

Stimulation of phospholipase C- β_2 by the Rho GTPases Cdc42Hs and Rac1

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Neutrophils contain a soluble guanine-nucleotide-binding protein, made up of two components with molecular masses of 23 and 26 kDa, that mediates stimulation of phospholipase C- β_2 (PLC β_2). We have identified the two components of the stimulatory heterodimer by amino acid sequencing as a Rho GTPase and the Rho guanine nucleotide dissociation inhibitor LyGDI. Using recombinant Rho GTPases and LyGDI, we demonstrate that PLC β_2 is stimulated by guanosine 5'-O-(3-thiotriphosphate) (GTP[S])-activated Cdc42Hs \times LyGDI, but not by RhoA \times LyGDI. Stimulation of PLC β_2 , which was also observed for GTP[S]-activated recombinant Rac1, was independent of LyGDI, but required C-terminal processing of Cdc42Hs/Rac1. Cdc42Hs/Rac1 also stimulated PLC β_2 in a system made up of purified recombinant proteins, suggesting that this function is mediated by direct protein-protein interaction. The Cdc42Hs mutants F37A and Y40C failed to stimulate PLC β_2 , indicating that the Cdc42Hs effector site is involved in this interaction. The results identify PLC β_2 as a novel effector of the Rho GTPases Cdc42Hs and Rac1, and as the first mammalian effector directly regulated by both heterotrimeric and low-molecular-mass GTP-binding proteins.

Keywords: Cdc42Hs/phospholipase C/Rac1/RhoA/signal transduction

Introduction

The signal transduction pathways responsible for activation of neutrophils by chemoattractants have been the subject of intense investigation over recent years. The binding of chemoattractants to their receptors on the neutrophil plasma membrane generates multiple intracellular second messengers and signalling molecules through the activation of phospholipases C, A₂ and D, as well as through the activation of lipid and protein kinases (Bokoch, 1995; Ye and Boulay, 1997; Baggiolini, 1998). Phospholipase C (PLC) hydrolyses phosphatidylinositol 4,5-bisphosphate (PtdInsP₂) to produce two second messengers, D-myo-inositol-1,4,5-trisphosphate and *sn*-1,2-diacylglycerol. D-myo-inositol-1,4,5-trisphos-

phate causes the release of Ca²⁺ from intracellular stores, whereas *sn*-1,2-diacylglycerol activates protein kinase C. Molecular cloning has revealed the existence of three major families of PLC: β , γ and δ . PLC β and PLC γ enzymes are activated by G protein-coupled receptors (GCRs) and tyrosine-kinase-linked receptors, respectively. Whether PLC δ enzymes are regulated by cell-surface receptors is unknown. PLC β exists in four isoforms, PLC β_{1-4} , which are activated by members of the α_q subfamily of the G protein α subunits and, except for PLC β_4 , by G protein $\beta\gamma$ dimers (Rhee and Bae, 1997; Singer *et al.*, 1997).

We have previously shown that cytosolic preparations of myeloid differentiated HL-60 cells and of bovine neutrophils contain a soluble phospholipase C, which is stimulated by the poorly hydrolyzable GTP analogue guanosine 5'-O-(3-thiotriphosphate) (GTP[S]) (Camps *et al.*, 1990; Illenberger *et al.*, 1997). A factor mediating GTP[S]-dependent stimulation of PLC in neutrophils was resolved from endogenous PLC, and functionally reconstituted with exogenous recombinant PLC β_2 (Illenberger *et al.*, 1997). The resolved protein mediated GTP[S]-dependent activation of PLC β_2 , but not PLC β_1 and PLC δ_1 . Stimulation of phosphatidylinositol-4-phosphate 5-kinase was not involved in this activation. Purification of the stimulatory factor revealed that it consisted of two proteins of apparent molecular masses of 23 and 26 kDa. The purified heterodimer stimulated a deletion mutant of PLC β_2 , PLC $\beta_2\Delta$, lacking a C-terminal region necessary for stimulation by G protein α_q subunits. These results led us to suggest that a GTP-binding protein distinct from α_q subunits, most likely a low-molecular-mass GTP-binding protein associated with a regulatory protein, is involved in activation of PLC β_2 .

Herein, we report the identification of the PLC β_2 -stimulating factor of bovine neutrophils as a member of the Rho subfamily of low-molecular-mass GTP-binding proteins associated with the Rho GDP dissociation inhibitor LyGDI. We demonstrate that GTP[S]-activated recombinant Cdc42Hs and Rac1, but not RhoA, stimulate the activity of PLC β_2 . While the presence of LyGDI is not necessary for PLC β_2 activation, both C-terminal processing of Cdc42Hs and Rac1 and integrity of their effector-regulating domains are required for this effect. These results identify PLC β_2 as a novel effector of the Rho GTPases Cdc42Hs and Rac1.

Results

The purification of the PLC β_2 -stimulating guanine nucleotide-binding protein from soluble fractions of bovine neutrophils revealed that it consists of two polypeptides of ~23 and 26 kDa (Illenberger *et al.*, 1997). The two proteins were subjected to protease

digestion and proteolytic fragments were obtained for amino acid sequence analysis. The sequences of two fragments of the 23 kDa protein (DQFPEVYVPTVFENY and DRLRPLSY) were identical as partial amino acid sequences of bovine RhoA (Ogorochi *et al.*, 1989) and human RhoC (Chardin *et al.*, 1988). Three peptides of the 26 kDa fragments were highly related in amino acid sequence to internal peptide fragments of human LyGDI (Scherle *et al.*, 1993) and D4 (Lelias *et al.*, 1993), but were clearly different from the corresponding fragments of bovine RhoGDI (Fukumoto *et al.*, 1990) (DESLTKYTKLLG, DPTAPNVK~~V~~TRLTLVXESAP, DKATFMVGSYGPRPE; italics, residues not present in human LyGDI/D4; underlined, residues not present in bovine RhoGDI). LyGDI and D4 are homologues of the Rho guanine nucleotide dissociation inhibitor RhoGDI, and are highly expressed in hematopoietic cells (Lelias *et al.*, 1993; Scherle *et al.*, 1993). Their amino acid sequences differ by only two out of 202 residues. Taken together, these results led us to conclude that the soluble PLC β_2 -stimulating guanine nucleotide-binding protein of bovine neutrophils is a heterodimer of a Rho GTPase and LyGDI/D4.

To obtain further functional support of this notion, recombinant glutathione *S*-transferase fusion protein of human LyGDI (GST–LyGDI) was produced in *Escherichia coli* in order to deplete the Rho GTPases from the PLC β_2 -stimulating factor purified from bovine neutrophils. To this end, matrix-immobilized GST–LyGDI was incubated with the PLC β_2 -stimulating factor. Following incubation, matrix and supernatant were separated by centrifugation, and bound proteins were eluted from the matrix with glutathione. Figure 1A (left) shows that the treatment of the PLC β_2 -stimulating factor with matrix-immobilized GST–LyGDI led to an almost complete loss of the GTP[S]-dependent stimulation of PLC $\beta_2\Delta$. Importantly, the stimulatory activity was recovered from the matrix by treatment with glutathione (Figure 1B, right). Thus, an almost 2-fold stimulation of inositol phosphate formation by GTP[S] was observed upon reconstitution of the eluate with PLC $\beta_2\Delta$. These results provide direct evidence that the PLC β_2 -stimulating factor specifically interacts with LyGDI and thus confirm its identity as a member of the Rho subfamily of Ras-related proteins.

