Phosphoinositides Affect both the Cellular Distribution and Activity of the F-BAR-containing RhoGAP Rgd1p in Yeast*

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Cell polarity is a key element of development in most eukaryotes. The Rho GTPase-activating protein Rgd1p positively regulates the GTPase activity of Rho3p and Rho4p, which are involved in bud growth and cytokinesis, respectively, in the budding yeast Saccharomyces cerevisiae. Rgd1p contains an F-BAR domain at its N-terminal end in addition to its RhoGAP domain at its C-terminal end. We demonstrate here that phospholipids discriminate between the GTPase activities of Rho3p and Rho4p through Rgd1p and specifically stimulate the RhoGAP activity on Rho4p. The central region of the protein contiguous to the F-BAR domain is required for this stimulation. The F-BAR region binds to phosphoinositides in vitro and also plays a key role in the localization of Rgd1p to the bud tip and neck during the cell cycle. Studies of heat-sensitive mutants lacking phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-biphosphate suggested that Rgd1p initially binds to Golgi membranes via phosphatidylinositol 4-phosphate and is then transported to the plasma membrane, where it binds phosphatidylinositol 4,5-biphosphate. We demonstrate here the dual effects of phosphoinositides on a RhoGTPase-activating protein. Phosphoinositides both regulate the recruitment and trafficking of Rgd1p to membranes via the F-BAR domain and specifically stimulate GTPase-activating protein activity, consistent with functional interplay between lipids, RhoGAP, and its related GTPases in yeast growth.

Small GTPases of the Rho family play various roles in the organization of diverse cell functions, such as polarity, motility, shape change, membrane trafficking, and gene expression (1–4). Rho proteins act through their endogenous GTP hydrolysis activity, which is controlled by several regulatory proteins (guanine nucleotide exchange factors, GTPase-activating proteins, guanine nucleotide dissociation inhibitors), and induce cell signaling through several effectors. Like most small GTPases, they cycle between inactive (GDP-bound) and active (GTP-bound) forms. GTPase-activating protein (GAP)3 accelerates the endogenous GTP hydrolysis rate of small GTPases (5). In Saccharomyces cerevisiae, six Rho GTPases (Cdc42p and Rho1p–Rho5p) have been described, and, as in most eukaryotes, these proteins exert their effects on various cellular processes through effects on the actin cytoskeleton, septin organization, exocytosis, cytokinesis, and the cell wall stress response (6–11). We previously assessed the RhoGAP activity of various proteins with Rho GTPases in S. cerevisiae (12, 13) and, more specifically, explored the role of Rgd1p in regulating the activity of Rho3p and Rho4p, together with other cellular functions of these GTPases. Rgd1p is the only RhoGAP shown to act on both Rho3p and Rho4p. These GTPases are both involved in actin cytoskeleton organization (7, 14). Rho3p is also involved in exocytosis (8–10), whereas it has been suggested that Rho4p is involved in cytokinesis (15). RGD1 inactivation does not result in a strong mutant phenotype, other than lethality during the stationary phase, in standard growth conditions (16). However, Rgd1p RhoGAP becomes essential in conditions of stress (17). Functional links between Rgd1p and the actin cytoskeleton and between Rgd1p and the cell wall integrity pathway have been demonstrated by studying synthetic lethal strains inactivated for RGD1 (16, 18–20). The role of Rgd1p GAP activity in these functional links was revealed through the implication of activated Rho3p and Rho4p in the underlying genetic interactions (15, 20).

Rgd1p has an FCH domain at its N terminus (amino acids aa 33–144), a coiled-coil domain (aa 139–172), and a RhoGAP domain at its C terminus (aa 486–666) (19). The region extending between amino acids 1 and 300 is similar to the F-BAR domain, which is known to be involved in membrane binding and tubulation (21–23). The members of the F-BAR family have a similar domain organization, with several having SH3 or RhoGAP domains (24–26).

We investigated the ability of phospholipids to bind Rgd1p and their effects on the cellular distribution and GAP activity of Rgd1p with the aim of improving our understanding of the role of the Rgd1p RhoGAP in S. cerevisiae growth and in relation to its GTPases. Indeed, phospholipids not only bind proteins to cell membranes; they can also regulate GAP catalytic function in vitro (27, 28). We therefore investigated the effects of lipids on Rgd1p activity with the Rho3p and Rho4p GTPases. We observed a specific stimulation of RhoGAP activity with one particular GTPase following the addition of phospholipids and identified a region within Rgd1p responsible for the modulation.

