Lipid Products of Phosphoinositide 3-Kinase Interact with Rac1 GTPase and Stimulate GDP Dissociation*

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# Lipid Products of Phosphoinositide 3-Kinase Interact with Rac1 GTPase and Stimulate GDP Dissociation#

A number of reports suggest that under different conditions leading to cytoskeleton reorganization the GTPase Rac1 and possibly RhoA are downstream targets of phosphoinositide 3-kinase (PI-3-kinase). In order to gain more insight into this particular signaling pathway, we have addressed the question of a possible direct interaction of PI-3-kinase products with the Rho family GTPases RhoA, Rac1, and Cdc42. Using recombinant proteins, we found that Rac1 and, to a lesser extent, RhoA but not Cdc42 were capable to selectively bind to phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P3) in a mixture of crude brain phosphoinositides. Nucleotide-depleted Rac1 was the most efficient, but the GDP- and GTP-bound forms retained significant PtdIns(3,4,5)P3 binding activity. This protein-lipid association involved electrostatic as well as hydrophobic interactions, since both phosphate groups located at specific positions of the inositol ring and fatty-acyl chains were absolutely required. Based on the sequence of Rac1, two potential binding sites were identified, one at the C terminus and one in the extra α-helical domain. Deletion of these two domains resulted in a complete loss of binding to PI-3-kinase products. Finally, PtdIns(3,4,5)P3 strongly stimulated GDP dissociation from Rac1 in a dose-dependent manner. In agreement, data obtained in intact cells suggest that PtdIns(3,4,5)P3 might target Rac1 to peculiar membrane domains, allowing formation of specific clusters containing not only small GTPases but other partners bearing pleckstrin homology domains such as specific exchange factors required for Rac1 and RhoA activation.

It is now well established that PI-3-kinases1 play an essential role in cell signaling and in the regulation of a number of cellular functions, including proliferation, differentiation, apoptosis, cytoskeleton organization, or membrane traffic. PI-3-kinase products. Finally, PtdIns(3,4,5)P3 strongly stimulated GDP dissociation from Rac1 in a dose-dependent manner. In agreement, data obtained in intact cells suggest that PtdIns(3,4,5)P3 might target Rac1 to peculiar membrane domains, allowing formation of specific clusters containing not only small GTPases but other partners bearing pleckstrin homology domains such as specific exchange factors required for Rac1 and RhoA activation.

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In conclusion, there is growing evidence that PI-3-kinase products and small G-proteins interact in a complex manner, leading to the activation of Rac1 and possibly RhoA. This interaction may play a key role in the regulation of cell signaling and cytoskeleton organization.

1. Three different classes of enzymes have been identified so far, each of them displaying an increasing complexity as well as diverse mechanisms of regulation. (1) Class III PI-3-kinase with a substrate specificity restricted to PtdIns appears to participate mainly in the regulation of constitutive membrane trafficking and vesicle morphogenesis pathway. Whereas little is known about class II PI-3-kinases, class I enzymes are clearly involved in cell signaling. Although they might be regulated by different mechanisms, they have in common two main features. (i) They are heterodimers containing a regulatory subunit (p85, p55, p50, p101) and a 110-kDa catalytic subunit, at least four isoforms of p110 being characterized so far; (ii) all of them use mainly PtdIns(4)P and PtdIns(4,5)P2 as substrates, leading to the formation of PtdIns(3,4)P2 and PtdIns(3,4,5)P3, respectively. These two D-3 phosphoinositides accumulate in cells stimulated by various agonists or transformed by oncogenes such as v-src, they are insensitive to phospholipases C, and are considered as potential second messengers (2).

Several recent studies reported a number of signaling proteins able to interact with D-3 phosphoinositides, resulting in their membrane targeting and/or in the modulation of their enzymatic activity. These include nonclassical protein kinase C (2) as well as some proteins bearing Src homology 2 (3) or PH domains (2, 4), the PH domain-containing protein kinase B (or Akt) being of special interest (1, 5). In addition, several lines of evidence previously suggested a possible link between PI-3-kinase and Rac1, a small GTPase of the Rho family (6–9). These small GTPases are known to regulate the organization of actin cytoskeleton (10). Interestingly, reorganization of actin cytoskeleton induced by PDGF or insulin is impaired upon inhibition of PI-3-kinase (11). Moreover, constitutively active mutants of PI-3-kinase α promoted a rearrangement of the actin filament system similar to that observed in response to activated Rac1 or RhoA (12). It is thought that PI-3-kinase increases guanine nucleotide exchange on Rac (6, 8). Although regulators of Rho GTPases bearing PH domains may be involved in these effects (13), the precise mechanism by which PI-3-kinase activates in vitro the GDP/GTP exchange on these small G-proteins remains poorly understood.

