Interaction of Ras with phosphoinositide 3-kinase γ

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Phosphoinositide 3-kinase γ (PI3K γ) can be activated *in vitro* by both α and $\beta\gamma$ subunits of heterotrimeric G-proteins and does not interact with p85, the regulatory subunit of PI3K α . Here we demonstrate the binding of Ras to PI3K γ *in vitro*. An N-terminal

region of PI3K γ was identified as a binding site for Ras. After co-expression with PI3K γ in COS-7 cells, Ras induced only a modest increase in PI3K activity compared with the stimulation of PI3K α by Ras in the same cells.

INTRODUCTION

The small GTPase Ras represents one of the central elements of intracellular signalling. It is involved in the regulation of proliferation and differentiation of mammalian cells by tyrosine kinase and G-protein-coupled receptors [1,2]. Agonists of these receptors are able to induce an exchange of Ras-bound GDP for GTP, which generates the activated form of the GTPase. Active Ras subsequently interacts with effector proteins and induces the cellular response via several signalling pathways.

Recently, GTP-dependent interaction of Ras with the phosphoinositide 3-kinase (PI3K) species α and β has been described [3,4]. PI3Ks represent a group of intracellular signalling proteins involved in the regulation of several cellular responses including proliferation, differentiation and secretory processes [5]. The heterodimeric species PI3K α and PI3K β , comprising p110 catalytic subunits and p85 adaptor molecules, are regulated by receptors with intrinsic or associated tyrosine kinase activity [6]. Interaction of PI3K α with Ras-GTP leads to a stimulation of lipid kinase activity *in vitro* and *in vivo* [3,4]. The Ras-binding site on the p110 catalytic subunit of PI3K α was shown to lie between amino acid residues 133 and 314 [7].

We recently cloned a novel form of PI3K, termed PI3K γ , that can be activated *in vitro* by α and $\beta\gamma$ subunits of heterotrimeric G-proteins [8]. Here we show that Ras specifically associates with PI3K γ *in vitro*. Other small GTPases, including Rap1A, RhoA and Rac1, did not interact with PI3K γ . An N-terminal domain of PI3K γ has been mapped as a binding site for Ras. Mechanistic and functional characteristics of the Ras interaction with PI3K γ will be discussed.

EXPERIMENTAL

Cell culture

Spodoptera frugiperda (Sf9) cells were maintained by standard methods [9]. All culture medium was incubated at 27 °C before use and the cells were routinely incubated at 27 °C. The culture medium used throughout was IPL41 (Gibco) supplemented with 10 % FCS. Routine culture, virus amplification and baculovirus infection for protein production were performed in the presence of 1 % fungizone and 0.1 % gentamycin. Sf9 cells were subcultured at least twice a week. For viral infection, confluent Sf9 cells were routinely dislodged by gently tapping the side of the flask against a surface; samples were split 1:3 into new flasks

with fresh medium. Cells were allowed to attach for 1 h at 27 °C and infected with the appropriate, empirically determined, amount of the corresponding baculovirus.

COS-7 cells were routinely maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % (v/v) fetal calf serum (FCS) in the absence of antibiotics in a humidified atmosphere of air/CO₂ (37:3) at 37 °C. Cells were subcultured every 4–5 days by trypsin treatment.

Antibodies

Anti-[glutathione S-transferase (GST)] (Z5) and anti-p110 α (N20) were from Santa Cruz, and anti-(pan-Ras) from Oncogene Science. Anti-(VSV-tag) antibody was purchased from SIGMA. The Rap1A, Rac1 and RhoA antisera were the same as described in [3]. The anti-Crk serum was a gift from Asim Khwaja, (Imperial Cancer Research Fund, London, U.K.).

