Rac GTPase interacts specifically with phosphatidylinositol 3-kinase

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The Rac GTP-binding proteins are members of the Rho family and regulate growth factor-stimulated actin assembly in a variety of cells. The formation of phosphorylated inositol lipids has been implicated in control of the processes initiating and regulating such actin polymerization. Associations of Rho family GTP-binding proteins with enzymes involved in lipid metabolism have been described. Here we demonstrate a direct and specific interaction of Rac proteins with phosphatidylinositol (PI) 3-kinase. This interaction is dependent upon Rac being in a GTP-

bound state and requires an intact Rac effector domain. In contrast, direct binding of RhoA to PI 3-kinase could not be detected. Rac-GTP also bound to PI 3-kinase in Swiss 3T3 fibroblast and human neutrophil lysates, and increased PI 3-kinase activity became associated with Rac-GTP in platelet-derived growth factor-stimulated cells. Interaction of Rac-GTP with PI 3-kinase *in vitro* stimulated the activity of the enzyme by 2–9-fold. A specific interaction of active Rac with PI 3-kinase might be important in regulation of the actin cytoskeleton.

INTRODUCTION

The actin cytoskeleton plays an important role in regulating a variety of cellular processes, including cell motility and tumour metastasis. Members of the Rho family of GTP-binding proteins regulate assembly of the actin cytoskeleton, with Rho stimulating the formation of actin stress fibres [1], Rac the process of membrane ruffling [2], and Cdc42 the extension of filopodia [3,4]. Actin regulatory proteins (i.e. gelsolin and profilin) whose ability to bind actin can be modulated by lipids such as phosphatidylinositol 4,5-bisphosphate (PIP₂) could play important roles in controlling the polymerization of actin in response to external signals [5,6]. Stimulation of the formation of PIP₂ via lipid kinases has been suggested to account for actin assembly mediated by Rac [6] and Rho [7].

The activity of phosphatidylinositol (PI) 3-kinase is stimulated by a wide variety of hormones, growth factors and chemo-attractants [8,9]. Through the use of inhibitors such as wortmannin and LY294002, as well as by mutagenesis of PI 3-kinase binding sites on growth factor receptors, the product(s) of this enzyme, particularly phosphatidylinositol 3,4,5-trisphosphate (PIP₃), have been shown to be necessary for mitogenic responses to growth factors, changes in actin assembly associated with membrane ruffling, and the production of superoxide anion by the phagocyte NADPH oxidase [8–13]. The last two activities are known to involve the action of the Rac GTP-binding proteins (GTPases) [14,15].

PI 3-kinase consists of a family of closely related heterodimeric proteins made up of 85 kDa regulatory subunits and 110 kDa catalytic subunits [8,9,16–18], although potentially novel forms of PI 3-kinase have recently been reported [19,20]. The 85 kDa subunit contains binding sites for the 110 kDa catalytic subunit and SH2 and SH3 domains that enable PI 3-kinase to interact with phosphotyrosine and proline-rich motifs in other proteins. Also present is a region of homology to the Bcr GTPase-activating protein (GAP) domain, which catalyses GTP hydrolysis by Rac proteins [21]. PI 3-kinase activity can be stimulated by GTP analogues, and several studies have implicated

Ras [22], Rho [23] and Cdc42 [24] as regulators of PI 3-kinase, with direct interactions between Ras or Cdc42 and PI 3-kinase being described. A functional interaction between Rac and PI 3-kinase has been established in certain cellular settings, where PI 3-kinase activity has been shown to be necessary for the activation of Rac-mediated events, particularly membrane ruffling [11]. It has been suggested that this results from PI 3-kinase regulation of a Rac guanine nucleotide exchange factor, leading to the formation of active GTP-bound Rac [25a]. Here we establish a specific and direct interaction between Rac GTPases and PI 3-kinase. These findings strengthen the case for a functional interaction between Rac activity and PI 3-kinase, and suggest that these proteins form a regulatory complex under appropriate conditions of cell activation.