Further evidence for a close relationship between PI-3-kinases or their products and small G-proteins of the Rho family came from in vitro experiments showing, for instance, a binding of PI-3-kinase α to the GTP-bound forms of Rac1 and Cdc42 (14). On the other hand, although D-3 phosphoinositides were not specifically considered, their precursor PtdIns(4,5)P2 was proposed to directly stimulate the dissociation of GDP from Cdc42 and possibly RhoA (15), suggesting a direct interaction of this phosphoinositide with these small G-proteins. Finally, both RhoA and Rac1 were shown to activate a PtdIns(4)P 5-kinase in different models, including platelets (16). Whether...
this contributes to the stimulation of PI 3-kinase by RhoA observed in platelet extracts (17) is presently unknown.

In order to gain more insight into the possible role of PI 3-kinase in regulating Rho GTPases, we have addressed the question of a direct interaction between D-3 phosphoinositides and RhoA, Rac1, or Cdc42. Using various assay conditions and recombinant proteins, we found that Rac1 and, to a lesser extent, RhoA were capable to selectively bind to PtdIns(3,4,5)P_3 via two potential binding sites. In contrast to the interaction observed with PH domains, both the specific positions of the phosphate group on the inositol ring and the fatty-acid chains were required for this binding. Moreover, PtdIns(3,4,5)P_3 but not PtdIns(4,5)P_2 had in vitro a potent GDP-releasing activity on Rac1, suggesting a potential cooperative effect of this phosphoinositide and of exchange factors like Vav (13) or other regulators. Finally, we provide evidence for a physiologic significance of these results in EGF-stimulated Vero cells.

EXPERIMENTAL PROCEDURES

Reagents—PtdIns, PtdIns(4)P, PtdIns(4,5)P_2, crude brain phosphoinositides, and PS were purchased from Sigma. The diC16-PtdIns(4,5)P_2 and diC16-PtdIns(3,4,5)P_3 were from Matreya, Inc. Ptd[2-3H]Ins(4)P, Ptd[2-3H]Ins(4,5)P_2 (1 Ci/mmol), [3H]GDP (12.9 Ci/mmol), [35S]GTP-[\gamma-S] (1 Ci/mmol), and [32P]orthophosphate were from Amersham Pharmacia Biotech, and [\gamma-32P]ATP (3000 Ci/mmol) from NEN Life Science Products (France). The EGF (receptor grade) was obtained from Calbiochem. TLC plates were from Merck. Transformed JM101 Escherichia coli containing cDNAs encoding human Rac1, RhoA, and Cdc42 were generous gifts from Prof. A. Hall (University College, London, United Kingdom). The cDNA encoding the insertion domain deletion mutant of Rac1 protein (18) was a generous gift of Dr. O. Lotan (Sackler Faculty of Medicine, Tel Aviv, Israel) and was examined by loading the latter (about 50 µg)onto nitrocellulose filters (Millipore). Filters were washed three times with 2 mM Tris-HCl, pH 7.0, 0.02% Nonidet P-40, and finally 1 ml of saline buffer containing 0.02% Nonidet P-40, 1 ml of saline buffer containing 0.01% Nonidet P-40, 1 ml of saline buffer containing 0.01% Nonidet P-40, and 1 ml of saline buffer containing 0.02% Nonidet P-40. The remaining radioactivity associated with the beads was counted by scintillation (TRI-CARB, 1900 TR, Packard) and specific binding was calculated by subtracting the non-specific interaction with GST alone (5–10%). For the lipid selectivity experiments, bound lipids were extracted following a Bligh and Dyer modified procedure and resolved by TLC using chloroform/acetone/methanol/acetic acid/H_2O (80/30/26/24/14, v/v) as a solvent. For experiments performed with physiological concentrations of divalent cations, radiolabeled phosphoinositides were resuspended and sonicated in 10 mM Hepes, pH 7.0. The GST fusion proteins were washed twice with saline buffer (10 mM Hepes, pH 7.0, 80 mM KCl, 15 mM NaCl, 0.466 mM CaCl_2, 2.1 mM MgCl_2) (4) and resuspended in 30 µl of 2× saline buffer. Then 30 µl of radiolabeled phosphoinositides were added to the fusion proteins. After 40 min of incubation at room temperature, the beads were quickly washed twice with 1 ml of HNE supplemented with 0.5% Nonidet P-40. The GST fusion proteins (about 15 µg) were washed twice with saline buffer (10 mM Hepes, pH 7.0, 80 mM KCl, 15 mM NaCl, 0.466 mM CaCl_2, 2.1 mM MgCl_2) (4) and resuspended in 30 µl of 2× saline buffer. Then 30 µl of radiolabeled phosphoinositides were added to the fusion proteins. After 40 min of incubation at room temperature, the beads were quickly washed twice with 1 ml of saline buffer, 1 ml of saline buffer containing 0.005% Nonidet P-40, 1 ml of saline buffer containing 0.01% Nonidet P-40, and finally 1 ml of saline buffer containing 0.02% Nonidet P-40. The remaining radioactivity associated with the beads was analyzed as described above.