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3 The abbreviations used are: GAP, GTPase-activating protein; aa, amino acids; GFP, green fluorescent protein; FCH, Fes/CIP4 homology; GST, glutathione S-transferase; DTT, dithiothreitol; PtdIns, phosphatidylinositol; PtdIns(4)P, phosphatidylinositol 4-phosphate; PtdIns(4,5)P2, phosphatidylinositol 4,5-bisphosphate.
of RhoGAP activity by phosphoinositides. We also demonstrated a requirement for the F-BAR domain for phospholipid binding \textit{in vitro}. Using Rgd1p tagged at its C-terminal end with three green fluorescent proteins (GFPs) in yeast mutants with impaired phosphoinositide biosynthesis, we showed that interaction with phosphoinositides was essential for the correct distribution of Rgd1p during bud growth \textit{in vivo}. We propose here a model taking into account the dual effects of phosphoinositides on GAP activity and the distribution of the Rgd1p RhoGAP in \textit{S. cerevisiae}.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains and media**—The \textit{S. cerevisiae} strains used in this study are listed in Table 1. Standard techniques were used, and the composition of rich and minimal media for yeast growth have been reported elsewhere (29). Yeast strains were generally grown at 30 °C with the exception of heat-sensitive mutants. The heat-sensitive AAY104 (pik1-83ts), AAY202 (mss4-102ts), and CTY1568 (stt4-4ts) strains and the SEY6210 reference strain were cultured overnight at permissive temperature (38 °C) for 3 h and then observed by fluorescence microscopy. In parallel, the viability of the cells shifted to the nonpermissive temperature was assessed by the methylene blue method (18). After 3 h at the nonpermissive temperature, 2% of control strain cells and 12% of both stt4-4ts and mss4-102ts cells had died. These very low mortality rates ruled out the possibility of a defect in cell viability accounting for the different distribution of Rgd1p in heat-sensitive mutants.

**Plasmid Constructs**—Plasmids for the purification from bacteria of unmodified GST-tagged Rho3p and Rho4p have been described by Doignon \textit{et al.} (12). GST-tagged Rho3p and Rho4p were also produced in yeast to obtain modified GTPases and, in particular, prenylated Rho proteins. For this purpose, the coding sequences of the \textit{RHO3} and \textit{RHO4} genes were inserted into pMG1 (30). The recombinant p783 plasmid for the production of GST-tagged Rgd1p in bacteria (12) was used as template for inverse PCR for the construction of bacterial plasmids encoding various truncated forms of Rgd1p: Rgd1Δ1–300, Rgd1Δ1–350, Rgd1Δ1–450, Rgd1Δ301–350, and Rgd1Δ1–156. For the localization of Rgd1p \textit{in vivo}, we generated various constructs tagged at the C terminus with green fluorescent protein, using the integrative yeast vector pRS305-3×GFP (generously provided by Isabelle Sagot and David Pellman), which contains three tandem copies of the GFP gene. For localization of the wild-type form of Rgd1p in the wild-type strain and in phosphatidylinositol kinase mutants, we inserted the last 800 bp before the stop codon into the plasmid and targeted integration to the \textit{RGD1} locus. For the localization of mutant forms of Rgd1p, we generated constructs, including the \textit{RGD1} promoter and coding sequences carrying deletions (Δ1–156, Δ486–660, and Δ301–350). These constructs were integrated into the \textit{RGD1} locus by targeting the residual promoter region of the \textit{rgd1Δ} mutant strain. The resulting \textit{RGD1} sequences of all recombinant plasmids were verified by sequencing, and integration into the \textit{RGD1} locus was checked by PCR. Using the sensitivity of \textit{rgd1Δ} mutant cells to low pH (17), we also checked the effect of GFP tagging. Adding 3×GFP to the carboxyl-terminal extremity of wild type and truncated Rgd1p forms did not change their functional behavior compared with untagged proteins. Full-length tagged Rgd1p entirely rescued the \textit{rgd1Δ} phenotype as the untagged one.

**Purification of GST Fusion Proteins**—The production of GST-tagged proteins in \textit{Escherichia coli} and their purification have been described elsewhere (12). The modified GST-tagged Rho3 or Rho4 proteins, hereafter described as prenylated, were produced in \textit{S. cerevisiae} cells and were obtained from membrane fractions by affinity purification. The MGY70 strain containing the pMG1 plasmid encoding the GST-tagged Rho3p or Rho4p was cultured overnight in YPGaccharose (2%) medium and then used to seed YPgalactose (2%) medium, to induce Rho expression for 8 h. Cells were harvested, washed with water and frozen at −80 °C. The pellets were resuspended with glass beads in lysis buffer (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1 mM EGTA, 5 mM MgCl\textsubscript{2}, 1 mM DTT, and 1 mM phenylmethylsulfonyl fluoride) supplemented with a protease inhibitor mixture (Sigma) at 4 °C, using a Mini-beadbeater (Biospec Products). Cell lysates were centrifuged at 550 × g for 20 min at 4 °C. The supernatant was collected and centrifuged at 22,000 × g for 45 min at 4 °C to pellet the membranes. The 22,000 × g supernatant was discarded, and the membrane pellet was resuspended in 3 volumes of solubilization buffer (50 mM Tris-HCl, pH 7.6, 50 mM NaCl, 0.6% Triton X-100, 1 mM EGTA, 5 mM MgCl\textsubscript{2}, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor mixture) and incubated at 4 °C for 90 min, with gentle shaking. GST-Rho proteins were allowed to bind to glutathione-Sepharose beads (GE Healthcare) equilibrated in wash buffer (50 mM Tris-HCl, pH 7.6, 50 mM NaCl, 0.1% Triton X-100, 5 mM MgCl\textsubscript{2}, 1 mM DTT) for 1 h. The beads were washed four times, and GST fusion proteins were collected in elution buffer containing 20 mM reduced glutathione, 50 mM Tris-HCl, pH 8, 0.1% Triton X-100, 150 mM NaCl, 5 mM MgCl\textsubscript{2}, and 1 mM DTT. The purified proteins were stored in small aliquots, at −80 °C. Purity was checked by SDS-PAGE, and protein concentrations were determined in Bradford protein assays (Pierce).