LyGDI/D4 has been reported to interact with RhoA, Cdc42Hs and Rac1, but not with Ras and Rap (Adra *et al.*, 1993; Scherle *et al.*, 1993). In order to identify the Rho GTPase(s) associated with recombinant GST–LyGDI upon incubation with the PLC β_2 -stimulating factor from bovine neutrophils, immunostaining of the samples was performed using polyclonal antisera specifically reactive against human RhoA, Cdc42Hs and Rac1. Figure 1B shows that the purified PLC β_2 -stimulating factor was highly reactive with antisera raised against RhoA and Cdc42Hs. No immunoreactivity was observed with the serum raised against human Rac1 (results not shown). The specificity of the antisera was verified using bacterially expressed human RhoA, Cdc42Hs and Rac1 (not shown). Analysis of the fractions obtained after incubation of the factor with matrix-immobilized GST–LyGDI revealed a reduction of both the RhoA and the Cdc42Hs immunoreactivity. Most importantly, both immunoreactivities were recovered upon glutathione elution of the matrix containing GST–LyGDI

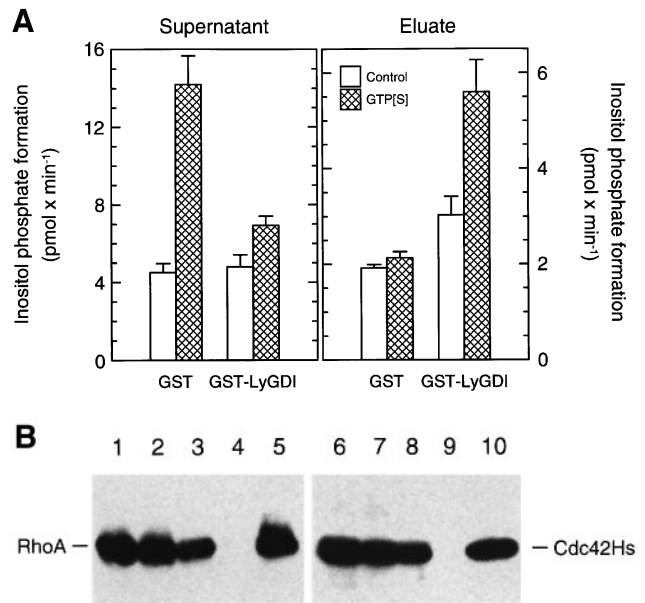


Fig. 1. Depletion of the PLC β_2 -stimulating activity from the purified soluble stimulatory factor with a GST fusion protein of LyGDI. The PLC β_2 -stimulating factor was incubated as indicated on the abscissa with GST or GST–LyGDI bound to Glutathione Sepharose 4B. (A) After removal of the matrix by centrifugation, 10 μ l aliquots of the supernatants were supplemented with soluble proteins of insect cells expressing PLC $\beta_2\Delta$ (0.2 μ g protein/sample) and incubated in the absence (open bars) or presence (hatched bars) of 100 μ M GTP[S] with phospholipid vesicles containing PtdInsP₂ (left panel). The proteins bound to the affinity matrix were eluted with glutathione and concentrated. Aliquots (10 μ l) of the concentrated solutions were supplemented with soluble proteins of insect cells expressing PLC $\beta_2\Delta$ (0.2 μ g protein/sample) and incubated in the absence (open bars) or presence (hatched bars) of 100 μ M GTP[S] with phospholipid vesicles containing PtdInsP₂ (right panel). The reaction was terminated by the addition of chloroform/methanol/concentrated HCl, and the mixture analysed for inositol phosphates. See Materials and methods for experimental details. (B) Aliquots of the sample containing the PLC β_2 -stimulating factor (lanes 1 and 6), of the supernatants obtained after incubation of the factor with matrix-immobilized GST (lanes 2 and 7) or GST–LyGDI (lanes 3 and 8), or of the concentrated solutions containing proteins bound to matrix-immobilized GST (lanes 4 and 9) or GST–LyGDI (lanes 5 and 10) were subjected to SDS–PAGE and immunoblotting was performed using antibodies reactive against either RhoA (lanes 1–5) or Cdc42Hs (lanes 6–10). The data are representative of three independent experiments with similar results.

(Figure 1B). No reactivity was detected in the eluate with serum raised against human Rac1 (not shown). These data demonstrate that at least two Rho GTPases, RhoA and Cdc42Hs, are present in the soluble PLC β_2 -stimulating factor from bovine neutrophils.

To determine whether LyGDI \times Cdc42Hs and/or LyGDI \times RhoA mediate(s) the GTP[S]-dependent stimulation of PLC β_2 , the proteins were produced as recombinant heterodimers for functional characterization. To this end, the heterodimers were prepared by solubilization of recombinant isoprenylated human Cdc42Hs or RhoA from membranes of baculovirus-infected insect cells by bacterially expressed, purified GST–LyGDI. The soluble fractions obtained after incubation of insect cell membranes with GST or GST–LyGDI were analysed by Western blotting. Figure 2A shows that GST–LyGDI was capable of extracting both Cdc42Hs and RhoA into the supernatant. The fact that only minor quantities of

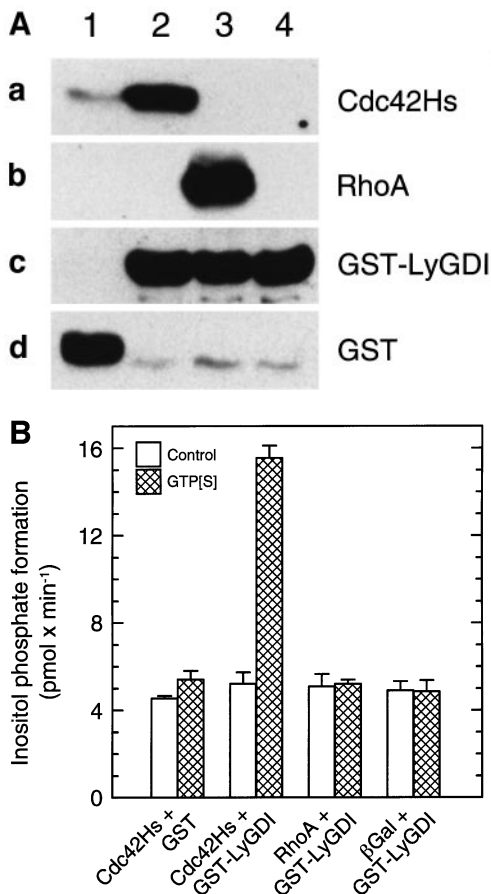


Fig. 2. Stimulation of PLC $\beta_2\Delta$ by recombinant heterodimeric GST-LyGDI \times Cdc42Hs. (A) Membranes of insect cells infected with baculovirus encoding Cdc42Hs (lanes 1 and 2), RhoA (lane 3) or β -galactosidase (lane 4) were incubated with 45 μ M purified, bacterially expressed GST (lane 1) or GST-LyGDI (lanes 2-4). The membranes were sedimented by centrifugation and the supernatants were concentrated. Aliquots of the concentrates were subjected to SDS-PAGE and immunoblotting was performed using antibodies reactive against (a) Cdc42Hs, (b) RhoA, (c) LyGDI or (d) GST. (B) Aliquots (10 μ l) of the concentrates were supplemented with soluble proteins of insect cells expressing PLC $\beta_2\Delta$ (0.2 μ g protein/sample). The samples were incubated in the absence (open bars) or presence (hatched bars) of 100 μ M GTP[S] with phospholipid vesicles containing PtdInsP₂.