Binding with Nucleotide-free or -bound Form of Rac1—Proteins were first depleted of nucleotide for 15 min at room temperature in a buffer containing 20 mM Tris-HCl, pH 7.4, 1 mM dithiothreitol (DTT), 2 mM EDTA, and 50 mM NaCl. When indicated, loading was started with GDP or GTPγS (100 µM) in HNE buffer (150 mM NaCl, 0.466 mM CaCl_2, 2.1 mM MgCl_2) (4). Rac1 was reconstituted with MgCl_2 at a final concentration of 1 mM. Binding with PtdIns(3,4,5)P_3 was carried out in HNE buffer as described above, except that 1 mM MgCl_2 was maintained during the time course of the experiment, accordingly the GTPases were in the expected forms (GDP- or GTP-bound, or nucleotide-free).

Determination of the Apparent Affinity Constant—For the determination of the dissociation constant, in vitro synthesized [\alpha-32P]PtdIns(3,4)P_2 was first TLC-purified and mixed with adequate amounts of unlabeled diC16-PtdIns(3,4)P_2 (about 100 pmol/dm), dried under nitrogen at 37 °C for 40 min, and resuspended in HNE buffer as described above. The concentration of diC16-PtdIns(3,4)P_2 was always assessed by phosphorus determination (24) and the purity of [\alpha-32P]PtdIns(3,4)P_2 by high performance liquid chromatography analysis (21).

GDP Dissociation Assay—The effect of phosphoinositides on the dissociation of [H]GDP from purified bacterial Rho family G-proteins were examined by loading the latter (about 5 µg) with 10 µM [H]GDP (20 Ci/mmol) for 15 min at room temperature in a buffer containing 20 mM Tris-HCl, pH 7.4, 1 mM DTT, 50 mM NaCl, and 1 mM EDTA (final volume 60 µl). Loading was stopped by adding MgCl_2 at 2.5 mM final (25). Then, 4 µl of [H]GDP-reconstituted proteins were incubated with 16 µl of buffer mixtures containing 20 mM Tris-HCl, pH 7.4, 1 mM DTT, 50 mM NaCl, 2 mM MgCl_2, and various lipids at different concentrations, for the indicated times at room temperature. Assays were stopped by dilution into 1 ml of ice-cold 20 mM Tris-HCl, pH 7.4, 50 mM NaCl, 2 mM MgCl_2 and were immediately filtered on 25-mm HAWP nitrocellulose filters (Millipore). Filters were washed three times with 2

PI 3-Kinase Products and Rho GTPases

Expression and Purification of GST Fusion Proteins—Transformed E. coli were grown in Luria-Bertoni medium containing 50 µg/ml ampicillin and induced for 3 h with 0.1 mM isopropyl-\beta-D-thiogalactopyranoside. Expressed G-proteins of the Rho family were then purified following the protocol described by Self and Hall (19); they were migrated as single bands at the expected molecular mass on SDS-PAGE. The protein concentration was determined on a 12.5% SDS-PAGE with bovine serum albumin as a standard and staining with a Colloidal Coomassie staining kit (Novex) as described (19). The quantification was performed by a densitometric analysis (Gel Doc 1000, Molecular Analyst, Bio-Rad).

Construction of Recombinant Mutants of Rac1—The C-terminal truncation mutant missing the last 33 amino acids (\DeltaCt Rac1) and the double mutant corresponding to the \DeltaCt Rac1 lacking the insert region (residues 205–267) (\DeltaDI-\DeltaCt Rac1). Rac1 was polymerase chain reaction using pGEX2T-Rac1 and pGEX2T-DHI to obtain the expected size on SDS-PAGE.

Synthesis of Radiolabeled D-3 Phosphorylated Inositol Lipids—The different phosphoinositides (40 µg) were mixed with PS (80 µg), dried under nitrogen, resuspended in 50 mM Tris-HCl, pH 7.4 (1 µM of phosphoinositides/µl), vortexed and sonicated (20 kHz for 3 × 10 s). Lipid vesicles were then incubated with immunoprecipitated PI 3-kinase for 30 min at 37 °C as described previously (21). The reaction was stopped by the addition of chloroform/methanol (1/1, v/v), and lipids were immediately extracted by an acidic Bligh and Dyer extraction procedure (22). This reaction allowed us to obtain about 2% of D-3 phosphoinositol-precursor. Lipids were then dried under nitrogen for 40 min at 37 °C and were resuspended in suitable conditions. In some experiments, lipids were decylated by incubation in methanol/methanol/NaOH (25:45:71/11.4, v/v) for 50 min at 53 °C (23), dried under nitrogen, and resuspended in appropriate buffers.