EXPERIMENTAL

Purification of PI 3-kinase

PI 3-kinase was purified from bovine brain by using sequential QAE-Sepharose anion-exchange chromatography, ammonium sulphate precipitation, hydroxyapatite adsorption chromatography, MonoS cation-exchange chromatography and MonoQ anion-exchange chromatography [25b]. The enzyme preparation (see Figure 1) was determined as being completely free of PI 4-kinase activity. The enzyme was routinely stored at 4 °C in 20 mM Tris/HCl, pH 8.3, containing 6 mM 2-mercaptoethanol, 0.1 mM EGTA, 1 mM MgCl₂, 1 µg/ml leupeptin and 1 µg/ml pepstatin A, and retained activity for 4–6 weeks under these conditions.

Preparation of recombinant proteins

Recombinant and fully isoprenylated Rac1, Rac2, Rac2 D38A and RhoA were prepared and purified from a baculovirus/Sf9 cell expression system as described previously [26]. Glutathione S-transferase (GST)–Rac1 or –p85 fusion proteins were expressed and purified from bacterial cultures as described [26]. The p85–GST constructs were gifts from Dr. Brian Schaffhausen, Tufts

Abbreviations used: GAP, GTPase-activating protein; GST, glutathione S-transferase; GTPase, GTP-binding protein; GTP[S], guanosine-5'-[γ-thio]-triphosphate; PI, phosphatidylinositol; PIP₂, phosphatidylinositol 4,5-bisphosphate; PIP₃, phosphatidylinositol 3,4,5-trisphosphate; PDGF, platelet-derived growth factor.

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Figure 1 Polyacrylamide gel analysis of the PI 3-kinase and Rac preparations used in these studies

PI 3-kinase (lane 1) was purified from bovine brain to essential homogeneity as described. Analysis was by Coomassie Blue staining on 8% (w/v) polyacrylamide gels. Rac2 (lane 2) and Rac1 (lane 3) were expressed in a baculovirus-Sf9 cell expression system and purified as described. The purity was assessed by silver staining after SDS/PAGE on 15% gels. The positions of molecular mass markers (kDa) are shown at the far left and the far right.

University Medford, MA, U.S.A.); GST-Rab1 was a gift from Dr. William Balch, The Scripps Research Institute.

Protein loading with guanine nucleotides

GTP-binding proteins in 20 mM Tris buffer, pH 7.5, containing 1 mM EDTA, 0.1 mM dithiothreitol and 5 mM MgCl_2 were incubated for 10 min at room temperature in the presence of 25 mM EDTA and 100 μM guanosine-5'-[γ -thio]-triphosphate (GTP[S]) or GDP. The binding was stopped by adding MgCl_2 to an excess of 5 mM, and the protein was stored on ice until use.

Co-immunoprecipitation analyses

PI 3-kinase (8 pmol), Rac1, Rac2 or RhoA (10–11 pmol), or a combination of PI 3-kinase and Rac/Rho were incubated in 100 μl of incubation buffer (30 mM Hepes, pH 7.5, containing 0.4 mM EGTA, 0.7 mM EDTA and 3 mM MgCl_2) for 10 min at 30 °C, followed by 20 min on ice. The GTPases were preloaded with GDP or GTP[S] before use, as described. Anti-p85 subunit antibody (UBI Product Number 06-195) (5 μl) was added and incubated overnight with shaking at 4 °C. A 1:1 suspension of Protein A beads (Sigma Chemical Co.) (25 μl) was added to each sample and incubated for an additional 45 min. The beads were then pelleted and washed five times with 1 ml of 150 mM NaCl, 50 mM Tris, pH 7.5, 0.1 mM sodium orthovanadate, 1% (v/v) Nonidet P40 (IP wash buffer). The washed precipitates were eluted with Laemmli sample buffer (100 μl), electrophoresed on 8% (w/v) polyacrylamide gels and transferred to nitrocellulose. Blotting was performed with a 1:2000 dilution of Rac1 antibody R785 and Rac2 antibody R786 [27] or a 1:2000 dilution of an RhoA monoclonal antibody [28]; detection was by alkaline phosphatase-coupled secondary antibody.