Cdc42Hs were observed in the supernatant after treatment of membranes with GST demonstrates that the extraction of the Rho GTPases was due to specific interaction with LyGDI. Figure 2B shows that the heterodimer made up of GST-LyGDI and Cdc42Hs led to a marked (~3-fold) stimulation of the activity of recombinant PLC $\beta_2\Delta$. In contrast, no stimulation of PLC $\beta_2\Delta$ was observed for heterodimeric GST-LyGDI \times RhoA or free GST-LyGDI. These results demonstrate that LyGDI \times Cdc42Hs, but not LyGDI \times RhoA or LyGDI alone, mediates the GTP[S]-dependent stimulation of PLC β_2 .

The next experiments were designed to examine whether both constituents of the Cdc42 \times LyGDI heterodimer are necessary for PLC β_2 stimulation or whether monomeric Cdc42Hs is sufficient to elicit this effect. Recombinant Cdc42Hs was solubilized from membranes of baculovirus-infected insect cells by extraction with detergent-containing buffer. As shown in Figure 3A, addition of GTP[S] greatly (~3.6-fold) enhanced the PLC $\beta_2\Delta$ activity in the

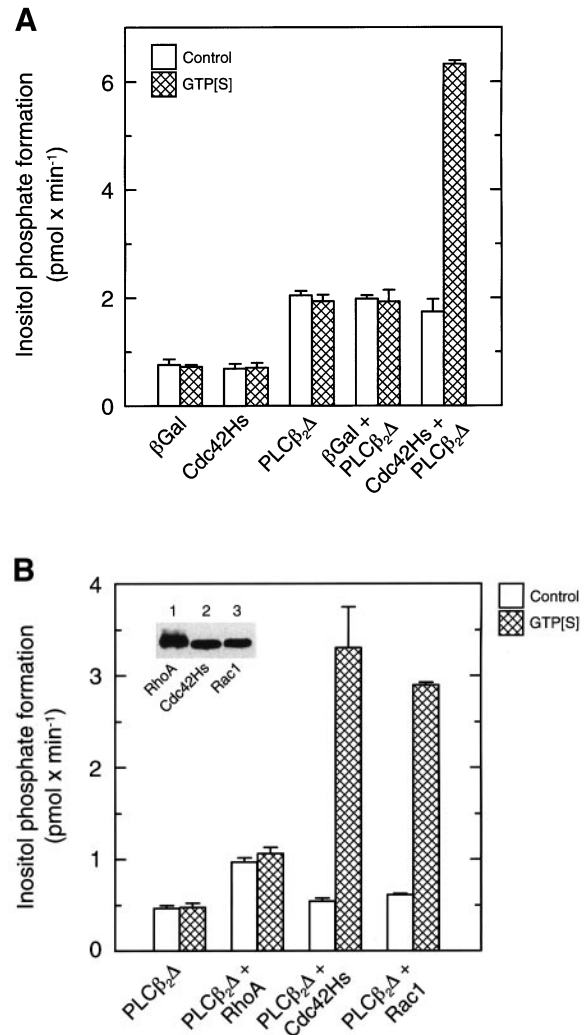


Fig. 3. Specificity of PLC $\beta_2\Delta$ stimulation by Rho GTPases.

(A) Membranes of insect cells infected with baculovirus encoding β -galactosidase (β Gal) or Cdc42Hs were extracted with buffer containing sodium cholate. Aliquots (10 μ l) of the detergent extracts were supplemented with soluble proteins of insect cells expressing PLC $\beta_2\Delta$ (0.4 μ g protein/sample) and incubated in the absence (open bars) or presence (hatched bars) of 100 μ M GTP[S] with phospholipid vesicles containing PtdInsP₂. The activities of the detergent extracts and the preparation containing soluble PLC $\beta_2\Delta$ alone in the absence or presence of 100 μ M GTP[S] is shown for comparison. In (B), membranes of insect cells infected with baculovirus encoding RhoA, Cdc42Hs or Rac1 were extracted with buffer containing sodium cholate. The abundance of the recombinant Rho GTPases in the detergent extracts was estimated by ³⁵S-GTP[S] binding. Aliquots of the detergent extracts containing equal amounts of the three Rho GTPases (12 pmol) were supplemented with soluble proteins of insect cells expressing PLC $\beta_2\Delta$ (0.5 μ g protein/sample) and incubated in the absence (open bars) or presence (hatched bars) of 100 μ M GTP[S] with phospholipid vesicles containing PtdInsP₂. The activity of the preparation containing soluble PLC $\beta_2\Delta$ alone in the absence or presence of 100 μ M GTP[S] is shown for comparison. The activities of the detergent extracts containing RhoA, Cdc42Hs or Rac1 were 0.39 \pm 0.13 pmol/min, not detectable, and 0.13 \pm 0.02 pmol/min, respectively, in the absence of PLC $\beta_2\Delta$, and were independent of the presence of GTP[S] (results not shown). The inset of (B) shows the results of the immunochemical analysis of the three detergent extracts. Aliquots of the three samples were subjected to SDS-PAGE and immunoblotting was performed using antibodies reactive against RhoA (lane 1), Cdc42Hs (lane 2) or Rac1 (lane 3).

presence of extracts containing Cdc42Hs. In contrast, no stimulation by GTP[S] was observed in the presence of extracts from control cells expressing recombinant β -galactosidase rather than Cdc42Hs. Thus, monomeric Cdc42Hs is sufficient to mediate GTP[S]-dependent stimulation of PLC β_2 .

Since it is well-established that a wide range of effectors is regulated by both Cdc42Hs and Rac1, but not by RhoA (Van Aelst and D'Souza-Schorey, 1997), the specificity of PLC β_2 stimulation by Rho family members was investigated. The recombinant GTPases Cdc42Hs, Rac1 and RhoA were extracted from membranes of baculovirus-infected insect cells with detergent-containing buffer and reconstituted with recombinant PLC $\beta_2\Delta$. In agreement with the results shown in Figure 2, the activity of PLC $\beta_2\Delta$ was not stimulated by GTP[S]-activated RhoA (Figure 3B). Additional experiments showed that RhoA failed to mediate GTP[S]-dependent stimulation of PLC $\beta_2\Delta$, even at concentrations up to 800 nM (not shown). In contrast, addition of both Cdc42Hs and Rac1 to PLC $\beta_2\Delta$ led to marked GTP[S]-dependent increases in inositol phosphate formation. Both GTPases, Cdc42Hs and Rac1, stimulated PLC $\beta_2\Delta$ activity to a similar extent (6.2- and 4.8-fold, respectively), when tested at the same concentration (200 nM). Thus, Rac1, although not detected by immunochemical means in the purified preparation from bovine neutrophils, may represent an additional Rho GTPase capable of stimulating PLC β_2 .