Overlay Binding Assay—Proteins were resuspended in the electrophoresis sample buffer, boiled for 5 min, separated by 10–15% gradient SDS-PAGE, transferred onto a nitrocellulose membrane (Gelman Scien-
ml of the same buffer and dried, and their radioactivity was counted.

Preparation of the Particulate Subcellular Fraction—The African green monkey kidney cells (Vero) were seeded in 175-cm² culture dishes and grown in Dulbecco's modified Eagle's medium as described previously (25) to about 80% confluence and serum-starved for 16 h. When indicated, Wortmannin (100 nM) was added 20 min before stimulation with EGF (100 ng/ml for 15 min). Cells were then scraped off the culture dishes, harvested in 800 μl of extraction buffer (25 mM Tris-HCl pH 7.5, 15 mM NaCl, 5 mM EDTA, 1 mM PMSF, 1 mM sodium orthovanadate, 20 μg/ml aprotinin, and 20 μg/ml leupeptin) and lysed with a Dounce homogenizer. Lysates were first centrifuged at 1,000 × g for 15 min to remove nuclei and debris and then for 30 min at 120,000 × g to obtain the total membranes and the cytosolic fraction. Membranes were resuspended in radioimmunoprecipitation buffer (10 mM Tris-HCl, pH 7.0, 150 mM NaCl, 2 mM EDTA, 1 mM sodium orthovanadate, 0.1% SDS, 1% Nonidet P-40, 1% sodium deoxycholate, 1 mM PMSF) and centrifuged at 13,000 × g to eliminate unsolubilized material. Protein concentration was determined using the micro-BCA protein assay reagent kit (Pierce). Eighty μg of proteins were resuspended in sample buffer, boiled for 3 min, separated on a 12.5% SDS-PAGE, and transferred onto nitrocellulose (Gelman Sciences) as reported previously (26, 27). Immunodetection was performed with the monoclonal anti-Rac1 antibody (Upstate Biotechnology), and the reaction was visualized using peroxidase-conjugated secondary antibody and the ECL system (Amersham Pharmacia Biotech). The various bands were quantified by a densitometric analysis measuring the pixel volume in each area (Gel Doc 1000, Bio-Rad).

Lipid Extraction and Analysis—Extraction and quantification of [32P]PtdIns(3,4)P2 and [32P]PtdIns(3,4,5)P3 produced upon EGF stimulation of Vero cells were performed as described previously (25).

Determination of Rac1 Activation State—Serum-starved Vero cells were washed once with phosphate-free Dulbecco's modified Eagle's medium and labeled for 5 h in the same buffer supplemented with 0.5 mM [32P]orthophosphate. Cells were then stimulated for 15 min with 100 ng/ml EGF and lysed in the lysis buffer (50 mM Heps, pH 7.5, 500 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 1 mg/ml bovine serum albumin, 5 mM MgCl2, 1% Nonidet P-40, 1 mM PMSF, 1 mM sodium orthovanadate, 10 μg/ml aprotinin, and 10 μg/ml leupeptin) for 20 min at 4 °C. After centrifugation at 13,000 × g for 15 min, the soluble fraction was incubated for 2 h at 4 °C with 10 μg of a specific anti-Rac1 antibody (Upstate Biotechnology). The immune complexes were collected with 30 μl of protein G-Sepharose (Sigma) and washed eight times with the washing buffer (50 mM Heps, pH 7.5, 500 mM NaCl, 0.1% Nonidet P-40, 0.005% SDS, 5 mM MgCl2, 1 mM sodium orthovanadate, 2 μg/ml aprotinin, and 2 μg/ml leupeptin). GDP and GTP were finally eluted in elution buffer (5 mM DTT, 5 mM EDTA, 0.25 mM GDP, and 0.25 mM GTP) at 68 °C for 20 min, separated on polyacrylamide-agarose gels (Schleicher & Schuell), and developed in 1.2 m ammonium formate, 0.8 m HCl as described (28). Fixed [32P]GDP and [32P]GTP were quantified with the PhosphorImager 445 SI.