### Table 1: Yeast strains

| Strain | Genotype | Source |
|--------|----------|--------|
| BY4742 | MATa his3Δ leu2Δ0 lys2Δ0 ura3Δ0 | Euroscarf |
| rgd1Δ | BY4742, rgd1Δ::KanMX4 | Euroscarf |
| SEY6210 | MATa, leu2-3,112, his3Δ2, lys2Δ0, trp1-1Δ901, lys2-801, suc2Δ9 | Ref. 39 |
| AAY104 | SEY6210, pik1::His3 with pRS314-pik1-83ts (LEU2) | Ref. 39 |
| AAY202 | SEY6210, mss4::His3 with pYClac11-102ts (LEU2) | Ref. 41 |
| CTY1568 | SEY6210, stt4::His3 with pYC-stt4-4ts (LEU2) | Ref. 41 |
| MGY70 | MATa ura3-1, trp1-28, leu2Δ0, lys2Δ0, his3Δ2, mss4::KanMX4, pep4Δ::LEU2 with pRS316-MOB1 | Ref. 30 |
Measurement of GTP Hydrolysis by Rho3p and Rho4p GTPases—Each measurement was performed in three successive steps (31). GTP loading was achieved by incubating the Rho proteins with $[\gamma-32P]$GTP in a buffer containing 20 mM Tris-HCl, pH 7.6, 25 mM NaCl, 0.1 mM DTT, and 10 mM EDTA for 10 min at room temperature. An amount of Rho protein suitable for all assays was added to a tube, together with 0.4–0.5 $\mu$Ci of $[\gamma-32P]$GTP (6000 Ci/mmol) per 15 pmol of GTPase. The reaction was stopped by adding 36 mM ice-cold MgCl$_2$, and the solution was kept on ice. GTPase activity was initiated by adding activated charcoal in 0.1 M NaH$_2$PO$_4$, and the amount of free GTP was measured. An aliquot of the loading mixture was also mixed with scintillation liquid and counted to determine precise the total radioactivity present in each assay. Once the hydrolysis assays to give a final concentration of 100 mM NaH$_2$PO$_4$, 1% Tween 20). All incubations were carried out at 22 °C, and the reaction was then stopped by adding 800 $\mu$L of an ice-cold suspension of 5% activated charcoal in 0.1 M NaH$_2$PO$_4$, and the amount of free $[\gamma-32P]$phosphate following GTP hydrolysis was determined in a scintillation counter after separation from $[\gamma-32P]$GTP by centrifugation. A control sample was used to determine the amount of inorganic $[\gamma-32P]$phosphate present in the supernatant without incubation. The radioactivity level determined for this sample, the blank, was subtracted from those obtained in GTP hydrolysis assays. An aliquot of the loading mixture was also mixed with scintillation liquid and counted to determine precisely the total radioactivity present in each assay. Once the value obtained for the blank had been subtracted, this value was considered to correspond to 100% $[\gamma-32P]$GTP availability, and the ratio of this value to the amount of radioactivity in the supernatant after incubation defined the percentage of GTP hydrolysis. We checked that the hydrolysis reaction had not reach the plateau at the end of incubation, using a control for which the incubation time was 15 min rather than 10 min. For each assay, the reaction was carried out in duplicate, and the mean value of the two assays was retained for analysis.

Lipid Preparation—Phosphatidylcholine from hens’ eggs (Sigma) was dissolved in ether and diluted to 1 mg/ml with incubation buffer. Phosphatidylinositol (PtdIns) from soybean (Sigma) was dissolved in chloroform and dried under a stream of nitrogen. Phosphatidylinositol-4-phosphate (PtdIns(4)P), and phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P$_2$) (Avanti), all from bovine or porcine brain, and PtdIns were rehydrated over a period of 2 h, and their concentration in incubation buffer was adjusted to 1 mg/ml. Vesicles were obtained by sonication of the lipid suspension with a VCX130 (Sonics) probe for a few s until the suspension became phase-separated. Vesicles were sonicated for 1–2 min at 80 °C and were added to GTP hydrolysis assays to give a final concentration of 100 μM.

Protein Lipid Overlay Assay—Overlay assays were performed as previously described (33). Nitrocellulose-immobilized phospholipids (PIP strips; Echelon Biosciences) were blocked by incubation for 1 h with 1% nonfat milk powder in PBST (137 mM NaCl, 2.37 mM KCl, 4.3 mM Na$_2$HPO$_4$, 1.4 mM KH$_2$PO$_4$, 1% Tween 20). All incubations were carried out at room temperature. We incubated 10 ml of PBST supplemented with 1% nonfat milk powder and 10 μg of either wild-type Rgd1p or one of its truncated forms, all of which were GST-tagged, for 1 h with the PIP strips. The PIP strips were washed four times with PBST and incubated for 1 h with a 1:10,000 dilution of anti-GST antibodies (Sigma) in PBST supplemented with 1% nonfat milk powder. The membrane was then washed four times and incubated with a 1:10,000 dilution of horseradish peroxidase-conjugated anti-mouse antibodies (Pierce). Bound antibodies were detected by chemiluminescence with the Lumi-lightPLUS substrate (Roche Applied Science) and the FluorChem 8800 Imaging System (Alpha Innotech).