In order to determine whether stimulation of PLC β_2 by Cdc42Hs or Rac1 is mediated by direct protein-protein interaction or whether additional proteins are required to mediate this effect, the protein components were purified from insect cells for functional reconstitution. PLC $\beta_2\Delta$ and LyGDI were purified from soluble fractions, and Cdc42Hs and Rac1 were purified from membranes of baculovirus-infected insect cells. Figure 4A shows the result of the analysis of the purified proteins by SDS-PAGE. The proteins were reconstituted and the inositol phosphate formation was measured in the absence and presence of GTP[S]. As shown in Figure 4B, purified Cdc42Hs and Rac1 indeed caused a marked (~3.4- and 4.3-fold, respectively) stimulation of purified PLC $\beta_2\Delta$.

Even at a concentration of 10 μ M, purified LyGDI had little, if any, effect on the activity of PLC $\beta_2\Delta$, regardless of whether GTP[S] was absent or present in the incubation medium. These data suggest that stimulation of PLC β_2 by Cdc42Hs and Rac1 is due to direct interaction between the GTPases and PLC β_2 . In additional experiments (results not shown), we found that purified LyGDI had no effect on the ability of GTP[S] to activate PLC $\beta_2\Delta$ in the presence of purified Cdc42Hs when present in up to 100-fold excess of the Rho GTPase. Only at very high concentrations of LyGDI (\geq 200-fold excess of Cdc42Hs or Rac1) was GTP[S]-dependent activation of the enzyme reduced.

The observation that LyGDI is apparently not required for PLC β_2 stimulation by GTP[S]-activated Cdc42Hs raises the question of whether the posttranslational modifications of Cdc42Hs, which are required for its interaction with GDIs (Leonard *et al.*, 1992), are necessary for PLC β_2 stimulation. To address this question, C-terminally modified Cdc42Hs extracted with detergent-containing buffer from membranes and soluble, unmodified Cdc42Hs

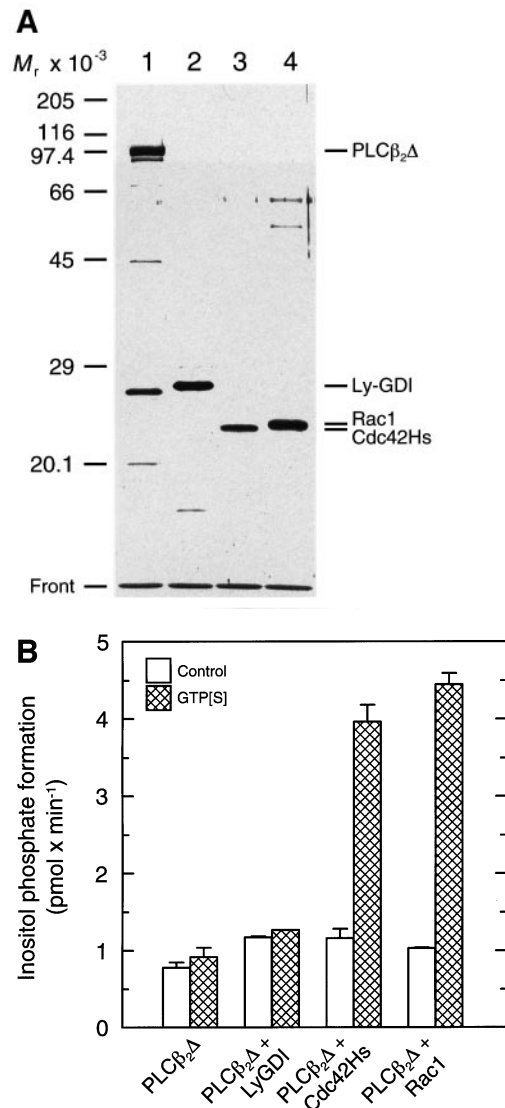


Fig. 4. Direct activation of PLC $\beta_2\Delta$ by Cdc42Hs and Rac1. Recombinant PLC $\beta_2\Delta$ and LyGDI were purified from soluble fractions of baculovirus-infected insect cells. Recombinant Cdc42Hs and Rac1 were purified from membranes of baculovirus-infected insect cells. (A) Aliquots of purified PLC $\beta_2\Delta$ (5 μ g of protein) (lane 1), purified LyGDI (1 μ g of protein) (lane 2), Cdc42Hs (0.5 μ g of protein) (lane 3) and Rac1 (1 μ g of protein) (lane 4) were subjected to SDS-PAGE. Proteins were visualized by staining with silver. The positions of PLC $\beta_2\Delta$, LyGDI, Rac1, Cdc42Hs and the molecular weight standards are indicated. We noticed a degradation of purified PLC $\beta_2\Delta$ upon storage and/or freezing and thawing, resulting in fragments migrating at ~90, 45 and 27 kDa. Attempts to resolve these fragments from full-length PLC $\beta_2\Delta$ by several chromatographic procedures were unsuccessful (results not shown). (B) Aliquots of purified LyGDI, Cdc42Hs and Rac1 were supplemented with purified PLC $\beta_2\Delta$ (150 ng protein/sample) and incubated in the absence (open bars) or presence (hatched bars) of 100 μ M GTP[S] with phospholipid vesicles containing PtdInsP₂. The final concentrations of LyGDI, Cdc42Hs and Rac1 were 10, 140 and 220 nM, respectively.

purified from cytosol of baculovirus-infected insect cells were compared in their abilities to stimulate recombinant PLC $\beta_2\Delta$. A marked ~3.6-fold stimulation of PLC β_2 activity by GTP[S] was observed in the presence of modified Cdc42Hs, but not in the presence of a similar quantity of soluble Cdc42Hs (Figure 5A). These results suggest that