RESULTS

Direct Interaction of Recombinant Rac1 and RhoA with PtdIns(3,4,5)P3 and PtdIns(3,4)P2—GST-RhoA, -Rac1, or -Cdc42 fusion proteins were immobilized on glutathione-Sepharose beads and incubated in HNE buffer with PS (66.7%) vesicles containing either [32P]PtdIns(3)P, [32P]PtdIns(3,4)P2, or [32P]PtdIns(3,4,5)P3 (0.3%) and their respective precursor (33%) in the presence of 0.02% Nonidet P-40 as described elsewhere (9). Fig. IA shows that, under these conditions, PtdIns(3,4,5)P3 and PtdIns(3,4)P2 were both able to interact with RhoA and Rac1 but not with Cdc42, whereas PtdIns(3)P appeared as a poor ligand for these small G-proteins. An overlay binding assay using either GST-RhoA, GST-Rac1, or GST-Cdc42 and [32P]PtdIns(3,4)P2 confirmed that RhoA and Rac1, but not Cdc42 could bind to this insoluble lipid (Fig. 1B). The same results were obtained with [32P]PtdIns(3,4,5)P3, but, again, [32P]PtdIns(3)P did not interact (data not shown).

Since divalent cations may modify intermolecular interactions, lipid binding assay to immobilized recombinant proteins was performed in a buffer containing physiological concentrations of divalent cations and lacking detergent (Fig. 1C). Although the binding efficiency was reduced, very similar results were obtained. In addition, in agreement with Zheng et al. (15), we found that [3H]PtdIns(4,5)P2 interacted significantly with Cdc42. Fig. 1C also shows that PtdIns(4,5)P2 was the phosphoinositide preferentially interacting with Cdc42 under our conditions, whereas its binding to RhoA and Rac1 was less efficient compared with PtdIns(3,4)P2 or PtdIns(3,4,5)P3. Finally, none of the proteins interacted significantly with PtdIns(4)P (Fig. 1C) or PtdIns(3)P data not shown.

To reveal a specific PI 3-kinase product that would preferentially associate with these small G-proteins, we have incubated a mixture of all [32P]labeled D-3 phosphoinositides in the presence of a large excess of crude brain phosphoinositides used as precursors. Both in HNE buffer (Fig. 2A) or in the presence of physiological concentrations of divalent cations and...
The D-3 phosphoinositides were synthesized in vitro from a mixture of crude brain phosphoinositides in order to have a population of phospholipids that was close to the natural proportions found in stimulated cells. Binding was performed in HNE buffer (A) or saline buffer (B) as described for Fig. 1. Bound lipids were finally extracted by an acidic Bligh and Dyer procedure and resolved by TLC. Lane 1, total [32P]labeled phosphoinositides incubated with GST proteins; lane 2, GST alone; lane 3, GST-RhoA; lane 4, GST-Rac1; lane 5, GST-Cdc42. The origin and the positions of the different ν-3 phosphoinositides are indicated. PI, PtdIns.

without detergent (Fig. 2B), [32P]PtdIns(3,4,5)P₃ selectively associated with GST-Rac1 and GST-RhoA, although the binding to RhoA was weaker. [32P]PtdIns(3)P did not bind and [32P]PtdIns(3,4)P₂ was much less efficient than [32P]PtdIns(3,4,5)P₃ in binding to these small G-proteins, indicating a preferential interaction with the latter lipid. Again, GST alone or GST-Cdc42 were unable to interact with PI 3-kinase products. In agreement with these data, diC₁₆-PtdIns(3,4,5)P₃ was the most efficient phosphoinositide able to compete for binding of [32P]PtdIns(3,4,5)P₃ to Rac1 (data not shown).

We have then determined whether PtdIns(3,4,5)P₃ would show the same pattern of association with different guanine nucleotide-bound forms of Rac1. Table I indicates that the depleted form is the most efficient in binding PtdIns(3,4,5)P₃, whereas both GTP-bound (or GTPγS-bound; data not shown) and GDP-bound forms were capable to interact with this particular lipid to a lesser extent.

The purification of sufficient amounts of [32P]PtdIns(3,4,5)P₃ is technically difficult; therefore, in order to estimate the apparent affinity constant, we have used various concentrations of diC₁₆-PtdIns(3,4)P₂ containing purified [32P]PtdIns(3,4)P₂ as a tracer (about 100 dpm/pmol). The mixture was sonicated in HNE buffer supplemented with 0.02% Nonidet P-40 in order to obtain micellar PtdIns(3,4)P₂. The binding curve of PtdIns(3,4)P₂ to GST-Rac1 shown in Fig. 3 allowed us to determine an apparent Kᵦ of 4.4 ± 0.4 μM (mean ± S.E., n = 3).

Both Electrostatic and Hydrophobic Interactions Are Involved in the Binding of ν-3 Phosphoinositides to RhoA and Rac1—Previous experiments depicted above with various phosphoinositides clearly demonstrated the pivotal role of phosphate groups at positions 3, 4, and 5 in the recognition of D-3 phosphoinositides by small G-proteins. However, deacylation of phospholipids drastically reduced their ability to bind to RhoA and Rac1 (Fig. 4). Similar results were obtained using the overlay binding assay with either purified GST-RhoA or GST-Rac1 and deacylated [32P]PtdIns(3,4,5)P₃ or [32P]PtdIns(3,4)P₂ (data not shown).

