LIPOSOMES COMPRISING ANIONIC BUT NOT NEUTRAL PHOSPHOLIPIDS CAUSE DISSOCIATION OF [Rac(1 or 2)-RhoGDI] COMPLEXES AND SUPPORT AMPHIPHILE-INDEPENDENT NADPH OXIDASE ACTIVATION BY SUCH COMPLEXES

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Running Title: Dissociation of [Rac-RhoGDI] complexes by anionic liposomes

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Activation of the phagocyte NADPH oxidase involves the assembly of a membrane-localized cytochrome b\textsubscript{559} with the cytosolic components p47\textsuperscript{phox}, p67\textsuperscript{phox}, p40\textsuperscript{phox}, and the GTPase Rac (1 or 2). In resting phagocytes, Rac is found in the cytosol as a prenylated protein in the GDP-bound form, associated with Rho GDP dissociation inhibitor (RhoGDI). In the process of NADPH oxidase activation, Rac is dissociated from RhoGDI and translocates to the membrane, in concert with the other cytosolic components. The mechanism responsible for dissociation of Rac from RhoGDI is poorly understood. We generated [Rac(1 or 2)-RhoGDI] complexes in vitro from recombinant Rac (1 or 2), prenylated enzymatically, and recombinant RhoGDI, and purified these by anion exchange chromatography. Exposing [Rac(1 or 2)(GDP)-RhoGDI] complexes to liposomes containing four different anionic phospholipids, caused the dissociation of Rac(1 or 2)(GDP) from RhoGDI and its binding to the anionic liposomes. [Rac2(GDP)-RhoGDI] complexes were more resistant to dissociation, reflecting the lesser positive charge of Rac2. Liposomes consisting of neutral phospholipid did not cause dissociation of [Rac(1 or 2)-RhoGDI] complexes. Rac1 exchanged to the hydrolysis-resistant GTP analogue, guanylylimidodiphosphate (GMPPNP), associated with RhoGDI with lower affinity than Rac1(GDP) and [Rac1(GMPPNP)-RhoGDI] complexes were more readily dissociated by anionic liposomes. [Rac1(GMPPNP)-RhoGDI] complexes elicited NADPH oxidase activation in native phagocyte membrane liposomes in the presence of p67\textsuperscript{phox}, without the need for an anionic amphiphile, as activator. Both [Rac1(GDP)-RhoGDI] and [Rac1(GMPPNP)-RhoGDI] complexes elicited amphiphile-independent, p67\textsuperscript{phox}-dependent NADPH oxidase activation in phagocyte membrane liposomes enriched in anionic phospholipids but not in membrane liposomes enriched in neutral phospholipids.

The production of reactive oxygen species represents one of the major microbicidal weapons of professional phagocytes. The primordial oxygen radical is superoxide (O\textsubscript{2}^{-}) and it is produced by the NADPH-derived one-electron reduction of molecular oxygen by an enzyme complex known as the NADPH oxidase (briefly “oxidase”) (reviewed in Refs.1-4). This consists of a membrane-bound heterodimeric flavocytochrome (cytochrome b\textsubscript{559}), consisting of two subunits, gp91\textsuperscript{phox} and p22\textsuperscript{phox}, and four cytosolic proteins, p47\textsuperscript{phox}, p67\textsuperscript{phox}, p40\textsuperscript{phox}, and the small GTPase Rac (1 or 2). In the resting phagocyte, there is no contact between cytochrome b\textsubscript{559} and the cytosolic components. In response to a variety of stimuli,
acting via membrane receptors, the cytosolic components engage in a complex set of protein-protein and protein-lipid interactions leading to their translocation to the membrane. The “purpose” of this process is to induce a conformational change in gp91^phox, which is the catalytic subunit of the oxidase containing all the redox stations and responsible for electron transfer from NADPH to oxygen. It is likely that the pivotal protein-protein interaction is between p67^phox and gp91^phox and there is good evidence for a key role of an “activation domain” in p67^phox, consisting of residues 199-210, in this process (5).

A model of oxidase assembly was proposed in which p47^phox and Rac1 serve as “organizers” by carrying or anchoring the p67^phox “activator” to the vicinity of gp91^phox or by promoting or stabilizing the interaction between p67^phox and gp91^phox (5-7). Oxidase assembly can be reproduced in vitro by exposing a mixture of phagocyte membranes and the cytosolic components p47^phox, p67^phox, and Rac to a critical concentration of an anionic amphiphile (8-12). O_2^- production can also be achieved in an amphiphile-independent manner in a system consisting of phagocyte membranes, p67^phox and the prenylated form of Rac, in the absence of p47^phox (13).

