Triglycine Motifs*

Jes K. Klarlund‡, William Tsiaras‡, John J. Holik, Anil Chawla, and Michael P. Czech§

From the Program in Molecular Medicine and Department of Biochemistry and Molecular Biology, University of Massachusetts Medical School, Worcester, Massachusetts 01655

GRP1 and the related proteins ARNO and cytohesin-1 are ARF exchange factors that contain a pleckstrin homology (PH) domain thought to target these proteins to cell membranes through binding polyphosphoinositides. Here we show the PH domains of all three proteins exhibit relatively high affinity for dioctanoyl phosphatidylinositol 3,4,5-triphosphate (PtdIns(3,4,5)P_3), with K_D values of 0.05, 1.6 and 1.0 μM for GRP1, ARNO, and cytohesin-1, respectively. However, the GRP1 PH domain was unique among these proteins in its striking selectivity for PtdIns(3,4,5)P_3 versus phosphatidylinositol 4,5-diphosphate (PtdIns(4,5)P_2), for which it exhibits about 650-fold lower apparent affinity. Addition of a glycine to the Gly_274-Gly_275 motif in GRP1 greatly increased its 650-fold lower apparent affinity. Addition of a glycine to the Hemagglutinin epitope-tagged PH domain of cytohesin-1 was not. These data indicate that the unique diglycine motif in the ARNO PH domain markedly reduced its binding affinity for PtdIns(4,5)P_2 but not for PtdIns(3,4,5)P_3. In intact cells, the hemagglutinin epitope-tagged PH domain of GRP1 was recruited to ruffles in the cell surface in response to insulin, as were full-length GRP1 and cytohesin-1, but the PH domain of cytohesin-1 was not. These data indicate that the unique diglycine motif in the GRP1 PH domain, as opposed to the triglycerine in ARNO and cytohesin-1, directs its remarkable PtdIns(3,4,5)P_3 binding selectivity.

Cell signaling processes are often initiated by the recruitment of protein complexes to the cytoplasmic face of the plasma membrane, where they act to elicit signaling events. Specialized regions or domains within such signaling proteins function as adapters in the recruitment process, linking these proteins to chemical motifs generated by receptor activation. For example, specific membrane-bound protein phosphotyrosine sites appear in response to activation of transmembrane receptor tyrosine kinases by growth factors and other stimuli, attracting Src homology (SH) domains within proteins that bind these sites (1). The proteins that contain SH2 domains are enzymes, regulator proteins, or simply adapters themselves that connect other proteins to the localized protein phosphotyrosines. This paradigm has been extended to a large number of protein domains and their respective ligands, and provides for an effective means of mobilizing cellular signaling machines (2).

A particularly interesting membrane localization domain that has been identified in over 100 proteins is the PH domain, which spans approximately 120 residues and contains an invariant tryptophan in its COOH-terminal region (3). Several PH domain structures have been solved by NMR and by x-ray crystallography, giving rise to the concept that their overall protein fold is formed from seven β sheets with connecting loops that form a ligand binding scaffold (4–10). The PH domain fold is similar to that of several other ligand binding protein domains that differ substantially in amino acid sequence, and has therefore been denoted as the prototype of a superfamily or superfold (11). The major class of ligands that bind most PH domains are the polyphosphoinositides (12–15), although some PH domains do not appear to bind these lipids (11). Other ligands have been reported to bind some PH domains as well, including βγ subunits of trimeric G proteins (16), actin (17), and protein kinase C (18). Results from the several PH domain structures that have been solved in association with a phosphoinositide headgroup show that the ligand binding site can vary in location. Thus, the spectrin and phospholipase C delta PH domains both bind inositol 1,4,5-triphosphate, but in different regions flanking the β3/β2 loop (4, 5).