DNA constructs

PI3K γ fragments were produced by PCR with primers containing appropriate restriction sites. The 32–434 fragment was prepared by PCR with the primers 5'-TCCTCCATGGAGTCATCCCC-ATC-3' and 5'-GCACTAGATCTGGAGGTTTCAG-3'. Primers included either a *Bam*HI or an *Eco*RI restriction site for cloning. *Bam*HI/*Eco*RI-cut fragments were ligated to the *Bam*HI/*Eco*RI-precut and calf intestinal phosphatase-treated pAcG2T vector for Sf9/baculovirus expression of GST fusion proteins (kindly provided by I. Jones). For expression in Sf9 cells the constructs were co-transfected into Sf9 cells with linearized baculovirus DNA (BaculoGold, Pharmingen) by following the manufacturer's protocol to produce infectious baculoviruses. Other baculoviruses used in this study have been described elsewhere [8,10]. For expression in COS-7 cells the PI3K γ cDNA was digested with *Bam*HI and *Xba*I and subcloned into pcDNA3 (Invitrogen).

Recombinant proteins

GST fusion proteins were purified from baculovirus-infected Sf9 cells as described in [11]. Briefly, baculovirus-infected Sf9 cells were mechanically dislodged from the flask 48–72 h (depending on the baculovirus used) after infection, pelleted at 500 g for

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; FCS, fetal calf serum; GSH, reduced glutathione; GST, glutathione S-transferase; PI3K, phosphoinositide 3-kinase.

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5 min and washed once with PBS; then the cell pellet was lysed in 5 vols. of lysis buffer A [50 mM Tris/HCl (pH 7.5)/150 mM NaCl/5 mM $MgCl_2$ /1 mM EGTA/1% (v/v) Triton X-100/1 mM dithiothreitol (DTT)/1 mM PMSF/1 mM benzamidine/10 μ g/ml leupeptin/200 k-i.u./ml aprotinin/10 mM Pefabloc] by vigorous vortex-mixing. After 15 min incubation on ice, the lysed cells were centrifuged at 10000 *g* and 4 °C for 15 min to pellet detergent-insoluble material. The supernatant, generally containing 80–90% of the fusion protein, was incubated for 2 h at 4 °C with 40 μ l of prewashed reduced glutathione (GSH)–Seph-*arose* (Pharmacia) slurry/ml of Sf9 pellet. The GSH precipitates were washed four times in lysis buffer A and once in 50 mM Tris/HCl (pH 7.5)/100 mM NaCl/5 mM $MgCl_2$ /1 mM EGTA/1 mM DTT, then stored in storage buffer [50 mM Hepes (pH 7.5)/50 mM NaCl/5 mM $MgCl_2$ /1 mM EGTA/1 mM DTT/50% (v/v) glycerol] at –20 °C. GST fusion proteins immobilized on GSH–Seph-*arose* (Pharmacia) were eluted from the matrix with 10 mM GSH by following the manufacturer's protocol, dialysed against storage buffer and stored at –20 °C.

Unmodified wild-type and V12 HaRas were purified from a detergent-free homogenate of recombinant baculovirus-infected Sf9 cells as described [12]. Briefly, unmodified Ras present in the 100000 *g* supernatant of mechanically disrupted Sf9 cells was subjected to anion-exchange chromatography on a MonoQ® HR10/10 column (Pharmacia). Fractions containing Ras (determined by silver stain and Western blot analysis) were loaded on a Superdex® 75HR10/30 column (Pharmacia). Ras fractions were 90–95% pure as judged by silver stain analysis. Ras fractions from the final gel-filtration step were divided into aliquots and stored at –80 °C.

The effector site mutant A38Ras, and the GTPases Rap1A, RhoA and Rac1, were purified from bacteria by following the protocol described in [10]. All proteins were full-length and lacked post-translational modifications. Proteins were stored at 1 mg/ml in 50 mM Hepes (pH 7.5)/50 mM NaCl/5 mM $MgCl_2$ /1 mM DTT/50% (v/v) glycerol at –20 °C.

Interaction assays *in vitro*

To load Ras and other GTPases with guanine nucleotides, purified recombinant protein was made 10 mM in EDTA and then mixed with 100 μ M (final concentration) GDP or GTP. After incubation at 37 °C for 5 min, $MgCl_2$ was added to a final concentration of 20 mM.