Interaction of Rac with recombinant p85 fusion proteins

Rac1 (0.75 μg) preloaded with GTP[S] was incubated in 25 mM Tris/HCl, pH 7.5, containing 1 mM EDTA, 1 mM dithiothreitol

and 5 mM MgCl_2 with 10 μl of the indicated GST fusion construct preassociated with glutathione-Sepharose beads. The incubation was carried out overnight at 4 °C with continuous agitation. The beads were then pelleted and washed five times with 1 ml of 25 mM Tris buffer, pH 7.5, containing 100 mM NaCl, 1% Nonidet P40 and 0.1% BSA. The washed beads were eluted with Laemmli sample buffer and analysed by Western blotting.

In vitro activation of PI 3-kinase

The effects of Rac on PI 3-kinase activity were measured as follows. Purified PI 3-kinase (2.5 $\mu\text{g}/\text{ml}$) was incubated in the presence or absence of the indicated GTP-binding protein in a buffer containing 20 μM [γ - ^{32}P]ATP (specific activity 8.8×10^3 c.p.m. per pmol; New England Nuclear), 30 mM Hepes, pH 7.2, 7 mM MgCl_2 , 0.4 mM EGTA, 1 mM EDTA, 0.33 mM Tris/HCl, pH 7.5, 400 $\mu\text{g}/\text{ml}$ PI and 40 $\mu\text{g}/\text{ml}$ PIP_2 . After a 10 min incubation at room temperature, 40 μl of 1 M HCl was added to stop the reaction, followed by 80 μl of chloroform/methanol (1:1, v/v). The mixture was vortex-mixed and centrifuged to resolve the phases, and the lipid-containing lower phase was removed to a clean vial. The upper phase was washed with an additional 50 μl of chloroform, and the combined lower phases were spotted on to potassium oxalate-treated Silica gel 60 thin-layer plates (EM Science) for analysis as described [29,30].

Cell culture and analysis of cell lysates

Swiss 3T3 fibroblasts were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Subconfluent Swiss 3T3 cells treated on ice for 5 min in the presence or absence of 3 ng/ml platelet-derived growth factor (PDGF)-BB were lysed in 25 mM Tris/HCl, pH 7.5, containing 1 mM EDTA, 0.1 mM EGTA, 5 mM MgCl_2 and 1 mM dithiothreitol (dilution buffer) plus 150 mM NaCl, 1% Nonidet P40, 10% (v/v) glycerol, 2 mM sodium orthovanadate, 0.1 mM PMSF, 100 units/ml aprotinin, 20 $\mu\text{g}/\text{ml}$ leupeptin, then diluted with one volume of cold dilution buffer. The lysates were clarified by centrifugation at 500 g in a Beckman rotor for 5 min at 4 °C. A 300 μl sample of each lysate was incubated with either 100 μl of GST-Rac1 or GST-Rab1 beads (1.1 mg/ml active Rac, 1.0 mg/ml active Rab1, as determined by GTP[S] binding) that had been preloaded with GTP[S] or GDP, or 5 μl of p85 antibody for 2 h at 4 °C with shaking. Protein A beads (50 μl ; 1:1) were added to the antibody incubation 30 min before washing. The precipitates were washed four times with 1 ml of IP wash buffer, then once with 1 ml of 25 mM Hepes buffer, pH 7.5, containing 1 mM EDTA and 7 mM MgCl_2 . The washed pellets were assayed for PI 3-kinase activity as described [29].

RESULTS

Rac binds to PI 3-kinase

The presence in the p85 subunit of PI 3-kinase of a region homologous with the Rac GAP domain of Bcr suggested that this protein could be a GAP for Rho family GTPases. We assayed purified bovine brain PI 3-kinase (Figure 1) for GAP activity towards Rac1 and Rac2 alone, in the presence of the lipid substrates PI and/or PIP_2 , or in the presence of these same lipids plus the cofactor ATP. We were unable to detect significant GAP activity under any conditions at concentrations of PI 3-kinase up to 100 nM. Similar negative results were obtained with RhoA, and have been reported for Cdc42 [24]. We therefore went on to examine whether PI 3-kinase was able to interact physically with Rac1 and Rac2. As shown in