The polyphosphoinositides represent diverse membrane targeting sites for PH domains because they include PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3, which appear upon cell stimulation by growth factors and other regulators of PI 3-kinases, as well as PtdIns(4,5)P_2, which is present in cells constitutively at relatively high abundance (19). Recent studies have suggested that many PH domains appear to show little or only modest ability to discriminate among the polyphosphoinositides with different binding affinities, while others show striking binding affinity preferences (15). Examples of the latter include the PH domain of phospholipase Cδ, which shows high selectivity for PtdIns(4,5)P_3, and the PH domain of the ARF guanine nucleotide exchange factor GRP1, which binds PtdIns(3,4,5)P_3 with much higher affinity than PtdIns(3,4)P_2 or PtdIns(4,5)P_2 (15, 20, 21). Consistent with these binding characteristics, the phospholipase C delta PH domain targets to cell plasma membranes under basal conditions (22), while the GRP1 PH domain is recruited to plasma membranes only upon PI 3-kinase stimu-
lation (23–25). Another example of such regulated recruitment is the activation of the protein kinases PDK1 and Akt/protein kinase B through their membrane localization in response to generation of 3'-phosphoinositides by PI 3-kinases (26). These results reinforce the concept that a major mechanism for signaling through the PI 3-kinase products PtdIns(3,4,5)P3 and PtdIns(3,4)P2 is the recruitment of signaling proteins to cell membranes via binding of their PH domains.

Based on the above considerations, it appears that the diversity of phosphoinositide binding properties characteristic of PH domains provides multiple modes by which signaling proteins containing these domains can be localized to membranes. A plausible hypothesis is that PH domains within a family of proteins containing a second common function may act to confer divergent membrane targeting modalities to this function. The aim of the present studies was to test this hypothesis with a family of proteins related to GRP1 (20). Like GRP1, the proteins ARNO (27) and cytohesin-1 (28) contain a single PH domain and a sec7 homology region that catalyzes GTP/GDP exchange on ARF proteins. The PH domains of these three proteins show high sequence similarity, but are not identical. We found that only GRP1 among these proteins binds the PI 3-kinase product PtdIns(3,4,5)P3 with high selectivity over PtdIns(4,5)P2, and much of this difference in binding specificity could be accounted for by a single additional glycine in the NH2-terminal region of the ARNO and cytohesin-1 PH domains. The high specificity of the GRP1 PH domain for binding PtdIns(3,4,5)P3 over the more abundant PtdIns(4,5)P2 species explains the highly sensitive membrane localization response of GRP1 to PI 3-kinase activation in intact cells.

EXPERIMENTAL PROCEDURES

Generation of GST PH Fusion Proteins—GST fusion protein containing the GRP1 sequence from residue 240 and through the remaining COOH-terminal part of the molecule was generated as described (20). The analogous constructs were made of cytohesin-1 and ARNO. Site-directed mutagenesis was performed by the Stratagene QuickChange protocol. Plasmid preparations were screened for the desired mutations, and all constructs were completely confirmed by DNA sequencing.

Lipid Binding and Competition Assays—Binding assays were performed exactly as described (14). Briefly, GST/PH domain fusion proteins were bound to glutathione immobilized on agarose beads (Sigma). Synthetic [3H]C8PtdIns(3,4,5)P3 (kindly provided by C.-S. Chen, University of Kentucky) were added, incubated with constant agitation, and the beads were separated from the supernatant by centrifugation after 1 h. The amounts of [3H]C8PtdIns(3,4,5)P3 bound to the PH domains were calculated by subtracting the amount of free [3H] present in supernatants from beads bound to the GST/PH domains from the amount of free [3H] in supernatants from beads containing only GST. To calculate apparent Kd values, the data were fitted to the equation, (bound) = Bmax [free]([Kd] + [free]), by a least squares curve fit. For the competition assays, the beads containing the GST fusion proteins were incubated with 2.5 μM [3H]C8PtdIns(3,4,5)P3, in the presence of different concentrations of unlabeled lipids. After 1 h of incubation at room temperature, the beads were washed and counted in a scintillation counter. The percentage of [3H]C8PtdIns(3,4,5)P3 bound was calculated based on the amount of [3H] bound to the beads in the absence of competitor. The total [3H] bound to the GST control was less than 0.5% of the total [3H] bound to GRP1 PH domain. The data were fitted to the equation, % bound = 100 – n × L/(Kapp + L), where n is the percentage of specific binding, L is the concentration of unlabeled lipid added, and Kapp is the apparent competitive dissociation constant. The ratios of the apparent dissociation constants equal the ratios of the true dissociation constants under these experimental conditions (14).