Potential PtdIns(3,4,5)P₃-binding Sites in Rac1—An important question then concerned the potential binding domain of Rac1 and RhoA that would be absent in the sequence of Cdc42 and thus could explain their specific interaction with PI 3-kinase products. In this respect, centaurin α (29), synaptojanin (30), neurophin, and neuromodulin (31) have already been described as PtdIns(3,4,5)P₃-binding proteins with a putative consensus motif responsible for such an interaction. This motif contains hydrophobic residues and a particular basic sequence. Interestingly, Table II indicates that a similar motif is present in the recently described insert domain of Rac1 and RhoA (32, 33), whereas Cdc42 displays a less conserved sequence. In addition, a cluster of basic residues is also found in the C-terminal region of RhoA (RRGKKK), Rac1 (KKRKKK), and, to a lesser extent, Cdc42 (KKR), and may play a role in phosphoinositide binding. No other obvious potential phosphoinositide binding domains were identified in these small G-proteins.

In order to check whether these two candidate regions could...
be involved in the association with PI 3-kinase products, a Rac1 deletion mutant lacking both the insert region and the C-terminal part was prepared. Coomassie Blue staining after SDS-PAGE indicated the stability of this protein, which appeared as a single band at the expected molecular weight. Fig. 5 shows that this double deletion fully abolished the association of Rac1 with PtdIns(3,4,5)P$_3$ and PtdIns(3,4)P$_2$. Furthermore, independent deletion of either the insert or the C-terminal region reduced binding by about 50%. It is noteworthy that addition of 5 μM peptide mimicking the insert region of Rac1 decreased the binding of PtdIns(3,4,5)P$_3$ by 46 ± 13% (mean ± S.E., n = 3).

**Stimulation of GDP Dissociation from Rac1 by PtdIns(3,4,5)P$_3$—**To test the effect of the above characterized phosphoinositide-protein interaction, we then measured the rate of [3H]GDP dissociation from Rac1 in the absence of free GTP, upon addition of increasing amounts of PtdIns(3,4,5)P$_3$. Fig. 6A shows that PtdIns(3,4,5)P$_3$ significantly stimulated the GDP dissociation in a dose-dependent manner, with a maximal effect obtained at 100 μM and a half-maximal effect occurring at 45 μM. In similar conditions, PtdIns(4,5)P$_2$, which interacts only weakly with Rac1, had a minor effect (Fig. 6B). Fig. 6C shows the time course of the dissociation in the presence of phosphatidylinolcholine or PtdIns(3,4,5)P$_3$. The strong stimulation of the initial rate of GDP dissociation by PtdIns(3,4,5)P$_3$ occurred with a half-time of about 3 min, which is consistent with the results obtained for Dbl-induced GDP dissociation (15). Similar data were obtained when the GDP dissociation assay was performed in the presence of 0.5 mM of free GTP. Interestingly, PtdIns(3,4,5)P$_3$ had only a weak effect on [35S]GTPγS dissociation from Rac1 (23% versus 91% for [3H]GDP, at 75 μM); however, it was not able to elicit the stimulation of [35S]GTPγS binding (data not shown). Finally, as shown in Fig. 6D, GDP release promoted by PtdIns(3,4,5)P$_3$ was maximal with Rac1 and weak with Cdc42, RhoA displaying an intermediate pattern.

**PI 3-Kinase Inhibition Impairs the Translocation of Rac1 to the Total Membranes and the [32P]GTP Loading on Rac1 Induced by EGF in Vero Cells—**In order to test the physiologic significance of the above described in vitro data, Vero cells were stimulated with EGF in the presence or in the absence of the PI 3-kinase inhibitor wortmannin. Both the membrane location and the [32P]GTP loading of Rac1 were then investigated. After 15 min of stimulation with EGF (100 ng/ml), the amount of [32P]PtdIns(3,4)P$_2$ and [32P]PtdIns(3,4,5)P$_3$ were increased by 3.9 ± 0.5- and 3.0 ± 0.2-fold, respectively. Treatment with 100 nM wortmannin fully inhibited the synthesis of these two lipids. In parallel, the levels of membrane Rac1 (Fig. 7A) and the amount of [32P]GTP loaded on immunoprecipitated Rac1 (Fig. 7B) significantly increased upon EGF treatment and wortmannin strongly inhibited these two events.

**FIG. 5.** The double Rac1 deletion mutant (ΔDI-ΔC) fails to interact with PtdIns(3,4)P$_2$ and PtdIns(3,4,5)P$_3$. GST-Rac1 and the indicated mutants (15 μg) were incubated with [32P]PtdIns(3,4)P$_2$ or [32P]PtdIns(3,4,5)P$_3$ in HNE buffer as described under “Experimental Procedures.” Results are expressed as percentages of specific binding and are means ± S.E. of three independent experiments.