Binding assays of immobilized GST fusion proteins (0.5–3 μ g) with free, unmodified Ras or Ras mutants, Rac1, Rap1A and RhoA (100–500 nM final concentration in assay) were performed in binding buffer A [100 mM sodium phosphate buffer (pH 7.5)/100 mM NaCl/5 mM $MgCl_2$ /1% (v/v) Triton X-100] at a final volume of 150–200 μ l. Assays were incubated for 90 min at 4 °C with shaking, and the beads were washed four times with binding buffer A. When the effect of antibodies on the binding was investigated, 5 μ g/ml antibody was added to the reaction mixture. For the detection of immobilized Ras binding to soluble PI3K, Ras proteins were coupled to Affigel-10 beads (Bio-Rad) in accordance with the manufacturer's protocol. Immobilized Ras proteins were loaded with nucleotide as described above and purified soluble GST–PI3K γ was added at a final concentration of 150 nM in a final volume of 200 μ l, made up with binding buffer A. After 90 min of shaking at 4 °C, beads were washed four times in binding buffer A. In some experiments 30 mM n-octylglucoside was used instead of Triton X-100.

Proteins associated with the beads were eluted by treating the beads for 5 min at 100 °C in Laemmli solution and subjected to SDS/PAGE. Proteins were transferred to a PVDF membrane

(Millipore) and analysed by Western blotting with appropriate antibodies. Immunocomplexes were detected by use of the enhanced chemiluminescence detection kit from Amersham.

To determine the K_d of the Ras–PI3K γ interaction, unmodified Ras was preloaded with 400 μ M GTP (final concentration) and 0.4 μ Ci (30 Ci/mmol) of [γ - 32 P]GTP per μ g of Ras. Various concentrations of Ras were incubated with 1 μ g of GST–PI3K γ in 50 μ l assays in 50 mM Hepes (pH 7.5)/100 mM NaCl/5 mM $MgCl_2$ /0.1 mg/ml BSA for 1 h at 4 °C. A 10 μ l portion of GSH–Seph-*arose* slurry (Pharmacia) was then added to each assay for 30 min at 4 °C with shaking and the beads were quickly washed four times with 1 ml of 50 mM Hepes (pH 7.5)/100 mM NaCl/5 mM $MgCl_2$ /0.1% BSA/0.1% Triton X-100. Pellets were subjected to Čerenkov counting and backgrounds without GST–PI3K γ were subtracted.

Lipid analysis in COS-7 cells

COS-7 cells were transfected, labelled metabolically with [32 P] P_i and PI3K lipid products were measured as described [8], except that cells were labelled with 100 μ Ci of [32 P] P_i and an ET 250/4 Nucleosil 100-5 SB column (Macherey-Nagel) was used for HPLC analysis. Confluent COS-7 cells were split 1:4 the day before transfection. Cells at 60–70% confluence were detached by trypsin treatment, washed once in 37 °C prewarmed DMEM/10% (v/v) FCS, twice in 37 °C prewarmed PBS and resuspended in 37 °C prewarmed PBS at 2×10^6 cells/ml. Samples (250 μ l) of the cell suspension were dispensed into electroporation cuvettes (0.4 cm gap; Invitrogen) and 5 μ g of each expression plasmid was added. The amount of plasmid in different transfections was made equal by using empty vector. The mix was subjected to a 250 V, 125 μ F pulse in a Bio-Rad Gene Pulser® and left for 10 min at room temperature. The content of an electroporation cuvette was routinely seeded into 4 ml of prewarmed DMEM/10% (v/v) FCS in a 60 mm dish and the medium was changed after 6 h. At 40–48 h after transfection, cells were washed with phosphate-free DMEM and labelled overnight with 100 μ Ci of [32 P] P_i per dish. Reactions were terminated by the addition, on ice, of 1 ml of ice-cold 2.4 M HCl. Cells were scraped off and transferred to a polypropylene tube. Then 1.5 ml of chloroform/methanol (1:2, v/v) [containing 10 μ g per point of phosphoinositide mix (Sigma)] and 1 ml of chloroform were added to the lysates, the mixture was thoroughly vortex-mixed and a phase separation was performed by a short centrifugation. The lower chloroform phase was transferred to a new vial and the upper phase was re-extracted twice with 1.5 ml of chloroform. Pooled chloroform phases were dried up in a Speed-vac and the lipids were deacylated by incubation for 1 h in methylamine [33% (v/v) in ethanol; Fluka] at 50 °C. Ethanol/methylamine was removed in the Speed-vac and the dried film was resuspended in 1 ml of water. The milky suspension was extracted twice with 1 ml of n-butanol. The aqueous phase containing the labelled lipid-head groups was analysed by HPLC as described [13]. Samples were standardized by loading equal radioactive counts of PtdIns(4,5) P_2 on the column.

