RhoA and a Cytosolic 50-kDa Factor Reconstitute GTPγS-dependent Phospholipase D Activity in Human Neutrophil Subcellular Fractions*

(Received for publication, April 15, 1995, and in revised form, August 1, 1995)

Jong-Young Kwak, Isabel Lopez, David J. Uhlinger, Sung Ho Ryu, and J. David Lambeth†
From the Emory University Medical School, Atlanta, Georgia 30322

Receptor activation of phospholipase D has been implicated in signal transduction in a variety of cells. Reconstitution of cell-free guanosine 5′-O-(3-thiotriphosphate)(GTPγS)-dependent phospholipase D activity from human neutrophils requires protein factors in both the plasma membrane and the cytosol. We previously proposed that one of the factors is a Ras-family small molecular weight GTPase of the Rho subtype (Bowman, E. P., Uhlinger, D. J., and Lambeth, J. D. (1993) J. Biol. Chem. 268, 21509–21512). Herein, we have used RhoGDI (GDP dissociation inhibitor), an inhibitory Rho-binding protein, to selectively extract Rho-type GTPases from the plasma membrane, and have used immunoprecipitation as well as chromatographic methods to remove cytosolic Rho. Depletion of RhoA from either the plasma membrane or the cytosol resulted in a partial loss in GTPγS-dependent activity, while removal of RhoA from both fractions resulted in a nearly complete loss in activity. Activity was nearly completely restored by adding purified recombinant RhoA, which showed an EC50 of 52 nM, while Rac1 showed little activity. Cytosol fractionated using DEAE-cellulose chromatography separated ADP-ribosylation factor and Rho from the major activating fraction. Gel exclusion chromatography of this fraction revealed an activating factor of 50 kDa apparent molecular mass. Using RhoA-depleted membranes, reconstitution of phospholipase D activity required both RhoA and the 50-kDa factor. Thus, RhoA along with a non-Rho, non-ADP-ribosylation factor 50-kDa cytosolic factor are both required to reconstitute GTPγS-dependent phospholipase D activity by neutrophil plasma membranes.

Phospholipase D (PLD)1 is activated via receptor-coupled mechanisms and by phorbol esters in a variety of cells (1–3). The enzyme catalyzes the hydrolysis of phosphatidylcholine to generate free choline and phosphatic acid. Phosphatic acid can be further metabolized via phosphatic acid phosphohydrolase to form diacylglycerol, and is the major source of signaling diacylglycerol in some cell types such as neutrophil (2, 4). Phosphatic acid and diacylglycerol have been implicated as second messengers involved in regulation of cell growth, differentiation, inflammation, and in a variety of cell-specific responses such as the respiratory burst of granulocytes (4, 5).

While the occurrence of receptor-activated PLD has been widely documented, its molecular mechanism of activation remains poorly understood. GTPγS activates PLD in cell-free systems including liver plasma membranes (6) and in lysates from human neutrophils and HL-60 cells (7, 8). Unlike the liver system, reconstitution of GTPγS- and phorbol myristate acetate-stimulated PLD from neutrophils requires protein factors in both the cytosol and the plasma membrane (7, 8). The calcium-dependent (7) activation by either GTPγS or phorbol myristate acetate has been reported to require the participation of a 50-kDa cytosolic factor (9) as well as unknown membrane components which include the PLD catalytic moiety (10). However, in other studies using HL-60 membranes, a requirement for a 50-kDa factor was not seen (10, 11), or was attributed to a 50-kDa complex between Rho GTPases and RhoGDI (12). Thus, there has been controversy regarding the existence and identity of the putative 50-kDa factor.

By pre-binding GTPγS to the plasma membrane followed by resolation to remove free GTPγS, the membrane was shown to contain the guanine nucleotide-binding site (9). The GTP-binding protein did not appear to be a heterotrimeric G protein, based on its magnesium dependence, lack of activation by fluoride, and lack of effect of G protein βγ subunits. Stimulation of GTP-dependent activation by GDP-dissociation stimulator, an exchange factor that functions on a variety of small GTPases (but not heterotrimeric G proteins), identified the activating GTP-binding protein as a member of the Rac superfamily. Inhibition of GTPγS-activated PLD by RhoGDI (GDP-dissociation inhibitor) further identified the GTPrase as a member of the Rho subfamily (9). It was later shown that RhoA can reconstitute GTPγS-dependent PLD activity in liver plasma membranes (13).

