Regulation of GRP1-catalyzed ADP Ribosylation Factor Guanine Nucleotide Exchange by Phosphatidylinositol 3,4,5-Trisphosphate

(Received for publication, September 15, 1997, and in revised form, November 25, 1997)

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Cellular levels of phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P$_3$) are rapidly elevated in response to activation of growth factor receptor tyrosine kinases. This polyphosphoinositide binds the pleckstrin homology (PH) domain of GRP1, a protein that also contains 200 residues with high sequence similarity to a segment of the yeast Sec7 protein that functions as an ADP ribosylation exchange factor (ARF) (Klarlund, J., Guilherme, A., Holik, J. J., Virbasius, J. V., Chawla, A., and Czech, M. P. (1997) Science 275, 1927–1930). Here we show that dioctanoyl PtdIns(3,4,5)P$_3$ binds the PH domain of GRP1 with a $K_D$ = 0.5 $\mu$M, an affinity 2 orders of magnitude greater than dioctanoyl-PtdIns(4,5)P$_2$. Further, the Sec7 domain of GRP1 is found to catalyze guanine nucleotide exchange on ARF1 and -5 but not ARF6. Importantly, PtdIns(3,4,5)P$_3$, but not PtdIns(4,5)P$_2$, markedly enhances the ARF exchange activity of GRP1 in a reaction mixture containing dimyristoylphosphatidylcholine micelles, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid, and a low concentration of sodium cholate. PtdIns(3,4,5)P$_3$-mediated ARF nucleotide exchange on GRP1 is selectively blocked by 100 $\mu$M inositol 1,3,4,5-tetrakisphosphate, which also binds the PH domain of GRP1. Taken together, these data are consistent with the hypothesis that selective recruitment of GRP1 to PtdIns(3,4,5)P$_3$ in membranes activates ARF1 and -5, known regulators of intracellular membrane trafficking.

The phosphoinositide 3-kinase (PI 3-kinase)$^1$ enzymes represent a major pathway by which biological signaling systems operate to control cell functions (1–4). Many cell surface receptor tyrosine kinases (5, 6) as well as certain GTP-binding protein-linked receptors (7, 8) acutely activate cellular PI 3-kinase activity, leading to the generation of 3'-polyphosphoinositides. Significant amounts of phosphatidylinositol 3-phosphate (PtdIns(3)P) are present in unstimulated cells, whereas phosphatidylinositol 3,4-bisphosphate (PtdIns(3,4)P$_2$) and phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P$_3$) are specifically produced upon cell surface receptor activation (6). A large number of biological processes have been implicated as targets of regulation by this pathway, including membrane ruffling, chemotaxis, secretion, insulin-stimulated glucose uptake, cell adhesion, cell growth, and apoptosis as well as the regulation of early endosome structure (7–13).

Recent work in this laboratory identified a potential effector protein, GRP1, containing a PH domain that binds PtdIns(3,4,5)P$_3$ but not PtdIns(3,4)P$_2$ or PtdIns(3)P (14). GRP1 also contains a 200-amino acid sequence with high similarity to a domain in the yeast Sec7 protein, which is known to be required for transport of polypeptides from the endoplasmatic reticulum to and through Golgi membranes (15). A similar Sec7 domain in the mammalian proteins cytohesin-1 and ARNO and in the yeast protein Gea1 were reported to catalyze guanine nucleotide exchange on the small GTP-binding protein ARF1 (16–18). ARF proteins in the GTP-bound form promote membrane vesicle trafficking pathways by recruiting coat proteins to membranes, causing membrane budding (19–21). A potential interaction between PI 3-kinase and ARF mutants had been suggested based on characteristic changes of endosome morphology elicited by wortmannin in various cultured cell lines (12). Taken together, these observations provide a framework for the hypothesis that GRP1 or its homologs may mediate cellular effects of 3'-polyphosphoinositides that require ARF proteins.

EXPERIMENTAL PROCEDURES

Recombinant GRP1 Protein—To map the active regions of GRP1, GST proteins fused to various domains of GRP1 were purified from lysates of bacteria expressing the relevant pGEX5X-3 constructs as described (14). For other experiments, GRP1 was cloned into pGEX-4T, and the recombinant protein was purified. The GST-GRP1 fusion protein was cleaved by incubation with 5 $\mu$g/ml thrombin in 200 $\mu$l of 20 mM Tris, pH 8.0, 2.5 mM CaCl$_2$, 150 mM NaCl overnight at 4 °C. Complete cleavage was verified by SDS-polyacylamide gel electrophoresis. The proteins were transferred to assay buffer (50 mM HEPES, pH 7.5, 1 mM MgCl$_2$, 100 mM KCl, 1 mM dithiothreitol) using Centricon 30 microconcentrators (Amicon) and stored at −20 °C in 50% glycerol.