Rac is absolutely required for the activation of the phagocyte oxidase. This was demonstrated by the ability of Rac1, purified from macrophages (14), and Rac2, purified from neutrophils (15), to support oxidase activation in a cell-free system and by the finding that recombinant Rac1 (14, 16) and Rac2 (17, 18) were as active as their counterparts purified from cytosol. The essential role of Rac in the regulation of phagocyte oxidase in intact cells has been amply demonstrated (19-21). Thus, a patient with a dominant negative mutation in Rac2 (D57N) suffered from a phagocyte immunodeficiency syndrome and his neutrophils produced markedly reduced amounts of O_2^- in response to some stimuli (22). Rac1 and Rac2 differ significantly in their C-terminal polybasic region; Rac1 contains six contiguous basic residues (183-KKRKKK-188) whereas Rac2 contains only three (183-RQQKRA-188). Rac1 has been reported to be the predominant isoform of Rac in monocytes (23) and macrophages (24) whereas Rac2 is predominantly expressed in neutrophils (25). In eukaryotic cells, Rac is subject to posttranslational modifications, consisting of prenylation (geranylgeranylation), carboxyl-methylation on the cysteine in the C-terminal CLLL (Rac1) or CSLL (Rac2) sequences, and cleavage of the last three residues (26, 27).

As a typical member of the Rho GTPase family, Rac acts as molecular switch cycling between inactive (GDP-bound) and active (GTP-bound) conformations. The GDP/GTP switch is controlled by guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs) (see Ref. 28, for review with emphasis on the role of Rac in phagocytes). In the resting phagocyte, Rac is found predominantly in the cytosol in the form of a heterodimer with the regulatory protein Rho GDP dissociation inhibitor (RhoGDI) (14, 17, 29). Prenylation is an absolute requirement for all members of the Rho family to form a complex with RhoGDI (for reviews on RhoGDI, see Refs. 30 and 31). Whether Rho GTPases have to be in the GDP-bound form in order to form complexes with RhoGDI is a less settled question. Thus, it was reported that the GTP-bound forms of Rac, RhoA and Cdc42Hs bind to RhoGDI with affinities lower (32, 33) or equal (34, 35) to that of the GDP-bound forms.

Three human RhoGDIs have been identified: the ubiquitously expressed RhoGDI-1 (or GDIα) (36, 37), the hemopoietic cell-specific Ly/D4GDI (or GDIβ) (38), and RhoGDI-3 (RhoGDIγ), expressed in lung, brain and testis (39, 40). RhoGDI-1 consists of a C-terminal domain that adopts an immunoglobulin-like fold and a highly flexible N-terminal regulatory region that becomes ordered into helixes upon complex formation with Rho proteins. The amino-terminal regulatory arm of RhoGDI-1 binds to the switch I and II domains of Rac, while the prenylated moiety of Rac inserts into a hydrophobic pocket within the immunoglobulin-like domain of RhoGDI (41-45).

RhoGDI regulates the activity of Rho family GTPases by the inhibition of GDP (and GTP) dissociation, inhibition of intrinsic and GAP-induced GTP hydrolysis, and by controlling the partitioning of Rho GTPases between cytosol and plasma membrane (46, 47). Moreover, structural and biochemical studies suggest that GEFs cannot act on Rho GTPases when these are bound to RhoGDI (48, 49).
Dissociation of prenylated Rac from RhoGDI was shown to be an obligatory step in the assembly of the oxidase, preceding translocation of Rac from cytosol to the plasma membrane (29, 50, 51). Thus, dissociation from RhoGDI represents an event linked to oxidase assembly for two reasons: enabling repartition of Rac from the cytosol to the membrane and making possible nucleotide exchange from GDP to GTP.