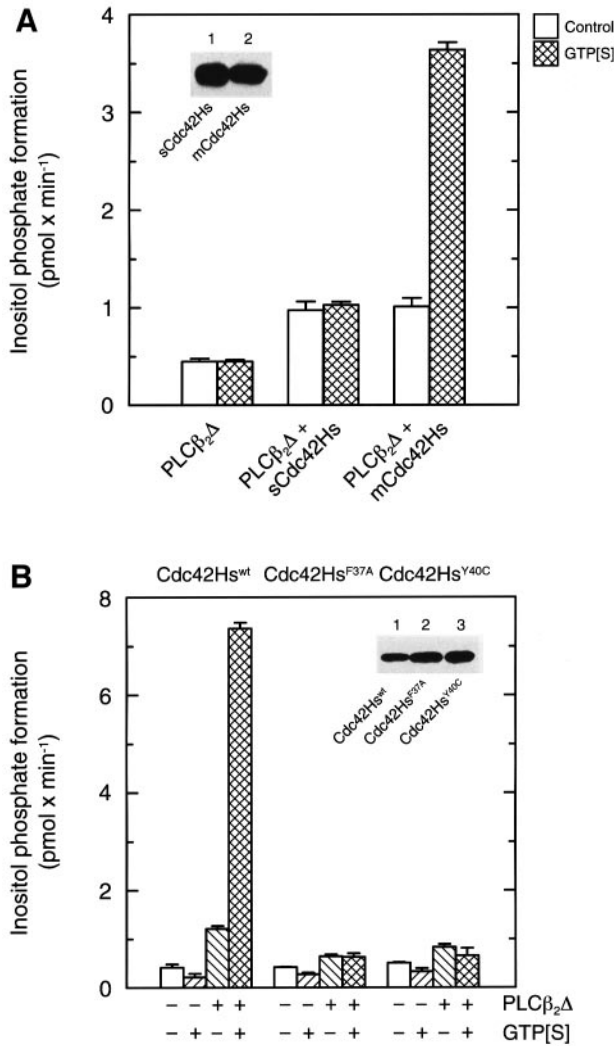


Fig. 5. Structural elements of Cdc42Hs involved in PLC $\beta_2\Delta$ stimulation. **(A)** C terminus. Cdc42Hs was either purified from cytosol or extracted with buffer containing sodium cholate from membranes of insect cells infected with baculovirus encoding Cdc42Hs. The abundance of recombinant Cdc42Hs in the two preparations was estimated by ³⁵S-GTP[S] binding. Aliquots of the two preparations containing 15 pmol soluble Cdc42Hs (*sCdc42Hs*) or 22 pmol membrane-bound Cdc42Hs (*mCdc42Hs*) were supplemented with soluble proteins of insect cells expressing PLC $\beta_2\Delta$ (0.5 μ g protein/sample) and incubated in the absence (open bars) or presence (hatched bars) of 100 μ M GTP[S] with phospholipid vesicles containing PtdInsP₂. Inset: aliquots of the two preparations containing 15 pmol soluble Cdc42Hs (lane 1) or 22 pmol membrane-bound Cdc42Hs (lane 2) were subjected to SDS-PAGE and immunoblotting was performed using antibodies reactive against Cdc42Hs. **(B)** Effector-regulating domain. Membranes of insect cells infected with baculovirus encoding wild-type Cdc42Hs (*Cdc42Hs^{wt}*), or the mutants *Cdc42Hs^{F37A}* and *Cdc42Hs^{Y40C}*, were extracted with buffer containing sodium cholate. The abundance of the recombinant Cdc42Hs proteins in the detergent extracts was estimated by ³⁵S-GTP[S] binding. Aliquots of the detergent extracts containing equal amounts of the three GTPases (30 pmol) were supplemented with soluble proteins of insect cells expressing PLC $\beta_2\Delta$ (0.5 μ g protein/sample) and incubated as indicated at the abscissa in the absence or presence of 100 μ M GTP[S] with phospholipid vesicles containing PtdInsP₂. The activities of the detergent extracts in the absence of PLC $\beta_2\Delta$ are shown for comparison. Inset: aliquots of the detergent extracts containing equal amounts (10 pmol) of *Cdc42Hs^{wt}* (lane 1), *Cdc42Hs^{F37A}* (lane 2) or *Cdc42Hs^{Y40C}* (lane 3) were subjected to SDS-PAGE and immunoblotting was performed using antibodies reactive against Cdc42Hs.

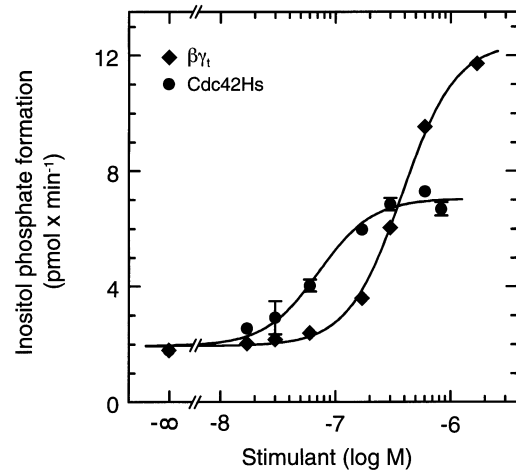


Fig. 6. Stimulation of PLC $\beta_2\Delta$ by $\beta\gamma_t$ versus Cdc42Hs. Purified recombinant PLC $\beta_2\Delta$ (120 ng protein/sample) was incubated at increasing concentrations of $\beta\gamma_t$ purified from bovine retinal rod outer segments (diamonds) or recombinant wild-type Cdc42Hs extracted with buffer containing sodium cholate from membranes of baculovirus-infected insect cells (circles) with phospholipid vesicles containing PtdInsP₂. The incubation was performed in the presence of 1.9 mM sodium cholate and 100 μ M GTP[S]. Cdc42Hs did not stimulate PLC $\beta_2\Delta$ in the absence of GTP[S] (not shown).

C-terminal processing of Cdc42Hs is a prerequisite for the ability of the protein to stimulate PLC β_2 .

Substitution of amino acids in a region (residues 26–45) of Cdc42Hs and Rac homologous to the effector region in Ras has been shown to interfere with their binding to and regulation of downstream effectors (Diekmann *et al.*, 1994; Lamarche *et al.*, 1996; Westwick *et al.*, 1997). To investigate whether the effector region in Cdc42Hs is involved in the stimulation of PLC β_2 , two single-amino-acid substitutions were introduced into the effector domain of Cdc42Hs. Both mutants, *Cdc42Hs^{F37A}* and *Cdc42Hs^{Y40C}*, were expressed in baculovirus-infected insect cells, and their ability to bind ³⁵S-GTP[S] was determined. *Cdc42Hs^{F37A}* and *Cdc42Hs^{Y40C}* were indistinguishable from wild-type Cdc42Hs in terms of their ³⁵S-GTP[S] binding parameters (not shown). However, in marked contrast to wild-type Cdc42Hs, which caused an ~6-fold stimulation of recombinant PLC β_2 , neither *Cdc42Hs^{F37A}* nor *Cdc42Hs^{Y40C}* affected the activity of PLC $\beta_2\Delta$ in the absence or presence of GTP[S] (Figure 5B). Therefore, the integrity of the effector-regulating region of Cdc42Hs is required for PLC β_2 stimulation.

A comparison of the effects of increasing concentrations of purified $\beta\gamma_t$ and GTP[S]-activated recombinant Cdc42Hs on purified PLC $\beta_2\Delta$ is presented in Figure 6. The results show that $\beta\gamma_t$ caused a higher maximal enzyme stimulation (~6.4-fold) than did GTP[S]-activated Cdc42Hs (~3.6-fold), but was required at ~5-fold higher concentrations to elicit this effect. Thus, half-maximal stimulation of PLC $\beta_2\Delta$ required ~400 nM $\beta\gamma_t$, but only ~70 nM Cdc42Hs. In additional experiments (not shown), we found that the potency of GTP[S]-activated Rac1 to stimulate PLC $\beta_2\Delta$ was ~20-fold higher than the potency of GTP[S]-activated Cdc42Hs. Consistent with earlier results (Illenberger *et al.*, 1997), we observed that wild-type recombinant PLC β_2 was stimulated to a lower maximal extent by Cdc42Hs or Rac1 (up to ~2.5-fold) than PLC $\beta_2\Delta$.

No evidence was obtained for synergism between either Cdc42Hs or Rac1 and $\beta\gamma$ upon co-stimulation of PLC β_2 or wild-type PLC β_2 .