Immunofluorescence—1 × 10^6 CHO-T cells were seeded on 18-mm coverslips in 12-well tissue culture plates. The next day, the cells were transfected with 4 μg of the indicated constructs and 0.5 μg of empty PCMV5 vector (to assure low levels of expression) using LipofectAmine (Life Technologies, Inc.) under serum-free conditions. The constructs were tagged at the COOH terminus with either the HA (YPYDVPDYA) or Myc (AEEQKLISEEDLLK) epitope tags, and they were detected using either an anti-HA epitope antiserum produced in American Type Culture Collection. Cells were stimulated with 0.1 μg insulin for 5 min or left untreated, fixed, and processed for immunofluorescence the following day as described (23).

RESULTS AND DISCUSSION

In order to characterize the polyphosphoinositide binding properties of the PH domains of GRP1 family proteins, GST fusion constructs were made for each of the GRP1, ARNO, and cytohesin-1 PH domains. Binding of each of these GST-PH domain fusion proteins to the PI 3-kinase product PtdIns(3,4,5)P3 was estimated by incubation of these proteins with various concentrations of water-soluble[3H]C8PtdIns(3,4,5)P3. As depicted in Fig. 1, each of the PH domains studied here bound the labeled 3'-polyphosphoinositide in a concentration-dependent manner. Binding of C8PtdIns(3,4,5)P3 to each of the PH domains also showed saturation kinetics, with similar stoichiometries of association at high concentrations. Assuming a single binding site for the polyphosphoinositide on each PH domain, the highest apparent affinity for the labeled C8PtdIns(3,4,5)P3 was exhibited by the GRP1 PH domain (apparent Kd = 0.05 μM). However, the PH domains of ARNO and cytohesin-1 also bound this lipid species with relatively high affinity (apparent Kd = 1.6 and 1.0 μM, respectively).

High affinity for PtdIns(3,4,5)P3 alone may not be sufficient to assure regulated membrane localization of a PH domain in response to generation of this lipid species in intact cells through the action of PI 3-kinase. This is because the abundance of PtdIns(4,5)P2, which is present constitutively in cell membranes, is much higher than the 3'-polyphosphoinositides even in stimulated cells. We thus evaluated the relative apparent affinities of the GRP1, ARNO, and cytohesin-1 PH domains for PtdIns(4,5)P2 by using this lipid as a competitive inhibitor of labeled C8PtdIns(3,4,5)P3 binding. Fig. 2 depicts this type of experiment and shows a striking difference in behavior between the GRP1 PH domain versus the PH domains of ARNO and cytohesin-1. For the latter two PH domains, C8PtdIns(4,5)P2 competed for binding of labeled C8PtdIns(3,4,5)P3 almost as well as unlabeled C8PtdIns(3,4,5)P3 itself. The concentration of PtdIns(4,5)P2 that half-maximally inhibits labeled PtdIns(3,4,5)P3 binding to these two PH domains is within a few-fold of the half-maximal inhibitory concentration measured for PtdIns(3,4,5)P3. In contrast, the GRP1 PH domain binding profile exhibits a striking difference between these lipids in respect to their ability to compete with labeled C8PtdIns-
Second, is there a functional difference in the activities of these PH domains that can be observed in intact cells, based on the high abundance of PtdIns(4,5)P$_2$ relative to PtdIns(3,4,5)P$_3$?