ARF Exchange Assay—Recombinant baculovirus encoding ARF1, -5, or -6 (ARF cDNAs kindly provided by Dr. R. Klausner) fused at the C terminus to a 9-amino acid sequence corresponding to the major antigenic determinant of influenza virus hemagglutinin was constructed. Sf9 cells were infected with the recombinant baculovirus, the cells were harvested 3 days later by centrifugation for 5 min at 3000 rpm, and the pellets were stored at −70 °C. A cell pellet corresponding to 50 ml of culture medium was dissolved in 1 ml of assay buffer supplemented with 1% Triton X-100, 1 mg benzamidine, 5 $\mu$g/ml leupeptin, 5 $\mu$g/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, and 50 $\mu$g/ml. After clarification by centrifugation at 20,000 rpm for 5 min, 50 $\mu$l of a rabbit antiserum that had been produced by immunization with a peptide

The abbreviations used are: PI 3-kinase, phosphoinositide 3-kinase; PtdIns, phosphatidylinositol; PH, pleckstrin homology; ARF, ADP ribosylation factor; GST, glutathione S-transferase; GTP, guanosine 5'-O-(thiotriphosphate); CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; PIP$_3$, phosphatidylinositol trisphosphate.

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Printed in U.S.A.
(YPYDVPDYA) conjugated to hemocyanin was added and incubated overnight on ice. The following day, 75 μl of Protein A conjugated to Sepharose CL-4B (Sigma) was added and incubated on an end-to-end mixer for 1 h. The beads were collected by centrifugation and washed five times with 1 ml of assay buffer. An additional 500 μl of Sepharose CL-4B was added as a carrier. Addition of ARF from Sf9 cells labeled with [3H]myristic acid by Western blotting and autoradiography revealed that at least 90% of the ARF molecules was modified by the lipid (22).

For the standard assay, 5 μl of assay buffer containing GRP1, 6 mM dimyristoylphosphatidylcholine (Avanti Polar Lipids Inc.), 0.2% cholate, 1% bovine serum albumin, 10 μM GTPγS, and 0.75 μCi of [35S]GTP was added to 5-μl beads containing immunoadsorbed ARF. Approximately 50% of the cholate molecules are incorporated into the micelles under these conditions, based on the rate of diffusion of [3H]cholate through a dialysis membrane in the presence versus absence of phospholipid micelles. After 40 min at room temperature, the beads were washed four times with 1 ml of assay buffer, and [35S]S was quantitated by liquid scintillation counting. Synthetic dipalmitoyl 3′-phosphoinositides were from Matreya Inc., and bovine brain PtdIns(4)P was from Calbiochem.

**Lipid Binding and Competition Assays**—Binding assays were performed as described (23). Briefly, GST fused to the PH region of GRP1 (amino acids 239–399) was bound to glutathione immobilized on agarose beads (Sigma). Synthetic [3H]labeled dioctanoyl PtdIns(3,4,5)P3 (kindly provided by C.-S. Chen (24)) was added, and after 1 h the beads were separated from the supernatant by centrifugation. The amount of [3H]PtdIns(3,4,5)P3 bound to the PH domain was calculated by subtracting the amount of free [3H] present in supernatants from GST-GRP1 from the amount of free [3H] in supernatants from control GST. The data were fitted to the equation, [bound] = Bmax × ([free]/Kd + [free]), by least squares curve fit. For the competition assays the beads containing the GST fusion proteins were incubated with 2.5 μM [3H]labeled dioctanoyl PtdIns(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 with buffer containing 0.5% Nonidet P-40 and 1% scintillation counter. The percentage of [3H]PtdIns(3,4,5)P3 bound was calculated based on the amount of [3H] bound to the beads in the absence of competitor. The ratio of the apparent dissociation constant (Kd) 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/Kd + (L), where n is the percent specific binding, L is the concentration of unlabeled lipid added, and Kd is the apparent dissociation constant. The ratios of the apparent dissociation constants accurately reflect the ratios of the true dissociation constants under the present experimental conditions (25).