The identification of the GTP-dependent activating factor has been confounded by recent studies implicating ADP-ribosylation factor (ARF) as the guanine nucleotide-dependent factor and as a cytosolic rather than a membrane factor (10, 11). These studies utilized permeabilized HL-60 cells or HL-60 plasma membranes (or their extracts) along with cytosol from bovine brain. In these heterologous systems, ARF was identified as a major cytosolic activating factor from brain. Using human neutrophil fractions, we recently showed that the major cytosolic activating factor is approximately 50 kDa by gel exclusion chromatography (9) and separates from fractions containing ARF. The cytosolic factor at a relatively high concentration activated PLD in the absence of added ARF. ARF alone produced a minimal stimulation of PLD when added to plasma membranes. However, in the presence of a low concentration of ARF-depleted cytosol, ARF and the cytosolic factor functioned synergistically to activate PLD (14). Similar results have been reported recently for HL-60 cells (12). In the latter study, it was proposed that the membrane GTP-binding factor is membrane-associated ARF rather than a Rho-family GTPase and that the
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EXPERIMENTAL PROCEDURES

Cell-free Assay of PLD—Human neutrophils were isolated from venous peripheral blood of healthy adult donors as described previously (17). Cells were labeled with \( ^{3}H \)alkyl-lysophosphatidylcholine (1.5 \( \mu Ci/10^7 \) cells/ml) for 90 min at 37°C. Plasma membranes and cytosol were isolated as described (7) except that triethanolamine buffer (25 mM triethanolamine, pH 7.4, 100 mM KCl, 5 mM MgCl\(_2\), 3 mM NaCl) was substituted for Hepes in the cation buffer. Incubations containing plasma membrane plus either cytosol or cytosol-derived fractions were carried out in the presence of 10 \( \mu M \) GTP-\( S \), 1 \( \mu M \) Ca\(_{2+}\), and 1.6% ethanol and the reaction was terminated by transfer to chloroform: methanol:acetic acid (90:10:10 by volume). Radioactivity was quantified using a Bio-scanner (Bioscan, Inc.) equipped with two-dimensional software. Phosphatidylethanol formed by transphosphatidyltransferase is expressed as the percentage of total counts in each lane.

Purification of Recombinant Proteins—The cDNA for RhoGDI was obtained from human B-cell cDNA library by polymerase chain reactions (9). The cDNA was inserted into the pGEX-2T vector (Pharmacia Biotech Inc.) by conventional techniques. The cDNA of RhoA (obtained from Alan Hall, University College, London) was also engineered into the pGEX-2T vector. Rac1(C189S) was previously engineered to include a BamHI and a 3' EcoRI restriction enzyme site and was inserted into the pGEX-2T vector (18). Fusion proteins containing an N-terminal glutathione S-transferase (GST) domain were induced in Escherichia coli by adding 100 \( \mu M \) isopropyl-\( \beta \)-D-galactopyranoside to the cultures. The RhoGDI-GST and RhoA-GST were purified by adsorption onto glutathione-agarose beads (Sigma) followed by elution with 10 mM glutathione, and were dialyzed overnight at 4°C in phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 4.3 mM NaH\(_2\)PO\(_4\), 7.5 mM KH\(_2\)PO\(_4\), pH 7.3). RhoA and Rac1 were released from the parent GST-fusion protein bound to the glutathione-agarose beads (Sigma) by exposure to elution buffer containing 0.1 M DTT and 10% glycerol. Gel filtration (Superose-12 column (1 x 30 cm) which had been pre-equilibrated with Buffer A (10 mM triethanolamine, pH 7.4, 10 mM MgCl\(_2\), and 3 mM NaCl), and was loaded onto a DEAE-cellulose column (2.5 x 20 cm) pre-equilibrated with Buffer A (pH 7.4). The column was washed with 50 ml of Buffer A, and eluted with a 200-ml linear gradient consisting 0–30 mM KCl in the same buffer, followed by a step elution which increased the KCl to 100 mM. Fractions (3 ml) were collected, and aliquots (20 \( \mu l \)) were analyzed for PLD stimulating activity and for the presence of ARF, Rho proteins, and RhoGDI, as described below. Fractions which showed phospholipase D stimulating activity but did not contain ARF, Rho, and RhoGDI were pooled and concentrated to about 400 \( \mu l \) using ultrafiltration (YM 10 membrane, Amicon) and microconcentration (Centricon 10, Amicon). The concentrated material was chromatographed on a Superose-12 column (1 x 30 cm) which had been pre-equilibrated with Buffer A containing 10 mM KCl, 20-\( \mu l \) aliquots of fractions (0.5 ml) were analyzed for PLD supporting activity. The five fractions showing the highest activity were pooled for further experiments.