We addressed the structural basis of the PtdIns(4,5)P$_2$ binding affinity differences revealed in Fig. 2 by examining the PH domain amino acid sequences derived from mouse GRP1 (20), human ARNO (29) obtained from the ATCC and sequenced by us, and mouse cytohesin-1 (30). Interestingly, while there are 18 locations containing residues that differ in at least one of the three PH domains, only one of these changes occurs within the first 60 residues of the NH$_2$-terminal region (Fig. 3). This one change is a deletion of a single glycine in the GRP1 PH domain relative to the two others, and it occurs near the predicted $\beta1/\beta2$ loop region known to bind polyphosphoinositide in other PH domain structures. A mutant GRP1 PH domain cDNA was thus engineered that contains an additional glycine in position 274, converting the normal diglycine at that position to triglycine. Conversely, a mutant ARNO PH domain was engineered in which the corresponding glycine was deleted, converting the triglycine motif to diglycine. Fig. 4 (left panels) shows the consequences of these mutations in the GRP1 and ARNO PH domains with respect to binding $[^{3}H]C_{8}$PtdIns(3,4,5)P$_3$. Mutant, triglycine GRP1 PH domain exhibits a severalfold lower affinity for the labeled 3'-polyphosphoinositide compared with native GRP1 PH domain, whereas mutant, diglycine ARNO PH domain shows an enhanced affinity compared with the native construct. In the case of the mutant GRP1 PH domain, the binding profile appears to be converted almost exactly to that characteristic of ARNO and cytohesin-1 (compare Figs. 1 and 4). These data are consistent with the hypothesis that the unique triglycine motif in GRP1 directs its higher affinity binding to PtdIns(3,4,5)P$_3$ as compared with that observed for ARNO and cytohesin-1, which display a third glycine in this position.

Additional experiments were conducted to evaluate the role of the diglycine in the GRP1 PH domain relative to its unique low affinity for PtdIns(4,5)P$_2$ (i.e. high selectivity for PtdIns(3,4,5)P$_3$). Fig. 4 (upper right panel) reveals that the mutant, triglycine GRP1 PH domain displays an apparent increase in its affinity for PtdIns(4,5)P$_2$ compared with the native protein. This conclusion is based on the enhanced ability of this lipid to compete with labeled PtdIns(3,4,5)P$_3$ for binding to the mutant protein compared with the native GRP1 PH domain. Thus, the relative apparent $K_b$ values for PtdIns(3,4,5)P$_3$ versus PtdIns(4,5)P$_2$ in these experiments with mutant GRP1 PH domain are different by only about 34-fold versus about a 650-fold difference for the native protein. Conversely, deletion of a glycine from the triglycine motif of ARNO PH domain leads to a much enhanced selectivity for binding PtdIns(3,4,5)P$_3$, due to a 34-fold decrease in affinity for PtdIns(4,5)P$_2$ (Fig. 4, lower right panel). Thus, this mutation dramatically increases the divergence between apparent $K_b$ values for PtdIns(3,4,5)P$_3$ versus PtdIns(4,5)P$_2$ (2.3-fold for native protein, 43-fold for mutant). The apparent $K_b$ values obtained in these experiments are presented in Table I. Taken together, these results indicate that the presence of two versus three glycines in the PH domains of these proteins contributes greatly to the divergent selectivity of these domains for PtdIns(3,4,5)P$_3$. Two glycines positioned in the native GRP1 PH domain (compared with the three in native ARNO or cytohesin-1) would appear to promote a structure that both slightly enhances affinity for PtdIns(3,4,5)P$_3$ and to a larger extent decreases affinity for PtdIns(4,5)P$_2$. Based on solved structures of PH domains associated with PtdIns(4,5)P$_2$ or PtdIns(3,4,5)P$_3$, it is difficult to predict what structural elements might be positioned to enhance binding of a triphosphate while at the same time decreasing affinity

![FIG. 2. Competition of binding $[^{3}H]C_{8}$PtdIns(3,4,5)P$_3$ by unlabeled dioctanoyl phosphoinositides.](image-url)
for the corresponding diphosphate. Nonetheless, this is what the structure of the GRP1 PH domain achieves when compared with the ARNO and cytohesin-1 PH domain structures. Understanding the molecular basis for this effect will necessarily await solving the three-dimensional structure of the GRP1 PH domain.

It is noteworthy that a previous study has claimed that the ARNO PH domain, like that of GRP1, is highly selective in its binding to PtdIns(3,4,5)P₃ in the presence or absence of single glycine residue in the PH domain. This would be the case in all tissues that differ in the presence or absence of this single glycine residue in the PH domain. This would provide all tissues the ability to bind phosphoinositides to otherwise identical proteins.