RESULTS

Phospholipids Strongly Activate GTP Hydrolysis by Rho4, but Not by Rho3, in the Presence of Rgd1p—Phospholipids can affect GTase-activating protein activity, and may favor the activity of some GTPases over others (28, 31, 32). We evaluated the effects of phospholipids on GTP hydrolysis by the Rho3p and Rho4p GTPases. We first measured the intrinsic GTase activity of Rho3p and Rho4p purified from yeast independently in the presence of individual phospholipids: phosphatidylycerine, phosphatidylcholine, PtdIns, PtdIns(4)P, and PtdIns(4,5)P$_2$. We decided to study phosphoinositides, because these molecules are involved in signal transduction and have different subcellular distributions (34). Phosphatidylycerine was studied, because it has been reported to bind the F-BAR domain (22), and phosphatidylcholine was studied as the main phospholipid component of cellular membranes (35). The addition of phospholipids to assays had no effect on the intrinsic GTase activity of prenylated Rho3p or Rho4p (data not shown). We then carried out the same experiment in the presence of Rgd1p (Fig. 1). To allow the detection of stimulation by phospholipids, we chose a GAP concentration that gave activation of GTP hydrolysis further from maximal activity of Rho proteins. For Rho3p, all of the phospholipids tested slightly increased GTase activity, by a factor of 1.1–1.5, the greatest stimulation being observed with PtdIns(4)P and the weakest stimulation with PtdIns(4,5)P$_2$. The effects of phospholipids on the GTP hydrolysis obtained with prenylated Rho4p in the presence of Rgd1p were clearly different from those on Rho3p. The addition of phosphatidylycerine had no effect, whereas GTase activity was strongly increased by PtdIns(4)P and phosphatidylycerine (by factors of 5 and 4, respectively) and by PtdIns and PtdIns(4,5)P$_2$ (by a factor of 3; Fig. 1). These results indicated a role for Rgd1p in the response to phospholipids and an effect of these molecules on RhoGAP activity. The prenylation of both Rho3 and Rho4 also appeared to be important for activation by phospholipid...
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FIGURE 1. Effect of phospholipids on the RhoGAP activity of Rgd1p with Rho3p and Rho4p. GTP hydrolysis was performed with 300 nM of prenylated Rho3p (top) or 400 nM of prenylated Rho4p (bottom) and with an Rgd1p concentration equimolar to that of the GTPase. Phosphatidylinerine (PS), phosphatidylycholine (PC), PtdIns (Pi), PtdIns(4)P (Pi(4)P), and PtdIns(4,5)P2 (Pi(4,5)P2) were added to the assay at a final concentration of 100 μM. Error bars, S.D. of duplicate assays in a given experiment. This profile is representative of several experiments.

ids. Indeed, phospholipids had a less marked effect if the Rho proteins used had been purified from bacteria. Indeed, no phospholipid stimulation of nonprenylated Rho3p was observed, and for the nonprenylated Rho4p with Rgd1p, hydrolysis was increased by a factor of 1.8–2.5 by PtdIns(4,5)P2, PtdIns, PtdIns(4)P, and PS (data not shown). These results are consistent with previous data demonstrating the importance of prenylation in promoting interactions between Rho-family small GTPases and their GTPase-activating proteins (28, 36). Two other experiments done with the prenylated Rho4p in different conditions were carried out to assess the strength of phosphoinositide stimulation. In the first experiment, a PtdIns(4)P concentration 1 order of magnitude lower than that originally used was tested (10 μM instead of 100 μM). In these conditions, stimulation was observed, with a doubling of activity despite the much lower concentration of phosphoinositide (data not shown). In the second experiment, the concentration of Rgd1p was decreased to one-tenth that of Rho4p. This resulted in a lower level of GAP activity, as expected, but the addition of PtdIns(4)P clearly stimulated (and indeed almost doubled) GTP hydrolysis by Rho4p (data not shown). These data indicate that phospholipids have a strong, specific effect on Rgd1p/Rho4p, potentially modulating GAP activation in different conditions intracellularly.

Definition of the Region of Rgd1p Mediating PtdIns(4)P-stimulated GAP Activity—We tried to identify the region of Rgd1p involved in the phospholipid-mediated regulation of GAP activity by constructing various N-terminally truncated forms of Rgd1p that nonetheless still contained the RhoGAP domain (Fig. 2A). We investigated the GAP activity of these truncated forms with nonprenylated and prenylated Rho4p in the presence and absence of PtdIns(4)P (Fig. 2B). As expected, all of the protein constructs displayed GAP activity. However, in the absence of phospholipid, the GAP activity observed with prenylated or unprenylated Rho4p was stronger with the truncated Rgd1p proteins than with the full-length protein. The Rgd1Δ1–450 form, consisting essentially of the RhoGAP domain, activated Rho4p GTPase activity the most strongly, suggesting that the N-terminal part of Rgd1p may in some way limit the activity of the RhoGAP domain.