Figure 2 Rac binds to PI 3-kinase

(A) Rac1 and RhoA were preloaded with GTP[S] (GTP γ S) and the interaction with purified bovine brain PI 3-kinase (PI3K) was probed by immunoprecipitation with p85 antibody, as described in the Experimental section. Each blot was analysed with the appropriate Rac or RhoA antibody. The recombinant Rac1 standard represents approx. 350 ng of Rac1 against approx. 350 ng of RhoA standard; both run at 22 kDa. By quantitative comparison of the amounts of each GTPase co-precipitated with PI 3-kinase and comparison with the standards, we estimate that approx. 200 ng or 9 pmol of Rac bound to approx. 8 pmol of PI 3-kinase (stoichiometry 1.12), whereas less than approx. 5 ng or 0.22 pmol of Rho bound to the same amount of enzyme (stoichiometry 0.028). The results shown are representative of at least five separate experiments. (B) Rac1 was preloaded with GDP or GTP[S] and equal amounts of active protein were analysed for binding to PI 3-kinase as described in the Experimental section. Rac standard 300 ng. (C) Rac2 or Rac2 D38A mutant was preloaded with GTP[S] and analysed for binding to PI 3-kinase as described in the Experimental section. The two Rac2 D38A lanes represent the use of 1 or 2 times the level of wild-type (WT) Rac2-GTP[S] respectively in the co-precipitation. Rac2 standard approx. 400 ng. The molecular mass markers (MWS) shown are 97, 66, 45, 31, 21 and 14 kDa respectively. Results shown in (B) and (C) are representative of at least two separate experiments.

Figure 2, when a highly purified preparation of bovine brain PI 3-kinase was incubated with Rac1-GTP[S] and then immunoprecipitated with an antibody directed against the p85 subunit of PI 3-kinase, the Rac1 protein was precipitated in association with PI 3-kinase. No Rac was precipitated by the antibody in the absence of added enzyme. We also performed the reciprocal experiment, in which we immunoprecipitated PI 3-kinase-Rac-GTP[S] complexes with an antibody directed against an epitope tag on Rac, with similar results (not shown). Rac2-GTP[S] also bound effectively to purified PI 3-kinase (Figure 2C). In contrast, we were unable to detect significant amounts of Rho-GTP[S] binding to the enzyme (Figure 2A), and controls with the Rab1 protein were similarly negative.

Rac binding to PI 3-kinase is GTP-dependent and requires the effector region of Rac

We evaluated whether the interaction of Rac1 with PI 3-kinase required Rac to be in a GTP-bound active conformation. As shown in Figure 2(B), binding of Rac1-GDP to PI 3-kinase was much lower than binding of Rac-GTP[S]. On the basis of the intensity of the Rac1 band in the precipitates in comparison with Rac standards on the same immunoblot, we determined that the amount of Rac-GTP[S] bound to PI 3-kinase was equimolar, indicating the formation of a 1:1 complex; this was verified by varying the amount of Rac added to the incubation in separate experiments (results not shown). In contrast, the binding of Rac1-GDP was always less than 10% of this level. Although we did detect a low level (approx. 10%) of Rho-GTP[S] binding to PI 3-kinase in some experiments, this was not observed consistently and was always 10% or less of the total amount of PI

Figure 3 Rac binds to the p85 subunit of PI 3-kinase

The binding of Rac1-GTP[S] to p85 fusion proteins was performed as described in the Experimental section. Lane 1, GST-p85; lane 2, GST bead control; lane 3, GST-p85 Bcr domain (residues 78–320); lane 4, GST-p85 SP 'spacer region' (residues 435–620); lane 5, approx. 300 ng of Rac1 standard.

3-kinase present. The specificity of the interaction with Rac was further established by using a Rac2 protein that had a mutation (D38A) in the 'effector' domain, which we have shown to prevent the ability of Rac to stimulate fibroblast membrane ruffling and phagocyte NADPH oxidase activation [26]. We observed (Figure 2C) that even when Rac2 D38A was preloaded with GTP[S], it was markedly diminished in its ability to bind to PI 3-kinase when compared with wild-type Rac2. This indicates that the classical 'effector' domain on Rac is required for binding to PI 3-kinase.