Discussion

We have previously shown that cytosolic preparations of bovine neutrophils contain a soluble, heterodimeric guanine nucleotide-binding protein that mediates stimulation of PLC β_2 . The molecular masses of the two components of the purified heterodimer, 23 and 26 kDa, suggested that a low-molecular-mass GTP-binding protein might mediate PLC β_2 activation. This notion was supported by the finding that the C-terminal region of PLC β_2 , which is necessary for stimulation by members of the α_q subfamily of the heterotrimeric G protein α subunits, was not required for PLC β_2 stimulation by the purified heterodimer (Illenberger *et al.*, 1997). In this study, we identified the constituents of the purified PLC β_2 -stimulating protein from bovine neutrophils as the Rho GTPase Cdc42 and its regulatory protein LyGDI. This result is consistent with our previous observations and demonstrates, for the first time, that PLC β_2 is regulated not only by heterotrimeric G proteins, but also by low molecular mass GTP-binding proteins of the Rho family. In addition, our experiments demonstrate that a recombinant heterodimer made up of human LyGDI and Cdc42Hs substituted for the heterodimer purified from bovine neutrophils in terms of its ability to stimulate PLC β_2 . Interestingly, the GTPase Cdc42Hs alone was sufficient to mediate GTP[S]-dependent stimulation of PLC β_2 . Analysis of the Rho family member specificity of PLC β_2 stimulation revealed that this stimulation was not limited to activated Cdc42Hs, but was also observed with activated Rac1. Importantly, activated RhoA was without effect, adding PLC β_2 to the growing list of targets regulated by Cdc42Hs and Rac1, but not by RhoA.

An important outcome of this study is the observation that stimulation of PLC β_2 by Cdc42Hs required C-terminal processing of Cdc42Hs (Glomset and Farnsworth, 1994). It should be noted that the inability of soluble unmodified Cdc42Hs to stimulate PLC β_2 was not due to its inability to bind GTP[S]. Both modified and unmodified Cdc42Hs possessed similar ^{35}S -GTP[S] binding activities when tested under a variety of conditions, including those used for measurement of their abilities to stimulate PLC β_2 (cf. legend to Figure 5A). Rho GTPases need to be C-terminally modified in order to interact with guanine nucleotide dissociation inhibitors (GDIs) and, at least in some cases, with guanine nucleotide exchange factors (GEFs) and effectors (reviewed in Takai *et al.*, 1995). The results reported here demonstrate that C-terminal modification of Cdc42Hs is not required for guanine nucleotide exchange, at least under the conditions used in this study, but is a prerequisite to its ability to mediate GTP[S]-dependent stimulation of PLC β_2 . The effector site substitutions Cdc42Hs^{F37A} and Cdc42Hs^{Y40C} resulted in mutants which failed to stimulate PLC β_2 , indicating that both C-terminal modification(s) and the integrity of the effector-regulating region of Cdc42Hs are required for PLC β_2 activation. In addition, our results obtained with purified proteins suggest that Cdc42Hs and Rac1 directly interact with PLC β_2 . Taken together, these data indicate

that PLC β_2 serves as an effector rather than a regulator of Cdc42Hs and Rac1.

It is likely that regulation of PLC β_2 activity by Cdc42Hs and Rac1 is subject to control of the GTPases by regulatory proteins, e.g. Rho family GDIs, GEFs or GAPs (Van Aelst and D'Souza-Schorey, 1997). Interestingly, GTP[S] is shown in this study to promote the Cdc42Hs-mediated stimulation of PLC β_2 activity, even in the presence of LyGDI and in the absence of any GEF. This implies spontaneous guanine nucleotide exchange under the conditions of our experiments, which is consistent with previous findings which indicate that such an exchange is feasible at low magnesium concentrations and/or in the presence of PtdInsP₂ (Self and Hall, 1995a; Zheng *et al.*, 1996). The existence of a specific GEF in neutrophils, which might enhance the Cdc42Hs/Rac1-mediated PLC β_2 stimulation, and the functional role of LyGDI will be investigated in future studies.

The findings of this study have important implications for the role of Cdc42Hs-mediated PLC β_2 stimulation in pathways controlled by Cdc42Hs in intact cells. Rho GTPases control the assembly and organization of the actin cytoskeleton in eukaryotic cells and regulate intracellular protein kinase cascades and gene transcription (Van Aelst and D'Souza-Schorey, 1997; Hall, 1998). Cdc42 and Rac1 mutants carrying effector site substitutions have previously been used to dissect the biochemical pathways involved in these cellular responses (Joneson *et al.*, 1996; Lamarche *et al.*, 1996; Westwick *et al.*, 1997). Results from these studies have provided evidence for bifurcating pathways controlling cytoskeletal changes and MAP kinase activation. Thus, amino acid substitution at position 40 of Cdc42 blocked c-Jun N-terminal kinase (JNK) activation without affecting activation of Rac1, while changes at codon 37 had the opposite effect. Both mutants, however, still induced DNA synthesis, and caused formation of filopodia and focal complexes (Lamarche *et al.*, 1996). Our own observation that both Cdc42Hs^{Y40C} and Cdc42Hs^{F37A} failed to stimulate PLC β_2 suggests that PLC β_2 may represent an additional independent effector moiety regulated by Cdc42Hs. Furthermore, PLC β_2 stimulation by Cdc42Hs is unlikely to be involved in Cdc42Hs-mediated activation of JNK, activation of Rac1, and formation of filopodia and focal complexes, at least in fibroblasts, since these pathways are activated by either Cdc42^{Y40C} or Cdc42^{F37A}.

An important issue concerning the role of Cdc42Hs and Rac1 in the regulation of PLC β_2 is how these low-molecular-mass GTP-binding proteins fit into the scheme by which GCRs control this enzyme in intact cells. Previous results provide strong evidence that GCRs activate PLC β_2 in neutrophils via $\beta\gamma$ dimers released from pertussis toxin-sensitive G proteins (Camps *et al.*, 1992; Jiang *et al.*, 1996). However, chemoattractant receptors have also been shown to cause the activation of Rho GTPases in intact leukocytes (Laudanna *et al.*, 1996). The mechanisms by which this activation takes place are largely unknown. Several scenarios are possible. First, GCRs could interact directly with Rho GTPases to catalyse their activation (Polakis *et al.*, 1989; Wieland *et al.*, 1990; Mitchell *et al.*, 1998). Secondly, Rho GTPases could be regulated through GCRs via direct interaction with free G protein $\beta\gamma$ dimers (Harhammer *et al.*, 1996; Alberts *et al.*, 1998). Thirdly, subunits of receptor-activated hetero-

trimeric G proteins could interact with and activate GEFs of Rho GTPases (Zhao *et al.*, 1995; Hart *et al.*, 1998; Nern and Arkowitz, 1998). Finally, leukocyte GCRs have been shown to cause alterations in the levels and/or activities of multiple intracellular second messengers and signalling molecules, some of which are known to modulate, either directly or indirectly, the activity of Rho GTPases (Hawkins *et al.*, 1995; Han *et al.*, 1998). Importantly, Cdc42 and/or Rac1 may also be activated by the latter mechanism(s) via receptors not coupled to G proteins, e.g. receptor tyrosine kinases. Signals emerging from both G protein-coupled receptors and tyrosine kinases may converge at the level of PLC β_2 . Of interest, signalling through CD5, a co-receptor and tyrosine kinase substrate of T lymphocytes, has recently been shown to cause an increase in intracellular Ca²⁺ through a process requiring phosphatidylinositol 3-kinase, Vav and Rac-1 (Gringhuis *et al.*, 1998).

In conclusion, we have shown that a heterodimer made up of Cdc42 and LyGDI mediates GTP[S] stimulation of PLC β_2 in soluble fractions of neutrophils. Our results identify PLC β_2 as a new target of the Rho GTPases Cdc42Hs and Rac1, but not RhoA. PLC β_2 may, therefore, represent an integral part of signal transduction pathways activated by Cdc42Hs and/or Rac1 in neutrophils and, possibly, other cells expressing this effector enzyme.