Immunoprecipitations

COS-7 cells were transfected with the appropriate vectors as described above; 48 h after transfection, COS-7 cells in 60 mm dishes were washed once with cold PBS and lysed on ice in 1 ml of ice-cold lysis buffer B [50 mM Hepes (pH 7.5)/150 mM NaCl/5 mM $MgCl_2$ /1 mM EGTA/1% (v/v) Triton X-100/1 mM DTT/1 mM PMSF/1 mM benzamidine/10 μ g/ml leupeptin/200 k-i.u./ml aprotinin]. Detergent-insoluble material was

precipitated out by centrifugation for 15 min at 10000 *g* and 4 °C. The lysates were treated with 5 μ g of antibody for immunoprecipitation/ml of lysate and 40 μ l of Protein G–Sepharose slurry (Pharmacia) for 1 h at 4 °C under constant mixing. Beads were washed four times in lysis buffer B and subjected to Western blot analysis.

RESULTS AND DISCUSSION

To investigate the interaction of Ras and PI3K γ *in vitro*, the lipid kinase was expressed in Sf9 insect cells as a GST fusion protein, purified and coupled to GSH–Sepharose. Ras preloaded with either GDP or GTP was incubated with the immobilized PI3K γ , and bound protein was detected by Western blotting with anti-Ras antibodies. As shown in Figure 1(a), Ras-GTP exhibited significant association with PI3K γ . Interaction of the p110 catalytic subunit of PI3K α with Ras-GTP has been demonstrated [3,7]. Using an alternative approach we immobilized Ras to Affigel-10 beads, loaded it with GDP or GTP and investigated the association of recombinant PI3K γ . The results in Figure 1(b) clearly demonstrate GTP-dependent binding of PI3K γ to immobilized Ras. The associated enzyme expressed PI3K activity (results not shown). Investigation of the affinity of Ras-GTP for PI3K γ revealed a dissociation constant of approx. 500 nM

(Figure 1c). This is in the range of the previously measured affinity of Ras for the catalytic subunit of PI3K α [7]. To elucidate the specificity of Ras binding to PI3K γ , we investigated the interaction of other GTPases of the Ras superfamily with PI3K γ . Using immobilized GST–PI3K γ we found no association of Rap1A, Rac1 and RhoA with the lipid kinase (Figure 1d).

Different mutants of PI3K γ (Figure 2a) and Ras were used to identify the domains required for the association of both proteins. Our results point to an essential role for an N-terminal region of PI3K γ involving amino acid residues 108–433 in the interaction with Ras-GTP (Figure 2b). A deletion mutant lacking this region failed to bind Ras-GTP, whereas deletions at the N-terminus (up to residue 123) or C-terminus (from residue 775) did not affect Ras-GTP binding. The fragment involving residues 32–434 bound very small amounts of Ras, which were visible after long exposure times. The Ras-binding site of PI3K γ proposed on the basis of our results corresponds to a stretch of similarity to the Ras-binding sites of the catalytic subunits of PI3K α and β [7]. This region includes a lysine residue that has been shown to be essential for the binding of Ras to PI3K α .