GTP hydrolysis by unprenylated Rho4p was monitored in the presence of PtdIns(4)P. In these conditions, the increase in GAP activity observed with Rgd1Δ1–300 was slightly smaller than that observed with the full-length Rgd1p (Fig. 2B and Table 2). The removal of the additional residues between positions 300 and 450 abolished the activation of GAP activity by PtdIns(4)P. Thus, amino acids 300–350 of Rgd1p seem to play a major role in the PtdIns(4)P-mediated stimulation of GAP activity. A same analysis was carried out with prenylated Rho4p, and the overall response was similar to that obtained with the unprenylated Rho protein. The Rgd1Δ1–300 protein displayed stimulation, as observed with the wild-type form (Table 2). However, with Rgd1Δ1–350 and Rgd1Δ1–450, which did not lead to PtdIns(4)P stimulation with the unprenylated Rho4p, lower levels of activation were observed with the prenylated Rho4p, with similar decreases in activation observed for both truncated forms (Fig. 2B and Table 2). These observations with prenylated Rho4p also showed that amino acids 301–350 of Rgd1p were important for PtdIns(4)P stimulation. The existence of residual activation not seen with unprenylated Rho4p suggested that over and above the action of the Rgd1p-specific region, prenylation of the RhoGAP domain played an important role in the stimulation mediated by PtdIns(4)P.

We explored the region of Rgd1p involved in the response to phospholipids in more detail, by constructing a RhoGAP protein lacking amino acids 301–350. We assayed the GAP activity of this mutant protein in independent experiments with PtdIns(4)P and prenylated or unprenylated Rho4p. This deletion halved the effect of PtdIns(4)P on unprenylated Rho4p, the PtdIns(4)P stimulation factor being 1.69 ± 0.37 rather than 2.97 ± 0.19, as for the wild-type Rgd1p. However, the effects of phospholipid were not entirely abolished, by contrast to what was observed with the truncated form Rgd1Δ1–350. The results obtained with prenylated Rho4p were similar to those
obtained with the unmodified GTPase. The PtdIns(4)P stimulation factor was 2.38 ± 0.13, versus 4.23 ± 0.56 for the wild-type Rgd1p. In both cases, deleting amino acids 301–350 of Rgd1p halved the effect of PtdIns(4)P. Thus, this internal sequence of Rgd1p is clearly involved in regulating the stimulatory effects of PtdIns(4)P on RhoGAP but probably acts together with other parts of the Rgd1p protein closer to the N terminus.

The N-terminal Region of Rgd1p Is Required for Interaction with Phosphoinositides—We investigated the physical interaction of Rgd1p with phospholipids through a protein lipid overlay assay. The GST-tagged Rgd1p and various GST-truncated forms of Rgd1p were used to probe nitrocellulose-immobilized phospholipid membrane (PIP strips), and bound proteins were visualized by Western blotting with anti-GST antibodies (Fig. 3). For the full-length Rgd1p, solid binding of the protein to all monophosphorylated forms of phosphatidylinositides (phosphatidylinositol 3-phosphate, PtdIns(4)P, phosphatidylinositol 5-phosphate) was observed. A strong interaction with PtdIns(3,5)P2 was also observed. In addition, a weak signal was obtained for the binding of Rgd1p to phosphatidylinositol 3,4-bisphosphate, PtdIns(4,5)P2, and phosphatidylinositol 3,4,5-trisphosphate. A similar binding pattern was observed with the deleted Rgd1Δ301–350 form. By contrast, no binding was observed with either Rgd1Δ1–300 or Rgd1Δ1–450.

Thus, amino acids 1–300 are clearly essential for the physical binding of Rgd1p to phospholipids and, more specifically, to phosphoinositides. Amino acids 300–350 of Rgd1p play a key role in PtdIns(4)P-stimulated GAP activity but do not seem to be involved in phospholipid binding. These data are consistent with the identification of an F-BAR domain (aa 1–300) at the N-terminal extremity of Rgd1p, which is known to interact with some phospholipids (21, 22). The F-BAR domain includes the FCH domain and coiled-coil regions. To carry on exploring phospholipid binding, we tested the GST-tagged Rgd1p only deleted for the FCH domain (aa 1–156). No binding was detected indicating the determining role of FCH in interaction with phospholipids (Fig. 3).

The N-terminal Region of Rgd1p Containing the F-BAR Domain Is Necessary for Correct Localization in Vivo—Phosphoinositides are concentrated at the cytosolic surfaces of membranes, and each has a unique subcellular distribution, being localized principally in certain subsets of membranes (37). Phosphoinositides are involved in direct signaling, through the binding of their head groups to cytosolic proteins or the cytosolic domains of membrane proteins (38). The presence of an F-BAR domain, a domain known to interact with phospholipids, at the N terminus of Rgd1p suggested that this region might be crucial for localization of the RhoGAP protein in yeast cells. We therefore investigated the effect of this region on the subcellular distribution of Rgd1p.

We localized Rgd1p in the wild-type BY4742 background by tagging the protein with three GFP molecules at its carboxyl
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terminus. Rgd1p was found mostly in areas of polarized growth during cell cycle progression (Fig. 4A). It was found in the bud emergence area in the G1 phase and in the bud tip during the S and G2 phases. During isotropic bud growth, Rgd1p was detected in dense patches resembling intensely fluorescent crescents under the cortex at the bud tip. During M phase and cytokinesis, Rgd1p was undetectable at the bud tip and was instead found only at the bud neck. At this location, Rgd1p formed a single, intensely stained ring. After cytokinesis, Rgd1p was distributed asymmetrically between mother and daughter cells. Many patches of staining were observed around the scar in the mother cell, whereas the fluorescent signal tended to take the form of a continuous line facing the scar in the daughter cell.