In light of the centrality of this issue, it is surprising that the mechanism(s) responsible for the dissociation of Rho GTPases from RhoGDI, in general, and of Rac from RhoGDI, in the context of oxidase assembly, in particular, are poorly understood. The following mechanisms were reported to regulate the balance between RhoGDI-bound and free GTPase: (1) Phosphorylation of Rho GTPases (RhoA and CDC42) by protein kinase A enhanced binding to RhoGDI (52, 53); (2) On the other hand, phosphorylation of RhoGDI by protein kinase C (54, 55) or by p21-activated kinase (Pak1) (56), facilitated the disruption of [Rac-RhoGDI] but not of [RhoA-RhoGDI] complexes; (3) Arachidonic acid and certain phospholipids, such as phosphatidic acid and some phosphoinositides, in the 0.5 to 50 μM concentration range, disrupted [Rac-RhoGDI] complexes (51); (4) Certain phosphoinositides were shown to cause a “partial opening” of [RhoA-RhoGDI] complexes, allowing GDP to GTP exchange on RhoA and its translocation to the membrane (57); (5) Several cellular components can act as displacement factors to release Rho GTPases from RhoGDI: thus, direct interaction of RhoGDI with the neurotrophin receptor p75 initiated the activation of RhoA by facilitating the release of prenylated RhoA from RhoGDI (58); proteins from the ERM (ezrin/radixin/moesin) family appear to act as displacement factors (59), and a role for integrins in promoting the dissociation of Rac-GTP from RhoGDI and thus enhancing membrane targeting of Rac was also reported (60). It was also proposed that, in the process of oxidase assembly, dissociation of the [Rac-RhoGDI] complex is correlated with GDP to GTP exchange on Rac, mediated by a membrane-bound GEF (61).

Our group purified and characterized a cytosolic component of the oxidase complex, present in macrophage cytosol, which was initially called σ₁ or “the third cytosolic component” (62). This was found to be a heterodimer of two proteins, 22 kDa and 24 kDa in size, which were later identified as Rac1 and RhoGDI, respectively (14, 29). Rac2 was also isolated from neutrophil cytosol as a complex with RhoGDI (17). It soon became apparent that purified Rac1 and Rac2 can support oxidase activation in vitro in the absence of RhoGDI and that non-prenylated recombinant Rac1 and Rac2 are both active in the amphiphile-dependent cell-free system (14, 18). These findings support a model in which RhoGDI is not actively involved in the process of oxidase activation and serves exclusively as a negative regulator, the dissociation of which from Rac is a precondition for activation to proceed. An alternative possibility is that an intact [Rac(GDP)-RhoGDI] complex, such as isolated from macrophage (62) and neutrophil (50) cytosol, participates in oxidase activation in the absence of complex dissociation and nucleotide exchange on Rac to GTP. Functional (63) and structural (44) arguments in favor of such a process have been put forward.

In the present study we provide experimental evidence for the proposal that the stability of [Rac-RhoGDI] complexes is regulated by the phospholipid composition of the phagocyte membrane, with emphasis on the electric charge of the phospholipids. We describe an in vitro system in which negatively charged phospholipid liposomes (a protein-free “membrane model”) are shown to be able to dissociate [Rac-RhoGDI] complexes. The influences of the isoform of Rac and of the type of guanine nucleotide bound to Rac, on the level of liposome-induced dissociation are also described. Finally, we show that [Rac-RhoGDI] complexes are capable of amphiphile-independent oxidase activation in a cell-free system centered on phagocyte membrane liposomes enriched in anionic phospholipids.

**EXPERIMENTAL PROCEDURES**

Chemicals and Reagents - The hydrolysis-resistant nucleotide analog guanylylimidodiphosphate (GMPPNP, tetralithium salt, 83%, < 0.2% GTP) was purchased from Roche Applied Science. The fluorescent hydrolysis-resistant GTP analogue 2′-(or 3′)-O-(N-methylanthraniloyl) - guanylylimidodiphosphate (mant-GMPPNP) and 2′-(or 3′)-O-(N-
methylanthraniloyl) - guanosine 5’-diphosphate (mant-GDP) were obtained from Jena Bioscience. The following phospholipids were purchased from Sigma-Aldrich: L-α-phosphatidylcholine (PC) (from soybean, 99%, product number P 7443); L-α-phosphatidyl-L-serine (PS) (from soybean, 98%, product number P 0474); L-α-phosphatidylinositol (PI) (ammonium salt, from soybean, 98%, product number P5954); L-α-phosphatidyl-DL-glycerol (PG) (ammonium salt synthetic, 99%, product number P 6956), and L-α-phosphatidic acid (PA) (sodium salt, from egg yolk lecithin, 98%, product number P 9511). Common laboratory chemicals were from Sigma or Merck.