Two mutants of Ras and two epitope-specific antibodies against Ras were used for further characterization of the interaction with PI3K γ . As shown in Figure 2(c), the GTP complex of the GTPase-negative mutant V12 expressed a full ability to

Figure 1 Association of Ras with PI3K γ *in vitro*

(a) Binding of Ras to PI3K γ and PI3K α . V12Ras (100 nM) preloaded with either GDP or GTP was incubated with 1.5 μ g each of immobilized GST, GST–p110 of PI3K α or GST–PI3K γ . Associated Ras was detected by immunoblotting with the pan-Ras antibody as described in the experimental section. (b) Binding of PI3K γ to immobilized Ras. Ras was immobilized on Affigel-10 (Bio-Rad) and loaded with either GDP or GTP. Immobilized and preloaded Ras (1 μ g) was incubated with 150 nM GST–PI3K γ . Associated protein was assayed by immunoblotting with anti-GST antibody. BSA (1 μ g) immobilized on Affigel-10 was used as a control. Recombinant GST–PI3K γ (10 ng) was loaded in the first lane as a Western blot control. (c) Affinity of Ras for PI3K γ *in vitro*. Ras was preloaded with [γ - 32 P]GTP and incubated at different concentrations with free GST–PI3K γ . Complexes were precipitated with GSH–Sepharose and washed, and associated radioactivity was measured by Čerenkov counting. The background obtained in the absence of GST–PI3K γ was subtracted for each point. Results shown are the average of duplicates. (d) Binding of Ras, Rap1A, Rac1 and RhoA to PI3K γ . The association of 1.5 μ g of immobilized GST–PI3K γ with 500 nM of each GTPase preloaded with GDP or GTP was investigated as in (a). One-hundredth of the total amount of each GTPase used in the assay was loaded in the last lane of each panel (Load).

Figure 2 Determination of the binding sites on PI3K γ and Ras

(a) Deletion mutants of PI3K γ used for binding studies with Ras. The shaded box represents a region of limited similarity to the Ras-binding domain identified in the p110 catalytic subunits of PI3K α and PI3K β [8]. (b) Binding of Ras to deletion mutants of PI3K γ . Immobilized GST-tagged fragments or GST-PI3K γ (2 μ g of each) were incubated with 500 nM Ras-GDP or Ras-GTP; association of the polypeptides was assayed by SDS/PAGE and immunoblotting. (c) Interaction of PI3K γ with mutants of Ras. GST or GST-PI3K γ (1 μ g of each) immobilized on GSH-Sepharose was incubated with the indicated unprocessed Ras mutants (200 nM). Interacting Ras proteins were detected by Western blotting. Abbreviation: wt, wild-type. (d) Effect of antibodies 259 and 238 on Ras binding to PI3K γ . The interaction of purified immobilized GST-PI3K γ with 100 nM V12Ras was investigated in the presence of equimolar concentrations of anti-RhoB, the non-neutralizing anti-Ras antibody 238 or the neutralizing antibody 259.

associate with PI3K γ . In contrast, Ras-GTP containing the A38 mutation in the effector region was unable to interact with the lipid kinase, suggesting an important function of this domain for the interaction with PI3K γ . In line with this result the antibody 259, which is known to neutralize the effector region of Ras [3,10], suppressed the interaction of Ras with PI3K γ (Figure 2d). Addition of the non-neutralizing antibody 238 or a Rho-specific antibody did not affect the binding of Ras-GTP to PI3K γ .

Several approaches were used to explore the effects of Ras binding on PI3K γ activity. In our hands, incubation of Ras-GTP with PI3K γ did not lead to a measurable increase in the lipid kinase activity (results not shown). This result contrasts with published data on a significant stimulation of PI3K α *in vitro* by Ras-GTP [5,8] and possibly hints at an essential role of additional regulatory proteins that affect the interaction of PI3K γ with Ras. Detailed recent investigations *in vitro* indicated an important role of the p85 regulatory subunit of PI3K α in the stimulation of its lipid kinase activity by Ras [8].