Immunocochemical Methods—Western blotting was carried out by standard methods (20), using alkaline phosphatase-conjugated secondary antibody. The RhoGDI antibody was raised in rabbit, using E. coli expressing recombinant RhoGDI as a soluble GST fusion protein. For the RhoA antibody, a mouse monoclonal antibody (1D9) from Dr. Richard Kahn. The RhoA and CDC42 antibodies were rabbit anti-peptide polyclonal antibodies (Santa Cruz antibodies) which were specific for RhoA and CDC42, respectively. Antisera were used at dilutions of 1:1000, 1:500, 1:100, and 1:500, respectively.

RESULTS

Depletion of Rho from Plasma Membranes—In preliminary studies, we used antibodies to RhoA, RhoB, Rac1 (1 and 2), Rac2, and CDC42 to determine which of these Rho family small GTPases were present in neutrophil lysates and plasma membranes. RhoA, Rac (1 and 2), Rac2, and CDC42, but not RhoB immunoreactivity were detected in lysates, but only RhoA was present in appreciable quantities in neutrophil plasma membranes. We therefore monitored RhoA immunocytochemically to determine our ability to remove Rho protein(s) from the plasma membrane.

The fusion protein RhoGDI-GST was used to extract membranes, since it was as effective as RhoGDI in inhibiting GTP-\( S \)-stimulated PLD activity. RhoGDI has been previously shown to be capable of binding to and extracting Rho proteins from plasma membranes of other cell types (21). Upon incuba-

2 H. Yang, unpublished data.
tion at 22°C for 5-10 min, less than half of the RhoA in neutrophil plasma membranes was released into the soluble fraction. However, as shown in Fig. 1, greater than 80% of the RhoA was extracted from membrane by incubation with 20 μM RhoGDI-GST for 20 min at 30°C and all detectable RhoA was extracted by incubation for 1 h. A mock incubation which did not contain RhoGDI-GST failed to extract RhoA.

Extraction of Rho Proteins from the Cytosol—RhoA has a dual distribution in neutrophil, with approximately 5% present in the plasma membrane fraction, and 95% in the cytosol. This cytosolic RhoA is expected to equilibrate into the plasma membrane when GTPγS is present. It was therefore necessary to deplete this cytosolic reservoir of Rho proteins. Since cytosolic Rho family proteins were shown previously to be associated with RhoGDI (22, 23), RhoGDI-bound RhoA was removed from the cytosolic fraction by immunoprecipitation of the RhoGDI-Rho complexes using a rabbit antibody to RhoGDI, as detailed under “Experimental Procedures.”

Fig. 1. Extraction of RhoA from neutrophil plasma membranes using recombinant RhoGDI-GST. Plasma membranes (1 mg/ml) were treated with (+RhoGDI-GST) or without (Mock) 20 μM RhoGDI-GST for the indicated times at 30°C. Membrane (M) and supernatant (S) fractions were resolved by sucrose density centrifugation as described under “Experimental Procedures.” Plasma membranes (18 μg) and supernatant (18 μl) were resolved by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and visualized using antibody to RhoA, as detailed under “Experimental Procedures.” Results are representative of 10 experiments.