**Preparation of Macrophage Membrane Liposomes** - Membranes were isolated from guinea pig peritoneal macrophages, as described previously (8). Membranes suspended to a concentration of 500x10^6 cell equivalents/ml were first solubilized by 40 mM octyl glucoside and then reconstituted into liposomes by dialysis against detergent-free buffer, as reported before (65). The specific cytochrome b_{559} heme content of membrane liposomes was measured by the difference in spectrum of sodium dithionite-reduced minus oxidized samples (66).

**Preparation of Neutral and Anionic Phospholipid Liposomes** - These were prepared essentially as described in Ref. 67. Briefly, PC, PS, PI, PG, and PA were dissolved in the buffer also used for membrane solubilization, containing 40 mM octyl glucoside, at a concentration of 5 mM. To facilitate solubilization, the phospholipids were first homogenized by using an ultrasonic processor (Vibra Cell, VCX 400 w, Sonics and Materials) at 20% amplitude for three 10 s cycles in ice-cooled tubes. The mixtures were stirred magnetically in ice-cooled glass vials till the appearance of a clear solution. Uncharged phospholipid liposomes were prepared from PC. Negatively charged liposomes were prepared by mixing 2-ml aliquots of a 5 mM solution of PC with 1-ml aliquots of a 5 mM solution of PS, PI, PG, or PA and dialyzing these against a 500-fold excess of detergent-free buffer, using dialysis membranes with a molecular weight cut-off of 25,000 (Spectrum Laboratories), for 18 h at 4 °C. The vesicles elute in the exclusion volume (corresponding to a M_w of ≥ 2x10^8) by gel filtration on a Superose 12 10/300 GL fast protein liquid chromatography (FPLC) column (Amersham Biosciences). Preliminary experiments, performed on a light scattering-based particle sizing apparatus (ALV-5000/EPP, ALV-GmbH, Langen, Germany) indicated that the vesicles were in the 250-300 nm diameter range.

**Enrichment of Macrophage Membranes in Neutral or Anionic Phospholipids** - The lipid composition of macrophage membranes was modified by mixing 1 volume of phagocyte membranes, solubilized by 40 mM octyl glucoside and brought to a concentration of 1200 pmol cytochrome b_{559} heme/ml, with 4 volumes of PC, PS, PI, PG, or PA, at a concentration of 5 mM, dissolved in buffer containing 40 mM octyl glucoside. The mixtures were reconstituted into liposomes by dialysis against detergent-free buffer, as described in the preceding section. The concentration of cytochrome b_{559} was measured after dialysis and was normally found to be close to 240 pmol heme/ml; that of the added anionic phospholipids was 4 mM. The final concentration of cytochrome b_{559} heme of phospholipid-enriched membranes in cell-free oxidase assays was 5 nM and the final concentration of anionic phospholipids in the assay was 80-85 μM.

**Determination of Membrane Phospholipid Concentration** - The concentration of total phospholipids in native macrophage membrane liposomes was measured as described in Ref. 68, following extraction by chloroform, as described in Ref. 69. The concentration varied from 3.2 to 3.5 mM.

**Preparation of Recombinant Proteins** - Non-prenylated Rac1 and Rac2, Rac1 mutants G12V and Q61L, and RhoGDI were expressed as glutathione S-transferase (GST) fusion proteins in E.coli BL21-CodonPlus™ competent cells (Stratagene) and purified by affinity chromatography on glutathione-agarose (Sigma-Aldrich), followed by cleavage by thrombin in situ, as described in Ref. 70. p67phox and p47phox were prepared in baculovirus-infected Sf9 cells, as described before (71).

**Protein Concentration** - This was estimated by the method of Bradford (72), modified for use with 96-well microplates (Technical Bulletin 1177EG, Bio-Rad), using the Bio-Rad protein assay dye reagent concentrate and bovine γ-globulin, as standard.
SDS-PAGE and Immunoblotting - These were performed as described in Ref. 62. The gels were stained with GelCode blue stain reagent (Pierce). Detection of Rac1 was performed with an affinity-purified rabbit polyclonal anti-Rac1 C-terminal peptide antibody (sc-217; Santa Cruz Biotechnology), at a dilution of 1:500. RhoGDI was detected with a mouse monoclonal anti-RhoGDI antibody (R26230; Transduction Laboratories), at a dilution of 1:2500. Second antibodies were affinity-purified alkaline phosphatase-conjugated goat anti-rabbit IgG (Sigma), used at a dilution of 1:1000, and affinity-purified alkaline phosphatase-conjugated goat anti-mouse IgG (Sigma), used at a dilution of 1:2000.