We next addressed the second question posed above related to the functional consequences of differential selectivity of binding to GST fusion proteins of the PH domains of GRP1-like proteins by PtdIns(3,4,5)P₃ and PtdIns(4,5)P₂ by the PH domains of GRP1 versus ARNO and cytohesin-1. First, experimental conditions were developed in which membrane localization of the GRP1 PH domain in intact cells could be demonstrated to depend on its ability to bind PtdIns(3,4,5)P₃. These studies are based on previous work by others (24, 25) as well as our laboratory (23) showing recruitment of either full-length GRP1 or ARNO to sites of membrane ruffles in response to insulin. The cells were then evaluated by immunofluorescence microscopy following staining with primary polyclonal anti-HA antibody and secondary fluorescein isothiocyanate-labeled anti-rabbit antibody. Fig. 5A shows a cytoplasmic disposition of native HA-tagged GRP1 PH domain in control cells and a clear recruitment of this protein to membrane ruffles upon treatment of the cells with insulin. As found previously (23–25), membrane localization of the GRP1 PH domain in response to insulin was abolished by the PI 3-kinase inhibitor wortmannin (data not shown). The mutant K273A GRP1 PH domain also displays a cytoplasmic disposition under basal conditions, but fails to respond to insulin (Fig. 5, B and C). These data demonstrate that under these experimental conditions the ability of the GRP1 PH domain to localize to plasma membranes is abolished by inhibitors of the enzyme. In the course of evaluating several mutant GRP1 PH domain constructs, we discovered that substituting alanine for lysine 277 virtually eliminated binding to PtdIns(3,4,5)P₃ versus PtdIns(4,5)P₂ (31). This may relate to a difference in amino acid sequence in the ARNO PH domain. As shown in Fig. 3, we have employed a native ARNO PH domain construct that displays the triglyceric motif. Interestingly, mouse cytohesin-1 has also been reported to contain either a diglycine (32) or triglyceric (30) by different laboratory groups. Even more remarkable is the recent observation by Vaughan and colleagues (33) that cDNA from the same sample of human brain contained sequences coding for both the di- and triglyceric motifs of ARNO, cytohesin-1 and GRP1. The ratios varied such that approximately 80% of GRP1 was found as the diglyceric variant, whereas the others were predominantly in the triglyceric forms. It is possible that different splicing variants or different alleles of these proteins are expressed in human and mouse tissues that differ in the presence or absence of this single glycine residue in the PH domain.

![Figure 3](image-url)

**FIG. 3.** Comparison of the primary sequence of GRP1, ARNO, and cytohesin-1. Residues that differ in the proteins are bold. The location of the additional glycine in GRP1 is indicated by the arrow. The asterisk indicates the position of the lysine that is necessary for phosphoinositide binding.

![Figure 4](image-url)

**FIG. 4.** Role of the GGG/GG motifs for binding and discrimination of phosphoinositides. The PH domain of GRP1 was mutated to code for an extra glycine at position 277, and the corresponding glycine was deleted in ARNO. Binding and competition was performed as described in Figs. 1 and 2. The values are means of duplicate determinations, and the error bars represent the range of the measurements. The data are from one of three experiments with essentially identical results.
requires the ability to bind PtdIns(3,4,5)P$_3$.

Comparative studies of this type were then conducted using HA-tagged constructs of the PH domains of GRP1, which shows high selectivity for binding PtdIns(3,4,5)P$_3$, and of Myc epitope-tagged cytohesin-1, which exhibits reasonably high affinity for both PtdIns(3,4,5)P$_3$ and PtdIns(4,5)P$_2$. Fig. 2 shows that, in these experiments, the PH domain of cytohesin-1 displays a cytoplasmic disposition whether or not insulin is added to CHO-T cells. This failure of the cytohesin-1 PH domain to localize to membranes occurs under conditions identical to those in which the GRP1 PH domain is recruited to membrane ruffles in response to insulin (Figs. 5 and 6). Interestingly, under these same conditions, full-length native cytohesin-1 also presents a cytoplasmic localization in the absence of insulin, but is markedly translocated to membrane ruffles in response to the hormone as is full-length GRP1 (Fig. 6). Recruitment of full-length cytohesin-1 to membrane ruffles in response to insulin is fully inhibited by wortmannin (data not shown), indicating this recruitment requires one or more lipid products of PI 3-kinase.