**RESULTS**

To test whether GRP1 functions as an ARF guanine nucleotide exchange factor, hemagglutinin-tagged ARF1, -5, or -6 proteins produced in Sf9 cells were incubated with recombinant GRP1 protein in the presence of [35S]GTPγS for various times prior to determination of ARF-bound label. Fig. 1 shows a nearly linear rate of labeled guanine nucleotide binding to these ARF proteins during this time course in the presence or absence of GRP1. A 4–10-fold stimulation of ARF1 binding to [35S]GTPγS was observed in response to GRP1, whereas no effect of GRP1 on ARF6 binding to nucleotide was detected. Up to 10 molecules of [35S]GTPγS could be bound to ARF1 per molecule of GRP1 under our experimental conditions, showing that GRP1 acts catalytically. Guanine nucleotide exchange on ARF6 was also significantly enhanced by GRP1, although to a lesser extent than that for ARF1. Fig. 1D indicates that GRP1 maximally activates labeled nucleotide binding to ARF1 and -5, but not ARF6, when present at 2.5 μM under the conditions of this assay.

Because the Sec7 homology domains of the related protein ARNO (16) have been shown to be sufficient for catalysis of guanine nucleotide exchange of ARF1, we analyzed the ARF1 exchange activity intrinsic to each of three segments of GRP1, including the Sec7 homology region (Fig. 2). GST fusion proteins of the N terminus and PH regions of GRP1 exhibited no effect on ARF1 binding to labeled nucleotide in our assay. In contrast, a GST fusion protein containing the Sec7 homology domain was as effective as the full-length GRP1 in catalyzing ARF1 exchange activity (Fig. 2).

GRP1 was initially identified based on its high affinity binding to PtdIns(3,4,5)P3 through its PH domain (14). We therefore questioned whether this polyphosphoinositide might regulate the ARF exchange activity of GRP1. Fig. 2 shows that PtdIns(3,4,5)P3 stimulated binding of [35S]GTPγS to ARF in a

\[ B_{\text{max}} = \frac{L}{K_d + L} \]

\[ \text{error bars} \] are standard deviations.

**FIG. 1.** GRP1 selectively catalyzes guanine nucleotide exchange of ARF1 and -5 (A–C). Isoforms of ARF were tagged with a 9-amino acid sequence corresponding to the hemagglutinin antigenic epitope, expressed in Sf9 cells, immunoprecipitated, and incubated in the presence of [35S]GTPγS and 0.15 μM GRP1 as indicated. Analysis of immunoprecipitates by SDS-polyacrylamide gel electrophoresis and subsequent autoradiography from cultures labeled with [3H]myristic acid revealed that similar amounts of the ARF isoforms were present in the precipitates. The values are means of duplicate determinations, and the error bars represent the range of measurements. Dose response of activation of ARF isoforms by GRP1 in the presence of phosphatidylcholine and cholate as described under “Experimental Procedures.” Immunoprecipitated ARF isoforms were incubated for 40 min with various concentrations of GRP1. The values are means of four determinations, and the error bars are standard deviations.

**FIG. 2.** Effect of PtdIns(3,4,5)P3 on ARF1 guanine nucleotide exchange activity mediated by GRP1 or various domains of GRP1 (A). Activation of guanine nucleotide exchange of ARF1 by varying concentrations of PtdIns(3,4,5)P3 is shown. ARF1 was incubated with 0.1 μM GRP1 for 40 min in the presence of various concentrations of PtdIns(3,4,5)P3 and the amount of [35S]GTPγS bound to ARF1 was determined. The graph shows the -fold stimulation of the GRP1-mediated exchange activity by the indicated concentrations of PIP3. Values are means of four determinations, and the error bars represent standard deviations (B). Immunoprecipitated ARF1 was incubated with 1 μM GST, 1 μM GST fused to amino acids 5–76 of GRP1 (N-term), 0.5 μM GST fused to amino acids 52–260 (Sec7), 1 μM GST fused to amino acids 239–399 (PH), or 0.15 μM GST fused to amino acids 5–399 (GRP1). The incubations were performed in the presence or absence of 25 μM PtdIns(3,4,5)P3, as indicated.
The data depicted in Fig. 2 suggest the hypothesis that receptor signaling through PI 3-kinases, which generates PtdIns(3,4,5)P3, might specifically regulate ARF proteins through GRP1. To further test this concept, the relative binding affinities of PtdIns(3,4,5)P3 versus PtdIns(4,5)P2 were determined. Approximately 160 pmol of GRP1 PH was present in each incubation. The line drawn represents a best fit assuming a single class of binding sites (B). Competition for binding of [3H]dioctanoyl PtdIns(3,4,5)-P3 by various phosphoinositides is shown. The lines are the best fit assuming simple competitive binding. The values are means of duplicate determinations, and the bars represent the range of the measurements.