Deletion of the N terminus of the protein (Rgd1Δ1–300 and Rgd1Δ1–156) abolished binding to phosphoinositides on PIP strips. We analyzed the subcellular distribution of Rgd1p from which the entire FCH domain and part of the coiled-coil region (aa 1–156) belonging to the F-BAR domain had been deleted. This deletion led to mislocalization of all of the Rgd1p-3×GFP protein present, throughout the cell cycle, demonstrating the importance of this sequence in controlling the distribution of Rgd1p in S. cerevisiae. No specific fluorescence was observed at the bud tip and neck. Instead, a diffuse, heterogeneous signal was observed over the entire cell surface, with many apparently randomly distributed dots of staining (Fig. 4B). By contrast, microscopic analysis of the RGD1Δ486–660 mutant showed that the RhoGAP domain was not required for the correct distribution of Rgd1p; indeed, this deleted form had a distribution identical to that of the wild type in yeast cells (Fig. 4B).

We also showed that the removal of residues 301–350 of Rgd1p decreased the enhancement by PtdIns(4)P of the stimulation by Rgd1p of the GTPase activity of both unprenylated and prenylated Rho4p. This internal region was not involved in phospholipid binding, but we nonetheless investigated whether the Rgd1Δ301–350 deleted protein with three GFP tags was correctly distributed, to determine whether this stimulatory region was involved in controlling the distribution of Rgd1p. The percentage of cells in which the Rgd1p mutant protein was located at the bud tip and neck was lower than for the wild-type protein (for the mutant protein, 38 ± 8% was at the bud tip and 8 ± 2% was at the bud neck, whereas for the wild-type protein, 58 ± 6% was at the bud tip and 12 ± 1.6% was at the bud neck). Consistent with these observations, the fluorescence signal seemed to be more diffuse and slightly stronger in the cytoplasm of other Rgd1Δ301–350 cells (54%), in which the tagged protein was located outside the bud tip and bud neck. A smaller proportion of the truncated Rgd1p than of the wild-type protein was correctly distributed, but our findings nonetheless indicate that the deletion of amino acids 301–350 did not prevent the truncated form from reaching sites of polarized growth, as in the reference strain. Thus, despite a lack of physical interaction with phospholipids, loss of the region stimulating GAP activity appears to play a role in controlling the localization of Rgd1p, perhaps by maintaining RhoGAP at sites of polarized yeast growth.

**PtdIns(4)P and PtdIns(4,5)P_2 Pools Are Required for Normal Rgd1p Localization**—We explored the role of phosphoinositides further by examining the distribution of Rgd1p in mutants with impaired phosphoinositide synthesis. We specifically focused on the impact of PtdIns(4)P levels, because this molecule had the strongest effect on GAP activity and a strong physical interaction with Rgd1p. In S. cerevisiae, most of the PtdIns(4)P is generated by two PtdIns 4-kinases, Pik1p and Stt4p, and cells carrying the heat-sensitive *pik1*-83ts or *stt4*-4ts mutation have very low PtdIns(4)P levels, about half those of the wild type (39). Each enzyme generates a discrete pool of PtdIns(4)P regulating different essential biological processes. Pik1p is thought to supply the pool of phosphoinositides required for the

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**FIGURE 3. Interaction of Rgd1p with phospholipids, as revealed by protein-lipid overlay assays.** PIP strips were spotted with 100 pmol of lysophosphatidic acid (LPA), lysophosphocholine (LPC), PtdIns (PI), phosphatidylinositol 3-phosphate (PI(3)P), PtdIns(4)P (PI(4)P), phosphatidylinositol 5-phosphate (PI(5)P), phosphatidylethanolamine (PE), phosphatidylycerol (PC), sphingosine 1-phosphate (S1P), phosphatidylinositol 4,5-biphosphate (PI(4,5)P2), phosphatidylglycerol (PG), and phosphatidylinositol 3,4,5-trisphosphate (PI(3,4,5)P3). GST-tagged Rgd1p or truncated forms were incubated with the membrane and bound proteins were detected by Western blotting with anti-GST antibodies, as described under “Experimental Procedures.”

**FIGURE 4. Localization of wild-type and mutant Rgd1-3×GFP proteins.** A, the wild-type Rgd1-3×GFP was observed by fluorescence microscopy in an asynchronous cell population. The various phases of the cell cycle were reconstructed based on cell morphology and bud size. B, microscopy of Rgd1p forms with ΔFCH (Δ1–156) and ΔGAP (Δ486–660) deletions. We observed three independent clones for each mutant, and the images obtained are representative of the entire cell population.
Rgd1p at the bud tip was slightly lower in the Rgd1p, particularly at the bud tip. The proportion of cells with tip and neck in 87% of mutant cells, Rgd1p were detected (Fig. 5). Rdg1p was found outside the bud (3 h at 38 °C), many highly fluorescent, mislocalized patches of the wild-type strain. By contrast, at nonpermissive temperature the distribution of Rgd1p was very similar to that observed in PtdIns(4)P and PdtIns(4,5)P2 synthesis. RDG1 and STT4 genes encode the enzymes that synthesize the pool of PtdIns(4)P used to maintain cell wall integrity and for actin cytoskeleton organization (39, 41). 