Identification and Quantification of Nucleotides - Nucleotides bound to native Rac1 or Rac1 exchanged to GMPPNP, present in highly purified [Rac1-RhoGDI] complexes, were released from the protein and identified by high pressure liquid chromatography (HPLC) on a Partisil 10 SAX anion exchange column (Whatman), as described before (73).

Enzymatic Prenylation of Rac1 and Rac2 - Recombinant non-prenylated Rac1 and Rac2 were prenylated in vitro by recombinant geranylgeranyltransferase type I, as described before (74).

Nucleotide Exchange - Rac1 and Rac2 were subjected to nucleotide exchange from the native GDP-bound form to mant-GMPPNP or mant-GDP, as described before (13). A nucleotide to protein ratio of 10/1 was used. The nucleotide exchange procedure was applied prior to prenylation.

Isolation of Pure [Rac1-RhoGDI] and [Rac2-RhoGDI] Complexes In Vitro - Non-prenylated Rac1(GDP) and Rac2(GDP) expressed in E. coli were prenylated in vitro and either used in native (= GDP-bound) form or exchanged to the guanine nucleotide analog GMPPNP or the fluorescent analogs mant-GMPPNP or mant-GDP. Then, prenylated Rac1 or Rac2 was mixed with RhoGDI at a ratio of 1:2 or 1:4 and the (Rac + RhoGDI) mixture was incubated in a rotary mixer (Thermomix Comfort, Eppendorf), set at 500 rpm, for 20 min at room temperature. Following incubation, the solution now containing [Rac(1 or 2)-RhoGDI] complexes, was subjected to ultrafiltration (using Amicon Ultra-4 or Ultra-15 centrifugal filter units (Millipore)) in order to remove any unbound fluorescent nucleotide and also to transfer the proteins to the loading buffer in the subsequent anion exchange chromatography step. Thus, following exchange to loading buffer (20 mM Tris-HCl, pH 7.5, 5 mM MgCl2), the (prenylated Rac(1 or 2) + RhoGDI) mixtures were applied to a Mono Q 5/50 GL anion exchange FPLC column (Amersham Biosciences), on an AktaBasic 10 HPLC system (Amersham Biosciences), and eluted at a flow rate 0.5 ml/min, at 4 °C. A positive linear gradient of NaCl (0 - 0.5 M) in 20 ml of loading buffer was applied, at a flow rate 0.5 ml/min. Fractions (0.5 ml) containing the [Rac(1 or 2)-RhoGDI] complex and those containing the excess free RhoGDI were collected and pooled, supplemented with 30% glycerol, divided into aliquots and stored frozen at -75 °C.

Assessment of Rac(1 or 2) Dissociation from RhoGDI by In Line Fluorescence Assay - [Rac (1 or 2)-RhoGDI] complexes (aliquots of 1 nmol), in which the Rac moiety was labeled with the fluorescent analogues mant-GMPPNP or mant-GDP, were mixed with either neutral or anionic phospholipid liposomes (on most occasions, 500 nmol phospholipid) in a total volume of 0.5 ml. The mixtures were incubated for 2 min in a rotary mixer (Thermomixer Comfort, Eppendorf) set to room temperature and 500 rpm, and injected immediately into a Superose 12 10/300 GL FPLC gel filtration column (Amersham Biosciences). Elution was performed with a buffer consisting of 50 mM Tris-HCl, pH 7.5, 5 mM MgCl2, and 150 mM NaCl. Chromatography was performed on an HPLC system (Waters), at a flow rate of 0.2 ml/min, at 4 °C, and both absorbance and the fluorescent signal (excitation = 361 nm; emission = 440 nm) were measured continuously by passing the column eluate through a diode array detector (MD-1510, Jasco) and a spectrofluorometer (model FP-750, Jasco), fitted with an HPLC flow cell (MFC-132, Jasco). We expected the dissociated prenylated Rac to bind to the phospholipid liposomes and elute in the exclusion volume whereas the intact [Rac-RhoGDI] complex, to elute in a volume corresponding to its molecular mass. In control experiments, we also assessed the binding of free prenylated and non-prenylated Rac1(mant-GDP) to neutral and anionic liposomes as well as the
binding of free RhoGDI to anionic liposomes. A fact complicating the quantitative assessment of fluorescence in these experiments was the occurrence of light scattering by the phospholipid liposomes (75). Consequently, all data obtained in these experiments were corrected for light scattering by subtracting the fluorescent signal recorded when phospholipid liposomes were injected alone from the signal recorded when injecting a mixture of the same amount of phospholipid liposomes and either [Rac-RhoGDI] complex, free Rac, or free RhoGDI.