FIG. 3. Specificity of binding of [3H]dioctanoyl PtdIns(3,4,5)P3 to the PH domain of GRP1 (A). A GST fusion protein of the GRP1 PH domain was bound to immobilized glutathione and incubated with various concentrations of [3H]dioctanoyl PtdIns(3,4,5)P3, and binding was determined. Approximately 160 pmol of GRP1 PH was present in each incubation. The line drawn represents a best fit assuming a single class of binding sites (B). Competition for binding of [3H]dioctanoyl PtdIns(3,4,5)-P3 by various phosphoinositides is shown. The lines are the best fit assuming simple competitive binding. The values are means of duplicate determinations, and the bars represent the range of the measurements.

A GST fusion protein of the PH domain of GRP1 was incubated with various concentrations of synthetic [3H]dioctanoyl PtdIns(3,4,5)P3, and binding was determined (Fig. 3A). These experiments show high affinity binding of this polyphosphoinositide, with a calculated Kd of 0.5 μM, assuming one binding site per GRP1 molecule. Fig. 3B reveals that PtdIns(3,4,5)P3 competes for [3H]dioctanoyl PtdIns(3,4,5)P3 binding to the PH domain of GRP1 with a Kd(app) that is 10–20-fold lower than for PtdIns(4,5)P2. Comparison of the competition profiles for these two polyphosphoinositides containing identical fatty acyl side chains (dioctanoyl) showed that PtdIns(3,4,5)P3 had a 50–100-fold higher affinity when compared with PtdIns(4,5)P2. PtdIns(3,4)P2 had also a 50-fold lower affinity than PtdIns(3,4,5)P3 (Fig. 3B).

The data depicted in Fig. 3 suggested that high specificity of PtdIns(3,4,5)P3-mediated activation of ARF exchange may characterize GRP1 function. We tested the specificity of GRP1-stimulated ARF exchange by these phosphoinositides under conditions of various charge densities in detergent-phosphatidylcholine micelles (Fig. 4). Interestingly, when a relatively high concentration of negative charge is present in the micelles (0.1% cholate), PtdIns(3,4,5)P3, PtdIns(4,5)P2, and PtdIns(3,4)P2 all stimulated ARF1 exchange activity in the presence of GRP1.

In the absence of charge (0.1% CHAPS, no cholate), none of these phosphoinositides were able to enhance the ARF exchange activity of GRP1 (Fig. 4). In contrast, PtdIns(3,4,5)P3 selectively stimulated [35S]GTPγS binding to ARF1 in the presence of GRP1 when a low level of negative charge (0.05% cholate) was present with the phosphatidylcholine in our assay. Under these conditions PtdIns(4,5)P2 and PtdIns(3,4)P2 had significantly less effect. These experiments establish an in vitro assay system that reveals selective regulation of GRP1-catalyzed ARF1 guanine nucleotide exchange activity by PtdIns(3,4,5)P3.

Previous results (16, 25, 26) and data in Fig. 4 suggest that guanine nucleotide exchange of ARF proteins requires an interface of ARF, exchange factor, and negatively charged phospholipid, indicating that recruitment of GRP1 to the phospholipid membrane may be a key element of the regulation of ARF guanine nucleotide exchange. Thus we reasoned that Ins(1,3,4,5)P4, the polar head group of PtdIns(3,4,5)P3, should compete for binding and recruitment of GRP1 to PtdIns(3,4,5)P3 in micelles, thus blocking GRP1-mediated ARF1 exchange. Fig. 5 shows results that are consistent with this prediction. Addition of 100 μM Ins(1,3,4,5)P4 to our assay virtually ablated the increased ARF1 guanine nucleotide exchange activity catalyzed by GRP1 in response to PtdIns(3,4,5)P3. In concert with data in Fig. 4 indicating high binding specificity of the GRP1 PH domain, other polar head groups tested, including Ins(1,3,4,6)P4, Ins(1,4,5)-P3, Ins(1,3,4,6)P4, and Ins(1,2,5,6)P4, failed to inhibit GRP1-mediated ARF1 guanine nucleotide exchange (Fig. 5).