We examined whether Rac bound to the p85 subunit of PI 3-kinase (Figure 3). Rac-GTP[S] bound specifically to a recombinant GST-(p85 subunit) fusion protein, but not to GST beads

Table 1 Rac-GTP binds PI 3-kinase activity in fibroblast lysates

Swiss 3T3 cell lysates were incubated with GST-coated beads alone or with GST-Rac1-GDP, GST-Rac1-GTP[S] or GST-Rab1-GTP[S] beads and the amount of PI 3-kinase activity specifically bound to the beads was determined as described in the Experimental section. The results shown represent the means $\pm$ S.E.M. for five experiments, except for the Rab control, which is the average of two determinations.

Bead ligand	PI 3-kinase activity bound (% of total activity)
GST alone	2.8 $\pm$ 1.0
GST-Rac-GDP	5.2 $\pm$ 2.0
GST-Rac-GTP[S]	13.6 $\pm$ 4.7
GST-Rab-GTP[S]	3.1

Figure 4 Effect of Rac on PI 3-kinase activity

The effect of the indicated form of Rac1 on phosphatidylinositol 3-phosphate (PI-3P) and PIP_3 formation by purified PI 3-kinase (PI3K) was determined as described in the Experimental section. The numbers in parentheses represent the nanomolar concentration of Rac added to the incubation. +GTP[S] and -GTP[S] indicates the presence or absence of 100 μM GTP[S] in the incubation as a control for possible effects of the unbound guanine nucleotide present in the preloaded Rac samples. In the complete absence of PI 3-kinase (-PI3K) there was no product formation in either the presence or absence of GTP[S] ($\pm$ GTP[S]) and/or Rac-GTP[S] ($\pm$ Rac[S]); therefore only one such data set is shown in the figure for brevity (lanes 1 and 2). Similarly, the basal activity of the PI 3-kinase was identical with or without GTP[S] in the absence of added Rac (lanes 3 and 4). This experiment represents a minimal activation response seen with Rac-GTP[S]; as indicated in the text, up to 9-fold activation was observed in some experiments.

alone. This interaction involves the Bcr homology domain of p85, as a similar level of binding was observed with the isolated Bcr domain expressed as a GST fusion protein.

Rac1-GTP[S] binds PI 3-kinase activity in cell lysates

We used a recombinant GST-Rac1 fusion protein to determine whether PI 3-kinase in Swiss 3T3 fibroblast lysates would interact with Rac in a GTP-dependent manner. A substantial portion of the total detectable PI 3-kinase activity in these cells could bind to GST-Rac-GTP[S] beads (10–15%), whereas there was little binding to control beads, GST-Rab1-GTP[S] beads or GST-Rac1-GDP beads (Table 1). Similar specific binding of GST-

Figure 5 Concentration-dependent activation of PI 3-kinase by Rac-GTP[S]

PI 3-kinase stimulation by Rac1-GTP[S] was determined as described in the legend to Figure 4. Stimulation of PI 3-kinase activity by Rac in the experiment shown, which was representative of two with similar results, was approx. 3-fold. The enzyme was present at a concentration of 12.8 nM in the reaction, supporting our determinations of a stoichiometric (1:1) interaction of the two proteins.

Rac1-GTP[S] to PI 3-kinase was observed in human neutrophil lysates (results not shown). The amount of PI 3-kinase activity that became bound to Rac-GTP was increased 4–8-fold in lysates from serum- or PDGF-stimulated Swiss 3T3 cells.