Fig. 2. Extraction of RhoA and RhoGDI from cytosol. The diagram to the lower outlines the extraction procedure described in the text and is keyed to the results shown on the upper. 10 mg of cytosol (1) was mixed with protein A-bound anti-RhoGDI antibody to obtain RhoGDI-depleted cytosol (3) or mock-treated cytosol (2) in which the specific antibody was omitted. These fractions were characterized by Western blotting using antibodies against either RhoGDI (upper panel) or RhoA (lower panels). The RhoGDI-depleted cytosol (3 and A) was then treated by incubating with RhoGDI-GST/glutathione-agarose to obtain Rho-depleted cytosol (B). For SDS-polyacrylamide gel electrophoresis, the amount of protein run per lane was 8 μg for lanes 1, 2, and 3 and 30 μg for lanes A and B. Transfer to nitrocellulose and immunostaining was carried out as described under “Experimental Procedures.” Results are representative of 10 experiments.

Fig. 3. Loss of GTPγS-stimulated PLD activity by extraction of RhoA. Plasma membranes (1 mg/ml) were mixed with or without 20 μM RhoGDI-GST for 20 min at 30°C and then resolated as described under “Experimental Procedures.” RhoA-depleted cytosol was prepared as in the legend to Fig. 2. Membranes (25 μg) were incubated with 50 μg of cytosol, 1 μM CaCl₂, and 1.6% ethanol in the absence or presence of 10 μM GTPγS as indicated for 20 min at 37°C. Groups tested were: A, mock-treated cytosol plus mock-treated membranes; B, mock-treated membranes plus Rho-depleted cytosol; C, mock-treated cytosol plus Rho-depleted membranes; and D, Rho-depleted cytosol plus Rho-depleted membranes. Phospholipase D-catalyzed transphosphatidylation was monitored by phosphatidylethanol formation (PETh), expressed as a percentage of the total radiolabeled lipids. The “mock treated” activity in this experiment was 60% of the GTPγS-stimulated activity seen using naive cytosol and plasma membrane. The result shown is representative of three experiments.
Rac is compared in Fig. 4B. As is shown, Rac1 was inefficient in reconstituting PLD compared with RhoA, although activation appeared to be slightly higher than control levels. The concentration dependence for RhoA reconstitution of PLD activity is shown in Fig. 5. The line shown is a theoretical fit to the Michaelis-Menten equation modified to include an added constant to account for the basal activity seen in this experiment. The EC50 for RhoA was determined to be 52 nM.

Separation of a 50-kDa Cytosolic Factor from ARF, Rho Proteins, and RhoGDI—In a previous study, we showed that ARF and a 50-kDa cytosolic factor synergized in the activation of plasma membrane phospholipase D (14), but these studies did not rule out the possibility that the 50-kDa factor was actually a complex between RhoGDI and a Rho-type small GTPase. In this study, we modified our DEAE-cellulose chromatography to utilize a shallow KCl gradient (Fig. 6, panel A), which effectively separated not only ARF but also RhoA from the major peak of PLD stimulating activity, ARF immunoreactivity (Fig. 6, panel C) eluted in early fractions corresponding to a small peak in PLD stimulating activity (less than 10% of the total, panel B). RhoA eluted at high salt in very late fractions (around fraction 117). This region contained significant PLD stimulating activity, but it is not clear whether this was due to RhoA or to a tailing artifact from the main peak. In any case, the majority (around 90%) of the PLD stimulating activity eluted at intermediate salt concentrations, around 15 mM KCl, and these fractions contained no detectable ARF or RhoA.

Although Rac2 and CDC42 were also detected in the second half of the major peak of PLD stimulating activity, their presence failed to correlate with PLD stimulating activity (compare Fig. 6, B and C). RhoGDI showed at least three peaks of immunoreactivity, and these corresponded to the presence of Rac2, CDC42, and RhoA. Thus, each of the small GTPases chromatographs as a complex with RhoGDI. The fractions (fractions 50–66) which showed the highest PLD stimulating activity, but which did not contain any detectable ARF, Rho-type proteins, or RhoGDI were pooled, concentrated, and chromatographed on a calibrated Superose-12 column. As shown in Fig. 7, PLD stimulating activity showed a major peak with an apparent molecular mass of 50 kDa. The five most active fractions were pooled and used in subsequent experiments. This fraction is designated the "50-kDa cytosolic factor."