**Discussion**

The data presented here demonstrate that the small G-protein Rac1 is able to specifically interact in vitro with PtdIns(3,4,5)P$_3$ and, to a lesser extent, with PtdIns(3,4)P$_2$. RhoA also displays significant, although weaker, binding to these two PI 3-kinase products. In contrast, we found that Cdc42 another small GTPases known to bind PtdIns(4,5)P$_2$ does not associate with D-3 phosphoinositides. Using different approaches, we found that the interaction of PtdIns(3,4,5)P$_3$ with either Rac1 or RhoA is stable and can resist to washing steps with buffers containing elevated concentrations of detergent (up to 0.5% Nonidet P-40). The apparent affinity constant of the binding of PtdIns(3,4,5)P$_3$ to Rac1 was 4.4 μM, and our results also suggest that PtdIns(3,4,5)P$_3$ has actually an even better affinity.

Deacylated lipids were no longer able to interact with Rac1 or RhoA, indicating that this binding requires hydrophobic interactions with fatty-acyl chains to be stable, in addition to the electrostatic effects of the phosphate groups at specific positions on the inositol ring. A similar behavior was previously observed for the assembly protein 3 (AP-3), another PtdIns(3,4,5)P$_3$-binding protein (34). Taking into account that both basic and hydrophobic residues may be involved, we found two potential binding sites on Rac1 and RhoA. One is the insert region of RhoA, which was originally observed in the crystal structure of Rac1, is unique to the Rho family (32, 33), and takes the form of a three-turn helix with several positive charges exposed together with a few hydrophobic residues. Antibodies directed against this region are no longer able to reach their epitope once RhoA is translocated to the membrane during Ca$^{2+}$ sensitization of smooth muscle (35), suggesting that this insert region may mediate the association of the protein with the membrane. We observed that this motif exhibits a good homology with the conserved putative PtdIns(3,4,5)P$_3$-binding domain previously identified in neurogranin (31) or centaurin α (29). A second candidate for such an interaction with D-3 phosphoinositide is the cluster of basic residues found in the C-terminal part of Rac1 and RhoA. Deletion of these two domains in Rac1 abolished its association with the lipid products of PI 3-kinase, whereas independent deletion of either the insert region or the C terminus of Rac1 led approximately to a 50% decrease in the binding efficiency. Whether these two regions cooperate for an efficient binding or whether they individually account for the interaction remains to be

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**TABLE II**

A putative PtdIns(3,4,5)P$_3$ consensus binding motif is found in the insert region of Rac1 and RhoA

Putative binding motifs identified on proteins that have been shown to directly interact with PtdIns(3,4,5)P$_3$ were compared to the potential candidate motif found in the insert region of RhoA and Rac1 and the corresponding region of Cdc42. Positively charged (in bold) and hydrophobic residues (Φ) are noted. The references to the papers describing this interaction are given. The protein sequences are given from the NH$_2$ terminus to the COOH terminus of the protein, except for the sequence of RhoA, which is in the reverse order.

| Proteins | Motifs | References |
|----------|--------|------------|
| SH2 domain of p85 | RKKXXKKΦΦKΦXΦH | (3) |
| Centaurin α | ΦRXXΦΦΦKΦXΦK | (29) |
| Screened peptide/ neuroginin | ΦKΦΦΦΦΦΦKΦXΦK | (31) |
| Rac1 | KΦΦΦΦΦΦΦΦΦΦΦΦΦΦ | (34) |
| RhoA (Nt → Ct) | ΦΦΦΦΦΦΦΦΦΦΦΦΦΦ | (29) |
| Cdc42 | ΦΦΦΦΦΦΦΦΦΦΦΦΦΦ | (3) |

*$N$-terminal → *C*-terminal.
established. However, we cannot exclude that the deletion of 33 amino acids at the C-terminal end induced a conformational change of the protein that may also indirectly modify its binding selectivity and efficiency. It is noteworthy that two PtdIns(4,5)P₂-binding domains have also been suggested in Arf (36).