FIGURE 5. Localization of Rgd1p in mutants with impaired PtdIns(4)P and PtdIns(4,5)P2 synthesis. Wild-type (WT) (SEY6210) and mutant (AAy104, AAy202, and CTY1568) cells producing Rgd1p were cultured at permissive temperature (26 °C) and shifted to nonpermissive temperature (38 °C) for 3 h. Bars indicate the S.D. of three measurements for each strain.

Golgi-to-plasma membrane steps of the secretory pathway (39, 40), whereas Stt4p is present in the plasma membrane, where it synthesizes the pool of PtdIns(4)P used to maintain cell wall integrity and for actin cytoskeleton organization (39, 41).

In the pik1–83ts mutant at permissive temperature (26 °C), the distribution of Rgd1p was very similar to that observed in the wild-type strain. By contrast, at nonpermissive temperature (3 h at 38 °C), many highly fluorescent, mislocalized patches of Rgd1p were detected (Fig. 5). Rgd1p was found outside the bud tip and neck in 87% of mutant cells, versus only 35% of wild-type cells. Rgd1p was located in the bud tip in far fewer mutant cells than cells of the reference strain (8 and 55%, respectively). By contrast, the percentage of cells in which Rgd1p was located at the bud neck was similar in the mutant and wild-type strains at nonpermissive temperature (8 and 10%, respectively). These results demonstrate the importance of the amount of PtdIns(4)P generated by PIKI1 for the correct distribution of Rgd1p, particularly at the bud tip. The proportion of cells with Rgd1p at the bud tip was slightly lower in the stta4–4ts mutant at permissive temperature than in the wild-type strain. Nevertheless, shifting the stta4–4ts cells to 38 °C for 3 h did not really amplify this difference with respect to the control strain, implying the absence of a clear role for STT4 in Rdg1p location. Thus, although PtdIns(4)P levels in the pik1–83ts and stta4–4ts mutants at 38 °C were about half those of the reference strain (39), our results highlight the specific effects of the PIKI1 gene in controlling Rdg1p location.

GAP activation in overall yeast growth has not yet been studied. The RhoGAP encoded by RGD1 in S. cerevisiae positively regulates the GTPase activity of both Rho3p and Rho4p (12). Its cellular distribution depends on cell cycle progression and is consistent with its RhoGAP function on Rho3p and Rho4p. Given the diverse biological processes in which Rho3p and Rho4p are involved (8, 14, 15, 43, 44), the activity of these GTPases is likely to be strongly and differently controlled by their regulatory proteins. Consistent with this hypothesis, some phospholipids were found to have specific effects, enhancing the stimulation by Rgd1p of Rho4p GTPase activity. However, the level of stimulation differed between the phospholipids tested. Phosphatidylserine and PtdIns(4)P gave the largest increase in activity, whereas phosphatidycholine had no effect on GAP activity. By contrast, none of the phospholipids tested enhanced the stimulatory effects of Rgd1p on Rho3p to any great extent. Phospholipids have already been reported to regulate GAP activity in vitro. The RacGAP activity of n-chimaerin is inhibited by some phospholipids and stimulated by others (32). Similarly, phospholipids strongly influence the GAP activity of the p190A and p190B RhoGAPs with both RhoA and Rac1 GTPases (28). In our study, lipids regulated the catalytic activity of the Rgd1p RhoGAP in a Rho-dependent manner, by changing both specificity and the extent of the stimulatory effect. This specific response must be linked to the structural properties of the Rho3p and Rho4p GTPases. Although both GTPases possess the typical regions for guanine nucleotide binding, Rho3p and Rho4p present some differences. Rho4p has a polybasic region (aa 281–287) preceding the CAAX box at the C-terminal end. The polybasic region present in Ras and Rho GTPases has been reported to control diverse functions, including binding to membranes, interactions with specific proteins, and localization in particular subcellular compartments (45, 46). Reciprocal replacement of the polybasic region of Rho4p by the equivalent region of Rho3p had no effect on the response of Rgd1p/Rho4p and Rgd1p/Rho3p to phospholipids. Other regions of Rho4p must therefore be involved in the stimulation effect.

The physiological significance of phospholipid-mediated GAP activation in overall yeast growth has not yet been studied.
Regulation of Rgd1p by Phosphoinositides

However, the principal consequence of the stimulation effect could be a decrease in the abundance of active Rho4p and, thus, in Rho4-dependent signal transduction. Rho4p has been reported to regulate the interaction between Hof1p and Bnr1p, two proteins involved in cytokinesis and localized at the bud neck, in a GTP-Rho4p-dependent manner (47). Decreasing the amount of active Rho4p might, therefore, rapidly restrict the interaction between Hof1p and Bnr1p proteins, leaving Hof1p free and allowing cytokinesis to progress. Indeed, Hof1p degradation at the end of mitosis is important for efficient contraction of the actomyosin ring and cell separation (48).