Materials and methods

Amino acid sequence analysis of the PLC $\beta_2\Delta$ -stimulating factor purified from bovine neutrophils

The PLC β_2 -stimulating factor present in soluble fractions of bovine neutrophils was purified as described previously (Illenberger *et al.*, 1997). For amino acid sequence analysis, the 23 and 26 kDa constituents of the purified protein were separated by SDS-PAGE and electroblotted onto ProBlott membranes (Applied Biosystems). The bands were excised and subjected to digestion with endoproteinase Asp-N (Boehringer Mannheim). The proteolytic fragments were fractionated by reverse-phase high-performance liquid chromatography prior to sequence analysis on an Applied Biosystems Model 470A automated gas phase sequenator.

Plasmids

The cDNAs of human Rho GTPases were kindly provided by Drs Martijn F.B.G. Gebbink, Wouter H. Moolenaar, Rainer Müller and Martin Bähler. The cDNA of human LyGDI (Scherle *et al.*, 1993) was amplified from poly(A)⁺ RNA of undifferentiated HL-60 cells by reverse-transcriptase-PCR (RT-PCR) using synthetic oligonucleotides complementary to the ends of the coding region and carrying recognition sites for BamHI and EcoRI on their 5' and 3' ends, respectively. The PCR product was ligated into the EcoRI/BamHI sites of the prokaryotic expression vector pGEX-2TK (Pharmacia) and the baculovirus transfer vector pVL1393 (Invitrogen). Point mutations were introduced into the cDNA of Cdc42Hs by the PCR overlap extension method (Horton and Pease, 1991) to generate the cDNAs of Cdc42Hs^{F37A} and Cdc42Hs^{Y40C}. The cDNAs encoding the Cdc42Hs mutants were ligated into the BamHI/EcoRI site of pVL1393. All cloned PCR products were sequenced using the T7 Sequenase version 2.0 DNA sequencing kit (Amersham).

Production of recombinant proteins in *E. coli*

GST fusion proteins of RhoA, Cdc42Hs, Rac1 and LyGDI were produced in *E. coli* DH5 α and purified by chromatography on Glutathione Sepharose 4B (Pharmacia) according to the method described previously (Self and Hall, 1995b). The purified GST fusion proteins were dialysed extensively against buffer containing 20 mM Tris-HCl pH 7.6, 2 mM MgCl₂, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride and 1 μ M GDP (GST-RhoA, GST-Cdc42Hs and GST-Rac1) or 20 mM Tris-HCl pH 7.5, 1 mM EDTA, 1 mM dithiothreitol and 0.1 mM phenylmethylsulfonyl fluoride (GST-LyGDI). The purity of the proteins was at least 90%, as judged by analysis on SDS-polyacrylamide gels stained with Coomassie Blue.

Production of recombinant proteins in baculovirus-infected insect cells

Recombinant baculoviruses were produced as described (Dietrich *et al.*, 1992). Recombinant proteins were produced by infecting *Spodoptera frugiperda* cells (Sf9 cells, Invitrogen) with recombinant baculoviruses as described (Dietrich *et al.*, 1994). PLC $\beta_2\Delta$, a deletion mutant of human PLC β_2 , lacking a C-terminal region necessary for stimulation by α_q subunits (F819-E1166) and carrying a serine to alanine replacement in position 2, was produced in baculovirus-infected Sf9 or *Trichoplusia ni* cells (High FiveTM cells, Invitrogen). PLC $\beta_2\Delta$ is indistinguishable from wild-type recombinant PLC β_2 in terms of its interaction with PtdInsP₂, Ca²⁺ and G protein $\beta\gamma$ dimers.

Purification of recombinant Rho GTPases and LyGDI from baculovirus-infected insect cells

Baculovirus-infected Sf9 cells were harvested and homogenized, and membranes and soluble proteins were prepared from the homogenate as described previously (Cerione *et al.*, 1995). Soluble Cdc42Hs was purified from the soluble fraction of baculovirus-infected insect cells by gel permeation chromatography using a HiLoad 26/60 Superdex 75 prep grade column (Pharmacia) (Illenberger *et al.*, 1997). Membrane-bound Cdc42Hs and Rac1 was solubilized by extracting the membranes with buffer containing 23 mM sodium cholate and the detergent-solubilized Rho GTPases were purified according to Cerione (1995), except that the proteins obtained by chromatography on Mono Q were diluted ~5-fold with buffer A (20 mM Tris-HCl pH 8.0, 23 mM sodium cholate) and applied to a column (0.5 \times 0.5 cm) of hydroxylapatite (Calbiochem, HPLC grade) connected to a SMART micropurification chromatography system (Pharmacia) which had been equilibrated with buffer A. The flow rate was 50 μ l/min. After application of the sample, the resin was washed with 1 ml of buffer A and eluted with a linear gradient (1 ml) starting with buffer A and ending with buffer B containing 20 mM Tris-HCl pH 8.0, 1 mM EDTA, 1 mM dithiothreitol, 100 mM K₂HPO₄/H₃PO₄, 40% (v/v) glycerol and 23 mM sodium cholate. Recombinant Rho GTPases eluted in single peaks at ~50 mM K₂HPO₄/H₃PO₄. LyGDI was purified from the soluble fraction of baculovirus-infected insect cells by sequential chromatography on Superdex 75 prep grade, and Mono Q as described for the purification of the soluble PLC $\beta_2\Delta$ -stimulating factor from bovine neutrophils (Illenberger *et al.*, 1997).

Purification of recombinant PLC $\beta_2\Delta$

The soluble fraction of *Trichoplusia ni* cells infected with baculovirus encoding PLC $\beta_2\Delta$ (10 ml, 200 mg of protein) was applied to a column (1.6 \times 8 cm) of Heparin Sepharose CL-6B (Pharmacia) that had been equilibrated with buffer A [20 mM Tris-HCl pH 8.0, 1 mM EDTA, 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 3 mM benzamide, 10 μ M leupeptin, 2 μ M pepstatin, 1 μ g/ml aprotinin, 16 μ g/ml L-1-chloro-3-(4-tosylamido)-4-phenyl-2-butanone (TPCK), 16 μ g/ml L-1-chloro-3-(4-tosylamido)-7-amino-2-heptanone hydrochloride (TLCK), 2 μ g/ml soybean trypsin inhibitor]. The flow rate was 1 ml/min. After application of the sample, the column was washed with 20 ml of buffer A and eluted with a linear gradient (60 ml) of NaCl (0–900 mM) in buffer A, followed by 10 ml of buffer A containing 900 mM NaCl. Fractions of 1 ml were collected and analysed by SDS-PAGE and immunoblotting using antibodies reactive against PLC $\beta_2\Delta$ (Camps *et al.*, 1992). Fractions containing maximal levels of PLC $\beta_2\Delta$ were pooled (15 ml, 40 mg of protein), concentrated ~10-fold by pressure filtration in a stirred cell equipped with an Amicon PM10 membrane, diluted 4-fold with buffer A and applied to a Mono Q HR 5/5 column (Pharmacia), which had been equilibrated with buffer A containing 100 mM NaCl. The flow rate was 0.5 ml/min. After application of the sample, the column was washed with 5 ml of buffer A containing 100 mM NaCl and eluted with a linear gradient (10 ml) of NaCl (100–900 mM) in buffer A, followed by 5 ml of buffer A containing 900 mM NaCl. Fractions of 500 μ l were collected into tubes containing 50 μ l of 10-fold concentrated protease inhibitors as listed above. Fractions containing PLC $\beta_2\Delta$ (1 ml, 10 mg of protein) were diluted 4-fold with buffer B (20 mM Tris-HCl pH 7.5, 100 μ M EDTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 3 mM benzamide, 16 μ g/ml TPCK, 16 μ g/ml TLCK). The sample (2 ml, 5 mg of protein) was applied to a column (0.5 \times 0.5 cm) of hydroxylapatite (Calbiochem, HPLC grade) connected to a SMART micropurification chromatography system (Pharmacia) which had been equilibrated with buffer B. The flow rate was 100 μ l/min. After application of the sample, the resin was washed with 0.5 ml of buffer B and eluted with a linear gradient (5 ml) of K₂HPO₄/H₃PO₄ pH 7.5, (0–500 mM) in buffer B. PLC $\beta_2\Delta$ eluted in a single peak at ~110 mM K₂HPO₄/H₃PO₄.