A regulatory function for Ras on PI3K α activity has previously been also demonstrated by transient transfection experiments in

COS-7 cells [4]. However, as shown in Figure 3 (upper panel), co-expression of Ras and PI3K γ in these cells produced only a small increase in PI3K product levels, as opposed to the strong stimulatory effect of Ras on PI3K α activity. In contrast with the relatively high level of PtdIns(3,4) P_2 production after co-expression of PI3K α and Ras, we generally observed an excess of PtdIns(3,4,5) P_3 over PtdIns(3,4) P_2 accumulation after co-expression of PI3K γ and Ras.

The expression levels of PI3K α and PI3K γ in these experiments were equivalent, as determined by Western blot analysis of total cell lysates (Figure 3, lower panel). The majority of both overexpressed PI3K isoforms were found to be associated with the detergent-insoluble fraction, in line with observations made in other laboratories indicating the interaction of both PI3K α and PI3K γ with the cytoskeleton [14–16]. We also ascertained that the overexpressed PI3K γ was enzymically active by testing *in vitro* the lipid kinase activity of immunoprecipitated VSV-PI3K γ (results not shown). We were unable to co-immunoprecipitate Ras with either of the two PI3K isoforms from COS-7 cells. Possibly under physiological conditions only a minor pool

Figure 3 Effects of Ras on the activities of PI3K α and PI3K γ *in vivo*

Upper panel: COS-7 cells were transfected with the indicated vectors and labelled with [32 P]P_i. Labelled lipids were extracted, deacylated and separated by HPLC anion-exchange chromatography. For standardization, 70 000 c.p.m. incorporated in PtdIns(4,5)P₂ were loaded on each HPLC run. The results shown are representative of three independent experiments. Lower panel: expression of PI3K α , PI3K γ and Ras was controlled in parallel by Western blotting with appropriate antibodies. Cells were lysed in lysis buffer B, and Triton X-100-soluble (S) and Triton X-100-insoluble (C) fractions were analysed. The distribution of the cytosolic protein Crk was assessed as a control. Abbreviations: kD, kDa; PI(3,4)P₂, PtdIns(3,4)P₂; PIP₃, PtdIns(3,4,5)P₃; α VSV, anti-VSV; α p110 α , anti-p110 α ; α Crk, anti-Crk; α Ras, anti-Ras.

of active Ras interacts with a small proportion of PI3K, making it an arduous task to detect an interaction between the two proteins *in vivo*.

Thus, Ras effects on PI3K γ and PI3K α lipid kinase activities differ significantly *in vitro* and *in vivo*. PI3K γ behaves as an effector of Ras as it binds to the effector site of GTP-loaded but not GDP-loaded HaRas. However, we have been unable to

detect a clearcut effect of Ras on the lipid kinase activity of PI3K γ under various experimental conditions where PI3K α has been shown to be activated by the GTPase. These results could point to an essential role for additional regulatory proteins that might be needed for the activation of PI3K γ by Ras. Alternatively, Ras could induce merely a GTP-dependent translocation of PI3K γ to the plasma membrane without affecting its kinase activity. Such a mechanism has been proposed for the interaction of Ras with Raf-1 [17]. Finally it is possible that, in contrast with PI3K α , PI3K γ is able to affect Ras activity. In line with this idea we previously found that transient expression of PI3K γ , but not PI3K α , in COS-7 cells produces a strong increase in mitogen-activated protein kinase activity, an effect that could be completely suppressed by dominant negative N17-Ras [18].

In conclusion, our results clearly indicate GTP-dependent binding of Ras to PI3K γ . Thus the signalling enzyme is able to interact with both heterotrimeric G-proteins and a small GTPase. This unique feature produces an urgent need for an analysis of the events produced by the interaction of PI3K γ with different combinations of these ligands *in vitro* and *in vivo*.

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