DISCUSSION

The results presented here demonstrate the specific binding and regulation of GRP1-catalyzed ARF1 guanine nucleotide exchange by PtdIns(3,4,5)P3, a product of receptor-regulated PI 3-kinase activity (Figs. 2, 4, and 5). These experiments (Fig. 2) also localize ARF exchange activity to an approximately 200-residue segment of the GRP1 protein that exhibits high sequence similarity to a region of the Saccharomyces cerevisiae Sec7 protein (27). GRP1 was found to catalyze nucleotide ex-
change on ARF1 and -5 but not ARF6 (Fig. 1). Interestingly, ARF5 was reported not to be a substrate for cytohesin-1 (17), but a detailed description of the specificities of the various isoforms of GRP1 is still lacking.

The identification of GRP1 based on its binding to PtdIns(3,4,5)P₃ in an expression cDNA library screen suggested a particularly high affinity for this polyphosphoinositide (14). Our present results confirm this expectation, revealing a $K_d = 0.5 \mu M$ for binding $[^{3}H]$dioctanoyl PtdIns(3,4,5)P₃ (Fig. 2). Importantly, the PH domain of GRP1 exhibits an apparent affinity for dioctanoyl PtdIns(3,4,5)P₃ that is about 2 orders of magnitude lower than that for dioctanoyl PtdIns(3,4,5)P₃ (Fig. 2). This extraordinary specificity for the 3′-phosphoinositide is a distinctive characteristic of the GRP1 PH domain among the many PH domains that have been characterized for phosphoinositide binding (28). Furthermore, this degree of specificity is consistent with that required of a protein regulated in intact cells by PtdIns(3,4,5)P₃ signaling, given that PtdIns(4,5)P₂ is much more abundant in cell membranes (6). Taken together, the data in Figs. 2–5 provide strong support for the hypothesis that GRP1 functions in intact cells in conjunction with membrane-localized PtdIns(3,4,5)P₃ generated by receptor-activated PI 3-kinase activity.

Particular attention in this study was focused on the relationship between the specificity of PtdIns(3,4,5)P₃ binding to GRP1 and the specificity of PtdIns(3,4,5)P₃-mediated activation of GRP1 exchange activity for ARF1 (Figs. 2 and 4). Previous work has emphasized the requirements for ARF myristoylation and phospholipid to obtain optimal nucleotide exchange rates for ARF proteins, suggesting exchange occurs at the membrane surface (26). In the case of ARNO, PtdIns(4,5)P₂ was found to be sufficient for its recruitment to membranes as well as activation of ARF exchange (16), suggesting specificity of its PH domain for binding this phosphoinositide or a nonspecific effect of PtdIns(4,5)P₂ to recruit ARNO to membranes based on a charge effect, or both. All three polyphosphoinositides tested were effective in stimulating GRP1-mediated exchange activity when a high charge density was present on micelles in the assay (Fig. 4), indicating that GRP1 can be nonspecifically bound to membranes under these conditions. Selectivity of PtdIns(3,4,5)P₃ in stimulating ARF1 nucleotide exchange by GRP1 over that observed for PtdIns(4,5)P₂ or PtdIns(3,4)P₃ was revealed at lower charge density (Fig. 4). These data are consistent with the hypothesis that binding of GRP1 to membranes containing ARF may include two components: specific interaction with PtdIns(3,4,5)P₃ through its PH domain and interactions with acidic phospholipids through one or more clusters of its basic amino acid residues. At concentrations of GRP1 that yield maximal [%35S]GTP₇S loading of ARF1, no stimulation by PIP₂ was observed, consistent with the concept that activation of GRP1 exchange activity results from recruitment to membranes (data not shown).

The high activity observed for ARF1 guanine nucleotide exchange catalyzed by the GRP1 Sec7 domain suggests that ARF1 function may be closely related to the physiological role of GRP1 (Fig. 1). ARF1 has been implicated in vesicle transport related to Golgi membrane function as well as in secretory and exocytosis pathways (19, 21). It is noteworthy that several targets of receptor-activated PI 3-kinase signaling involve intracellular membrane-trafficking systems, including mast cell secretion and insulin-sensitive GLUT4 glucose transporter translocation to the plasma membrane in muscle and adipocytes (9, 11, 29). Thus it is tempting to hypothesize a role for GRP1 in regulating such processes in response to localized synthesis of PtdIns(3,4,5)P₃.

Acknowledgments—Numerous helpful discussions with Dr. David Lambright are greatly appreciated. We thank Kristin Kwan for excellent technical assistance and Jane Erickson for expert assistance in the preparation of this manuscript. Dr. Andrew Cherniack provided expert assistance in preparation of the figures.

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