Depletion of Rho GTPases from the PLC β 2-stimulating factor with matrix-immobilized GST-LyGDI

Two hundred μ l of PLC β 2-stimulating factor partially purified from bovine neutrophils by gel filtration [Illenberger *et al.* (1997)] fraction 11 of the preparation shown in figure 2 therein] containing 1.2 mg of protein were supplemented with 200 μ l of a 50% slurry of Glutathione Sepharose 4B loaded with GST-LyGDI or GST as described in the GST Gene Fusion System Manual (Pharmacia) and rotated end-over-end for 15 h at 4°C. The beads were sedimented by centrifugation at 600 g for 5 min at 4°C and washed three times with 1 ml each of ice-cold buffer containing 20 mM Tris-HCl pH 7.5, 1 mM EDTA, 100 mM NaCl, 1 mM MgCl₂ and 1 μ M GDP. Proteins were eluted from the matrix by adding to the wet cake 400 μ l of 10 mM reduced glutathione in 40 mM Tris-HCl pH 7.5, 1 mM EDTA, 1 mM MgCl₂ and 1 μ M GDP. The eluate was concentrated ~6-fold using Nanosep-10 microconcentrators (Pall Filtron, Dreieich).

Solubilization of membrane-bound recombinant Rho GTPases with soluble GST-LyGDI

Membranes of insect cells expressing Cdc42Hs, RhoA, Rac1 or β -galactosidase (3.5 mg of protein each) were rotated end-over-end for 15 h at 4°C in 200 μ l of 20 mM Tris-HCl pH 7.5, 1 mM EDTA, 1 mM dithiothreitol and 0.1 mM phenylmethylsulfonyl fluoride containing either 40 μ M GST or 40 μ M GST-LyGDI. The samples were centrifuged at 100 000 g for 10 min at 4°C and the supernatants used for further analysis.

Phospholipase C assay

Phospholipase C activity was determined as described previously (Illenberger *et al.*, 1997). In brief, 10 μ l of either native or recombinant heterodimeric LyGDI \times Rho were supplemented with 5 μ l of the soluble fraction of PLC β 2 Δ -baculovirus-infected insect cells and incubated for 1 h at 25°C in a volume of 70 μ l containing 28 μ M ³H-PtdInsP₂ (5 Ci/mol), 280 μ M phosphatidylethanolamine, 0.9 mM sodium deoxycholate, 50 mM Tris-maleate pH 7.4, 150 mM NaCl, 3 mM EGTA, 10 mM LiCl and 10 mM 2,3-bisphosphoglycerate. CaCl₂ was added to adjust the final concentration of free Ca²⁺ to 100 nM. The abilities of detergent-solubilized recombinant Cdc42Hs and Rac1 as well as soluble insect cell-expressed Cdc42Hs to stimulate PLC β 2 Δ were determined according to Hepler *et al.* (1993), with some modifications. Ten microlitres of either crude or purified Cdc42Hs or Rac1 were supplemented with 10 μ l of either the soluble fraction of PLC β 2 Δ -baculovirus-infected insect cells or purified PLC β 2 Δ , and incubated for 1 h at 25°C in a volume of 60 μ l containing 33 μ M ³H-PtdInsP₂ (5 Ci/mol), 50 mM HEPES-NaOH pH 7.2, 70 mM KCl, 3 mM EGTA, 2 mM dithiothreitol and 150 nM free Ca²⁺. The final concentration of free Mg²⁺ was \leq 35 μ M. The samples were analysed for inositol phosphates as described previously (Gierschik and Camps, 1994).

³⁵S-GTP[S] binding

Binding of ³⁵S-GTP[S] was assayed as described previously (Illenberger *et al.*, 1997) with minor modifications. In brief, samples containing 0.06–0.2 μ g of protein were incubated for 60 min at 30°C in 40 μ l of 25 mM HEPES-NaOH pH 8.0, 1 mM EDTA, 1 mM dithiothreitol, 100 mM NaCl, 20 mM MgCl₂, 0.1% (v/v) GENAPOL® C-100 and 0.5 nM ³⁵S-GTP γ S (1250 Ci/mmol). Unlabelled GTP[S] was added at concentrations ranging from 10 pM to 10 μ M. The incubation was terminated by rapid filtration through nitrocellulose membranes with a pore size of 0.45 μ m, and three washes with 2 ml each of ice-cold buffer containing 50 mM Tris-HCl pH 8.0, 100 mM NaCl and 5 mM MgCl₂. The radioactivity retained on the membranes was determined by liquid-scintillation counting. Non-specific binding was defined as the binding not competed for by 1 mM unlabelled GTP[S]. Homologous competitive binding curves were constructed to estimate the binding parameters K_D and B_{max} using the GraphPad Prism Version 2.01 software package as described previously (Motulsky, 1996). The K_D values determined for binding of GTP[S] to Cdc42Hs^{wt}, Cdc42Hs^{F37A} and Cdc42Hs^{Y40C} extracted with cholate from membranes of baculovirus-infected insect cells were 8.3 \pm 0.6 nM, 8.3 \pm 0.4 nM and 6.5 \pm 0.4 nM (means \pm SEM), respectively. The B_{max} values of these preparations were 610, 220 and 190 pmol/mg protein, respectively. By comparison, the B_{max} values obtained for GTP[S] binding to detergent extracts of membranes from β -galactosidase-baculovirus-infected cells were $<$ 20 pmol/mg protein. In some experiments, the number of GTP[S] binding sites was determined by using ³⁵S-GTP[S] at a single concentration (100 nM) in the binding assay. The number thus obtained corresponds to 85–90% of B_{max} .

Miscellaneous

The method used to purify $\beta\gamma_i$ from bovine retinal rod outer segment membranes is described in Gierschik and Camps (1994). Protein concentrations were determined according to Bradford (1976) using bovine IgG as standard. SDS-PAGE and immunoblotting were performed according to standard protocols (Harlow and Lane, 1988), except that immunoreactive proteins were visualized using the ECL Western blotting detection system (Amersham). The method used to stain the gels with silver is specified in Oakley *et al.* (1980). Polyclonal antibodies reactive against RhoA (sc-179), Cdc42Hs (sc-87), Rac1 (sc-217), LyGDI (sc-359) and GST (sc-459) were from Santa Cruz Biotechnology. A baculovirus encoding *E.coli* β -galactosidase was from Dr M. Ruffing. All other materials were from standard vendors or from sources described previously (Illenberger *et al.*, 1997).

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