At a first glance, binding of PtdIns(3,4,5)P₃ to Rac1 and RhoA suggest that this D-3 phosphoinositide may act to anchor these small GTPases to the membrane (37). In this respect, it is noteworthy that PI 3-kinase, which interacts with elements of actin filament system and of focal adhesions (27), can also bind in vitro to Rac1 in a GTP-dependent manner (14). If such an association also occurs in vivo, one can speculate that it should favor secondary interaction of Rac1 with the membrane. In addition, similar membrane associations could also concern some regulators of Rho family GTPases bearing PH domains, as recently reported (12, 13). For instance, diC₈-PtdIns(3,4,5)P₃ was found to enhance Lck-dependent tyrosine phosphorylation of Vav, which then promotes the exchange of bound GDP for GTP on Rac (13). It is interesting to note that a similar dual role of β-3 phosphoinositides has been already observed for the activation mechanism of protein kinase B (5). In the case of Rac1, the picture might even be more complex when considering the fact that Rac1 can activate a PtdIns(4)P 5-kinase (16). The subsequent accumulation of PtdIns(4,5)P₂ may thus contribute to the reorganization of actin cytoskeleton via interactions with proteins like gelsolin or profilin, and provide a down-regulation mechanism of Vav activity, since PtdIns(4,5)P₂ displays opposite effects to those of PtdIns(3,4,5)P₃ (13).

The specific dual interactions observed between PtdIns(3,4,5)P₃ and Rac1/Vav are actually similar to those previously reported for Arf1. Indeed, this small GTPase interacts with PtdIns(4,5)P₂, whereas its exchange factor Arno activates GDP/GTP exchange of Arf following binding of its PH domain to PtdIns(4,5)P₂ (36, 38). On the other hand, the general receptor for phosphoinositides (GRP1), another exchange factor for Arf1, is also a target for PtdIns(3,4,5)P₃ via its PH domain (39). Finally, PtdIns(4,5)P₂ was reported to promote interaction of Arf with GTPase-activating proteins (40). It thus appears that phosphoinositides begin to emerge as general regulators of various small GTPases acting by similar mechanisms, although some strict specificity might be brought by the nature of the phosphoinositide involved.

Under these conditions, what could be the biological significance of GDP release induced upon interaction of PtdIns(3,4,5)P₃ with Rac1? Obviously, this reflects a specific interaction of the protein with the phospholipid, as deduced from the comparison between Rac1 and Cdc42. A similar effect of PtdIns(4,5)P₂ was already shown for Cdc42 (15) and Arf (36), further illustrating the specificity of the recognition of different phosphoinositides by various small GTPases. Since PtdIns(3,4,5)P₃ did not alter the dissociation of [³⁵S]GTP·S from Rac1, this suggests that its interaction has mainly a role in the limiting step of GDP release and may act in concert with exchange factors, as discussed above. However, the fact that GDP exchange is not accompanied by GTP loading brings some doubts about the real biological significance of this effect, although we cannot exclude that the complex PtdIns(3,4,5)P₃-unloaded Rac1 or RhoA may directly regulate some specific targets, which remain to be identified. It is noteworthy that

![Figure 6: GDP dissociation from Rac1 is stimulated by PtdIns(3,4,5)P₃.](image-url)

The guanine nucleotide dissociation from Rac1 in the absence of free nucleotide was determined as indicated under “Experimental Procedures.” GDP-dissociation from Rac1 was measured in the presence of increasing concentrations of PtdIns(3,4,5)P₃ (A) or PtdIns(4,5)P₂ (B). The GDP dissociation assay was terminated 10 min after addition of phosphoinositides. The time course of [³⁵S]GDP release from Rac1 (C) was performed in the presence of 60 µM of phosphatidylcholine (■) or PtdIns(3,4,5)P₃ (□). A comparison of PtdIns(3,4,5)P₃ (60 µM)-induced GDP dissociation from Rac1, RhoA, and Cdc42 is shown in D. Results are expressed as percentage of [³⁵S]GDP remaining on Rac1 and are means ± S.D. of three different experiments. PI, PtdIns.
human blood platelet homogenate revealed an even stronger binding of [32P]PtdIns(3,4,5)P3 to a single protein that matched Rac1. 2 This suggests that the isoprenoid moiety may actually enhance the interaction. Our results, obtained in intact Vero cells stimulated with EGF, are also in agreement with our in vitro data and strongly suggest a role for PI 3-kinase products in the recruitment and the activation of the geranyl-geranylated form of Rac1 at the membrane, as also observed in other models (6, 8, 43). Recently, Azma et al. (43) reported that both Rac1 and gelsolin are recruited to the sites of ruffling activity and suggested a physical interaction between these two proteins with membrane lipids in these particular regions. Based on literature and on our present data, there is clearly much to learn about the role of phosphoinositides as regulators of small GTPase activity and their contribution in different physiological situations.

In conclusion, we have shown that small GTPases of the Rho family may directly and differentially interact with phosphoinositides, Cdc42 preferentially with PtdIns(4,5)P2, Rac1 and RhoA with PI 3-kinase products, i.e. PtdIns(3,4,5)P3 and, to a lesser extent, PtdIns(3,4)P2. In contrast to PH domains, this binding involves both electrostatic and hydrophobic interactions, it leads to GDP release, and, in concert with exchange factors or other regulators, it may play an important role in the PI 3-kinase-dependent activation of Rac1 and RhoA.

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