Reconstitution of GTP\(\gamma\)S-activated PLD with RhoA and the 50-kDa Cytosolic Factor—In the absence of cytosol, RhoA-depleted plasma membrane and mock-treated plasma membrane showed low basal or GTP\(\gamma\)S-stimulating PLD activity (Fig. 8). The addition of RhoA-GTP\(\gamma\)S alone to Rho-depleted and mock-treated membranes failed to reconstitute activity. Using the mock-treated membranes (which still contain RhoA), the 50-kDa factor was able to restore GTP\(\gamma\)S stimulated activity. However, using the RhoA-depleted membranes, neither RhoA nor the 50-kDa factor alone (with or without GTP\(\gamma\)S) restored activity. Activity was restored only when GTP\(\gamma\)S-preloaded RhoA and the 50-kDa factor were both present.

**DISCUSSION**

We have developed a Rho-depleted cell-free PLD system consisting RhoA-depleted membrane and cytosol. The depleted system has minimal GTP\(\gamma\)S-stimulated PLD activity, and provides an optimal system for investigating the role of small GTP-binding proteins in activation of the enzyme. It has the advantage that other membrane and cytosolic components in this complex system are not removed, minimizing the chance of artifact. It was possible to extract almost all of the detectable membrane-associated RhoA using RhoGDI-GST at 30 °C for 1 h. Less efficient extraction was seen at room temperature, presumably due to a less fluid membrane. Partial extraction at the lower temperature was reported previously (13, 21). Because Rho in the cytosol was already largely associated with RhoGDI, extraction with RhoGDI-GST alone failed to deplete RhoA. We therefore initially used an antibody against RhoGDI to immunoprecipitate the RhoGDI-RhoA complexes. Following this treatment, there remained a small quantity of soluble RhoA, which could be effectively removed by RhoGDI-GST plus glutathione-agarose. We propose that the small amount of residual RhoA present in the cytosol is due to free RhoA in equilibrium with RhoGDI-bound RhoA, or possibly
RhoA which is produced in excess of RhoGDI binding sites. The material is not likely to be nonprenylated RhoA, since only prenylated Rho proteins bind to RhoGDI (15, 24). RhoA was the only Rho family protein found in appreciable quantities in the membrane, so the efficiency of the extraction method for other Rho family proteins is not known, although others are reportedly extracted in other systems (13, 21). We also monitored the depletion of the small GTPase Rac (using a general antibody to both Rac1 and Rac2) from the cytosol, and found that this methodology was also effective in depleting Rac. Therefore, it appears that the depletion protocol is generally useful for removing Rho family proteins, and will likely find a variety of applications in other systems. The methodology allowed us herein to initially investigate the role of RhoA in GTPγS-dependent activation of phospholipase D in granulocytic cells.

Using the Rho-depletion system, depletion of RhoA is associated with loss of GTPγS-dependent activity, and recombinant RhoA (but not Rac1) reconstituted GTPγS-dependent PLD activity. It is usually assumed that isoprenylation of small GTP-binding proteins is essential for their effector functions (15, 24). Recombinant RhoA and Rac1 were expressed in E. coli, in which isoprenylation fails to take place. It was recently shown for Rac activation of the NADPH oxidase that isoprenylation was necessary for recognition by exchange proteins, but was not needed for the effector function (18). We therefore used a GTPγS-preloading protocol in which chelation with EDTA of magnesium permits release of bound GDP and subsequent binding of GTPγS. GTPγS-preloaded RhoA reconstituted a rate of PLD activity similar to that seen in untreated preparations. The relatively low EC₅₀ (50 nM) for RhoA further indicates that the activation is specific, and that isoprenylation is not essential, although it is not clear whether an increase in potency would be seen with isoprenylation. Reconstitution by RhoA is consistent with our previous studies which showed that RhoGDI inhibited GTPγS-dependent activation in a neutrophil cell-free system (plasma membranes plus cytosol) (9), and with subsequent studies in liver (13) which showed that RhoA can activate PLD in isolated plasma membranes. The latter system differs in several respects from the neutrophil/HL-60 system.
Rho effects on phosphatidylinositol 5-kinase but not PLD are likely due to the ATP requirement for lipid phosphorylation. Further stimulated by ATP (as is seen in U937 cells, presumably due to the ATP requirement for lipid phosphorylation).