We showed, with various truncated forms of Rgd1p and assays of RhoGAP activity and PIP strips, that the N-terminal part of this protein plays a determinant role in interactions with phospholipids. The N-terminal end of the protein (aa 1–156) is essential for physical interactions with the various phospholipids in PIP strip assays. This protein-lipid interaction was also detected by the identification of Rgd1p as a phosphoinositide-binding protein in another approach based on yeast proteome chips (49). In addition, the need for the N-terminal region (aa 1–156) for phosphoinositide binding is consistent with the identification of this sequence belonging to an F-BAR domain. Removal of the internal region (aa 301–350) also halved the stimulatory effect on GAP activity. Finally, phosphoinositides act at two different levels: binding to Rgd1p through the F-BAR domain and stimulating GAP activity, mostly via the internal region (aa 301–350). Elimination of the F-BAR domain (aa 1–300) was not sufficient to abolish the stimulation effect (see Table 2). We therefore suggest that the internal region may also interact with phospholipids but that this interaction is too weak for detection in PIP strip assays. The enrichment of the internal sequence in basic residues is consistent with this hypothesis. This suggests that, after lipid binding, the internal region transmits a structural modification to the C-terminal part of the protein. This structural change favors the activation of GTP hydrolysis specifically for Rho4p, either stabilizing the interaction between Rgd1p and Rho4p or increasing the rate of catalysis. The results obtained with the unprenylated GTPase are consistent with this interpretation. However, analyses with the prenylated Rho4 GTPase showed that the C-terminal region (aa 350–666) nonetheless responded in part to the lipid environment. The known role of prenylation in strengthening the actomyosin ring and cell separation (48).

Members of the F-BAR family are recruited from the cytoplasm to trigger the formation of plasma membrane extension, invaginations, tubular organelles, and transport intermediates, including endocytic vesicles (21–23, 51). In silico Rgd1p analysis showed that the N-terminal region adopted the conformation typical of F-BAR domains, with five helices. The mislocalization of the mutant RhoGAP with a deletion of the FCH domain and of part of the coiled-coil domain is consistent with the known role of F-BAR in interactions with cellular membranes. Our data show that this domain is essential for the localization of Rgd1p to cellular membranes at the bud tip and neck. This domain may be the key element for the correct targeting of the RhoGAP domain close to GTPase sites, where GAP activation takes place. However, deletion of the internal region (aa 301–350) led to a decrease in the proportion of correctly distributed proteins. However, this sequence does not seem to be necessary for directing the protein to the correct location, since some of the mutant protein nonetheless reached the bud tip and neck. We hypothesize that, in the absence of this sequence, the interaction between the RhoGAP and GTPases may be weakened, resulting in the more rapid release of the RhoGAP into the cytoplasm.

The considerable mislocalization of Rgd1p observed in the temperature-sensitive msst4-102 mutant, which had very low levels of PtdIns(4,5)P₂ in the plasma membrane (42), indicates that PtdIns(4,5)P₂ is essential for the correct location of Rgd1p at the bud tip. Our data indicate that Rgd1p is localized at the plasma membrane through binding to PtdIns(4,5)P₂ mediated by the F-BAR domain. However, this may be the case only in the bud tip, since the localization defect in the msst4-102 mutant appears limited to this area. We demonstrated that the F-BAR domain is also essential for the localization of RhoGAP at the bud neck. This domain may interact with other phosphoinositides or act in other ways to localize the protein at the bud neck. PtdIns(4)P depletion also disrupted the polarized localization of Rgd1p in the bud tip. This defect, which was observed only for the pik1 mutant and not in stt4 mutant, cannot be an indirect effect due to the disturbance of the cellular lipid homeostasis. Thus, given the clear role of Pik1p and Stt4p PtdIns 4-kinases, our observations in the pik1 mutant suggest that Rgd1p must initially bind to the cytoplasmic face of Golgi membranes via the binding of F-BAR to PtdIns(4)P, being then transported to the plasma membrane via the secretory pathway. At the plasma membrane, the protein can bind to the biphosphorylated phosphatidylinositol. Several proteins specifically require functional Pik1p and PtdIns(4)P and thus are selectively recruited to the Golgi apparatus in vivo (42, 52, 53), as is the Kesi1p protein, which is mislocalized to the cytoplasm in the pik1 mutant but not in stt4 cells (54). In agreement with our interpretation, Rgd1p was correctly localized in a mutant blocked in endoplasmic reticulum to Golgi transport.⁴

Phosphoinositides have a structural and a signaling role via their recruitment of proteins. Recently, it was reported that the amount of phosphoinositides increases at low pH, and it was proposed that PtdIns(4,5)P₂ facilitates actin repolarization and up-regulates fluid phase endocytosis after low pH shock (55). Given these data and the sensitivity of rgd1Δ cells to acidic environment, we can imagine that a PtdIns(4,5)P₂ increase facilitates recruitment of Rgd1p to plasma membrane through F-BAR domain. This recruitment would result in decrease of cell signaling and growth mainly through Rho3p, located at the bud tip, to allow physiological adaptation of yeast cells at low pH.

This work provides the first demonstration of a dual mode of action of phosphoinositides on a RhoGTPase-activating pro-
tein containing an F-BAR domain. Phosphoinositides regulate the trafficking of Rgd1p and its recruitment to membranes and stimulate GAP activity, suggesting a functional interplay between lipids, the RhoGAP, and the GTPases with which it interacts. Heath and Insall (56) recently described abnormal vacuoles in the rgdΔ mutant. It would therefore seem possible that Rgd1p controls other biological processes. Further analyses are required to investigate the multifunctional aspects of this RhoGAP in more detail and to discover the fine structural determinants of Rgd1p interacting with phosphoinositides.

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