Effect of Rac binding on PI 3-kinase activity

It was important to determine whether the interaction of Rac-GTP with PI 3-kinase directly affected the activity of the enzyme. We observed that both Rac1-GTP[S] and Rac2-GTP[S] stimulated PI 3-kinase activity *in vitro* routinely by 2–3-fold (Figure 4). Similar results were obtained with a GTPase-deficient Rac2 Q61L mutant loaded with GTP. Activation was concentration-dependent, with a half-maximal concentration of 10–20 nM Rac1- or Rac2-GTP[S] (Figure 5). The level of stimulation ranged from 2-fold to 9-fold in individual experiments, but the reason for this variation could not be determined. The degree of stimulation was independent of the enzyme or GTP-binding protein preparation used. However, we observed no effect on enzyme activity when recombinant RhoA-GTP[S] was added at similar concentrations. The binding of Ras [31] and Cdc42 [24] to PI 3-kinase has also been reported to produce modest GTP-dependent stimulation of enzyme activity *in vitro* (2-fold and 2–4-fold respectively).

DISCUSSION

We demonstrate that the Rac1 and Rac2 GTP-binding proteins specifically bind to the lipid kinase PI 3-kinase to form a 1:1 complex. This binding is markedly enhanced when Rac is in a GTP-bound state, indicating that PI 3-kinase may be a target of activated Rac; indeed, we observed that binding was substantially reduced in a Rac2 effector domain mutant that does not produce downstream signalling [26]. We were unable to detect significant binding of Rho-GTP[S] to PI 3-kinase, suggesting that previous reports of the regulation of PI 3-kinase activity by Rho may be a result of an indirect regulatory interaction. Our recent observation that Rho regulates the formation of PIP_2 via effects on

a PI 5-kinase raises the possibility that Rho modulates PI 3-kinase activity by affecting the levels of PIP₂ substrate [7]. The current data demonstrating the specific binding of Rac-GTP to PI 3-kinase are consistent with the recent reports by Zheng et al. [24] and Tolia et al. [32], but differ from the observations of Rodriguez-Viciana et al. [22], who did not detect binding of PI 3-kinase to Rac-GTP.

The ability of Rac to interact with the p85 subunit of PI 3-kinase in a highly specific manner, at least partly through binding to the Bcr homology domain on the p85 subunit (Figure 3) [24], suggests that this interaction may be relevant to both the regulation of PI 3-kinase activity and/or Rac activity in stimulated cells. We observed stimulation of PI 3-kinase activity by up to 9-fold in the presence of GTP[S]-bound Rac in some experiments, indicating that this interaction can increase the formation of phosphoinositides modified at the 3-position under certain circumstances. In initial experiments, however, we were unable to detect consistent increases in total PI 3-kinase activity in cells transfected with an activated form of Rac, as also reported by Rodriguez-Viciana et al. [22]. These data suggest that Rac-regulated PI 3-kinase activity may represent a small proportion of total PI 3-kinase, and this is consistent with the recent report of Tolia et al. [32], who reported that less than 1% of total PI 3-kinase activity co-precipitated with Rac in PDGF-stimulated fibroblasts.

The suggestions of regulatory effects of PI 3-kinase on Rac activation via a guanine nucleotide exchange factor raise the somewhat confusing scenario of Rac being both an upstream regulator and a downstream target of PI 3-kinase. The existence of a positive feedback loop is possible, and the ability of other GTPases (such as Ras and Cdc42) to interact with PI 3-kinase might allow cross-talk to occur at this level [3]. Another possibility is that regulation of overall PI 3-kinase activity is not the major consequence of its interaction with Rac-GTP. Rac-GTP might target PI 3-kinase to specific sites within the cell (i.e. membrane ruffles) where both proteins act to regulate cell function. The converse could also be true: PI 3-kinase could act to target Rac to specific intracellular locations. The p85 subunit has many of the protein interaction motifs characteristic of an adapter molecule, including SH3 domains, and the ability of these domains to direct subcellular localization has been demonstrated [33]. Furthermore the existence of p85 that is not associated with the p110 catalytic subunit in cells has been reported [34,35]. There is a good correlation between the regulation of cellular functions by Rac and the requirement for PI 3-kinase activity in these same systems [11]. The extent to which Rac-PI 3-kinase interactions occur in intact cells under different activating conditions, and the localization of these proteins to specific sites in the cell, will be important goals of further investigations.

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