The immunodepletion protocol removes Rho-type small GTPases, and demonstrates the need for non-Rho cytosolic components. However, the depleted cytosol may contain multiple activating factors (e.g., ARF, others). To further characterize the nature of the cytosolic activity, we developed chromatographic methods to resolve ARF, Rho-type GTPases, and RhoGDI from other activities. Using DEAE-cellulose chromatography, we were able to resolve these factors from a major peak of PLD stimulating activity. Subsequent gel exclusion chromatography indicated that the major activity migrates with an apparent size of 50 kDa. Using the Rho-depleted plasma membranes, it was clear that reconstitution of GTPγS-stimulated PLD activity required both the 50-kDa factor and RhoA.

The mechanism of activation of PLD by Rho is not yet clear. Signal transducing GTPases (both heterotrimeric G proteins and small GTPases) frequently function as molecular switches, binding to their effectors in their GDP (or GTPγS)-bound forms. In GTPγS pre-binding experiments, we showed that GTPγS binding to a component in the plasma membrane was sufficient to activate PLD, but that pretreatment of cytosol with GTPγS failed to activate PLD (9). Thus, it appears that the membrane-associated form of RhoA is the form relevant to PLD activation, although cytosolic Rho probably serves as a mobile reservoir to provide additional RhoA to the membrane. Earlier work (10) suggests that the PLD catalytic moiety itself resides in the plasma membrane. Thus, one model is that GTP association and translocation of RhoA results in its complexation and activation of a membrane-associated PLD, as is the case for activation of some forms of adenyl cyclase by Gαs. Ras, which is bound to the plasma membrane via its isoprenyl and other groups, provides an attractive alternative model. An effector enzyme for Ras is Raf-1. The latter is a protein kinase which is located in the cytosol, and which translocates to the membrane by binding to GTP-Ras where it is active (25). RhoA and the 50-kDa cytosolic factor are resolved as different proteins and both are necessary to activate PLD. Thus, we can speculate that membrane-associated GTP-Rho complexes with the essential 50-kDa cytosolic factor in neutrophils, permitting it to translocate to the plasma membrane where it participates in PLD activation. Consistent with this model, in U937 cells, which normally show a similar requirement for both plasma membrane and cytosol for PLD activity, pretreatment of permeabilized cells with GTPγS renders PLD activity in subsequently isolated plasma membranes independent of cytosol (26). Thus, GTPγS appeared to induce translocation from the cytosol to the membrane surface of a required PLD component.

Finally, Rho-GTP may be functioning by inducing the generation of an important cofactor such as PIP2. The latter lipid has been shown to be essential for PLD activity in brain (27) and U937 cells (28), and Rho reportedly activates an isofrom of phosphatidylinositol 5-kinase (29) which generates PIP2. While regulated production of PIP2 may also be important, three findings argue against the possibility that Rho activation of PIP2 generation explains PLD activation in our system. First, Rho effects on phosphatidylinositol 5-kinase but not PLD are inhibited by ADP-riboseylation of Rho with botulinus C3 exoenzyme (13, 29). Second, GTPγS activation in our system is not further stimulated by ATP (as is seen in U937 cells, presumably due to the ATP requirement for lipid phosphorylation).

Third, addition of PIP2, while causing a modest stimulation, does not eliminate the ability of RhoA or cytosolic factor to stimulate high levels of activity. If Rho were acting by increasing the synthesis of PIP2, then we would expect the activating lipid to eliminate the ability of Rho to further activate.

Rho family GTP-binding proteins have been implicated in mediating receptor-coupled regulation of the actin cytoskeleton, and appear to be involved in Ras-induced mitogenic transformation (30). Microinjected RhoA results in the production of actin stress fibers (31), while microinjected Rac1 results in membrane ruffling, the latter mediated by submembrane actin cytoskeleton. It is tempting to speculate that PLD may play a role in cytomechanical changes. However, stress fiber formation is inhibited by C3 exoenzyme ADP-ribosylation of Rho, but PLD activation is not affected. Thus, a more tenable model is that Rho represents a common coordination point for several cellular responses, with one of these being lipid signal transduction and another the regulation of the actin cytoskeleton. A role of Rho-activated PLD in transformation also remains a possibility. Rho may be important in coordinating diverse cellular responses, for example, as occurs during cytokinesis or locomotion.

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