Taken together, these data indicate that, in the case of GRP1, its PH domain is sufficient to explain regulated membrane localization, whereas the PH domain cannot account for this property of cytohesin-1. Another domain of cytohesin-1 such as its coiled coil region or its sec7 domain must direct its regulated membrane targeting. This may occur either directly or indirectly through binding to another cellular component which is responsive to PI 3-kinase activation. It is likely that the same is the case for ARNO, which like cytohesin-1 also binds both PtdIns(3,4,5)P$_3$ and PtdIns(4,5)P$_2$ (Fig. 2).

It is important to note that the conditions of these experiments depicted in Figs. 5 and 6 are critical to obtaining the results described. Most important is that the HA-tagged constructs expressed in the CHO-T cells are present at low levels, which is achieved by using low concentrations of expression vector during transfection of cells. At high expression levels, significant membrane targeting of all constructs shown in Fig. 6 is observed (data not shown). This is presumably due to the action of lower affinity interactions that are promoted by the high protein concentrations. A report (34) showing regulated membrane targeting of the cytohesin-1 PH domain in intact cells may be due to higher expression levels or the different cell type used. In addition, whereas localization in the present study was determined after fixation using PH constructs with short sequence tags, the previous report employed GFP fusion proteins in live cells. It is interesting that the PH domain of cytohesin-1 does not display a definitive plasma membrane disposition in unstimulated cells (Fig. 6), given its reasonable affinity for PtdIns(4,5)P$_2$ (Fig. 2) and the high abundance of this lipid in plasma membranes. In this regard, the cytohesin-1 PH domain is unlike that of phospholipase C$\delta$, which does localize to plasma membranes of unstimulated cells (22). The cytohesin-1 PH domain is thus similar to some other PH domains, including those in $\beta$-adrenergic receptor kinase 1 and...
pleckstrin itself, which do not discriminate greatly between binding PtdIns(4,5)P$_2$ and PtdIns(3,4,5)P$_3$, but also do not localize to plasma membranes in intact cells (15). This is likely due to the somewhat lower affinity that these three PH domains exhibit for PtdIns(4,5)P$_2$ compared with the PH domain of phospholipase C$_5$.

The data presented here show that the PH domains of the GRP1 family proteins GRP1, ARNO, and cytohesin-1 are heterogeneous with respect to their relative binding affinities for the polyphosphoinositides PtdIns(3,4,5)P$_3$ and PtdIns(4,5)P$_2$. The results are consistent with the hypothesis that this heterogeneity functions to confer diverse membrane localization mechanisms to these ARF exchange factors. Thus, the PH domain of GRP1 is sufficient to respond to the presence of the PI 3-kinase product PtdIns(3,4,5)P$_3$ in cell membranes, while the PH domains of ARNO and cytohesin-1 are not. These latter proteins may employ their PH domains in conjunction with other membrane localization motifs to affect recruitment to cell membranes. Thus, these PH domains may be necessary but not sufficient for membrane targeting. It is also possible that the GRP1 versus ARNO and cytohesin-1 PH domains function in the localization of these proteins to distinct plasma membrane microdomains not resolved by the microscopy methodology employed here or in other studies to date. Further refinement of imaging techniques will be required to test this idea. In any case, our results indicate that a single glycine in the amino acid sequences of the ARNO and cytohesin-1 PH domains, not present in GRP1, contributes greatly to the heterogeneity of phosphoinositide binding and thus to the divergent membrane localization properties of these proteins.

Acknowledgments—We thank Dr. David Lambright for helpful discussions and critical reading of this manuscript. We appreciate the excellent assistance of Jane Erickson in preparation of the manuscript.

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