**Cell-Free NADPH Oxidase Activation Assay** - In these experiments, oxidase activation by [Rac1-RhoGDI] complexes was assessed. The cell-free system consisted of phagocyte membrane liposomes in native form or enriched with either neutral or anionic phospholipids (at a concentration equivalent of 5 nM cytochrome b<sub>559</sub> heme), [Rac1(GDP)-RhoGDI] or [Rac1(GMPPNP)-RhoGDI] complexes, generated in vitro and purified on Mono Q (200 nM), and p67<sup>phox</sup> (300 nM), with or without the addition of p47<sup>phox</sup> (300 nM). No amphiphilic activator was added. Incubation was performed in 96-well microplates, in a total volume of 200 µl of assay buffer per well, for 90 s at 25 °C, before the addition of 240 µM NADPH, to initiate O<sub>2</sub><sup>•-</sup> production. This was quantified by the kinetics of cytochrome c reduction, as described earlier (76).

**RESULTS**

**Generation of [Rac1(GDP)-RhoGDI] and [Rac2(GDP)-RhoGDI] Complexes In Vitro** - The only posttranslational modification on Rac required for interaction with RhoGDI is the presence of a geranylgeranyl moiety at the C-terminus (77). Additional post-prenylation modifications of Rac1, such as carboxyl-methylation and proteolysis, are not required for complex formation with RhoGDI (77). On the contrary, lack of carboxyl-methylation enhanced binding of Rac1 to RhoGDI in endothelial cells (78). Thus, in order to be able to conduct quantitative biochemical studies on Rac-RhoGDI interactions, we created stable [Rac1(GDP)-RhoGDI] and [Rac2(GDP)-RhoGDI] complexes in vitro by mixing recombinant Rac1 or 2(GDP), expressed in E. coli and prenylated in vitro, with a two or four-fold excess of recombinant RhoGDI, also expressed in E. coli. The [Rac(1 or 2)(GDP)-RhoGDI] complexes were purified by anion exchange chromatography of Mono Q, which separates the complex from free RhoGDI (Fig. 1). For most of the experiments to follow, complexes were constructed from Rac1 or 2 exchanged to mant(GDP) before prenylation. These exhibited properties identical to those prepared from prenylated native Rac1 or 2, which is in the GDP-bound form (73). Under the chromatographic conditions used, Rac1 (pI = 8.77) and Rac2 (pI = 7.52) did not bind to the column. The [Rac1(GDP)-RhoGDI] complex eluted from the column at a conductivity of 11 mS/cm (Fig. 1A); the [Rac2(GDP)-RhoGDI] complex eluted at a conductivity of 15.5 mS/cm (results not shown graphically). Free RhoGDI eluted from the column at a conductivity of 19 mS/cm (Fig. 1A). The recovery of prenylated Rac1(GDP), in complex with RhoGDI, varied between 46 to 49% (free prenylated Rac, not bound to the column, could not be measured but was unlikely to exist in the presence of a large excess of RhoGDI). The recovery of RhoGDI (represented by the sum of free RhoGDI and RhoGDI in complex with Rac) varied between 56 and 58%. As evident from the clear distinction between the [Rac-RhoGDI] and free RhoGDI peaks and the SDS-PAGE analysis of the fractions (Fig. 1B), highly purified [Rac-RhoGDI] complexes were obtained and used in the experiments to follow.

**Disruption of the [Rac1(GDP)-RhoGDI] Complex by Anionic Phospholipid Liposomes** - The C-terminus polybasic sequence of both non-prenylated and prenylated Rac1, consisting of six contiguous basic residues, serves as a membrane targeting signal, mediated by its interaction with negatively charged membrane phospholipids (79-82, 67). Structural studies revealed the geranylgeranyl-binding C-terminal immunoglobulin-like pocket of RhoGDI comprises an acidic patch created by Gln<sup>130</sup>, Thr<sup>142</sup>, Tyr<sup>144</sup>, Glu<sup>163</sup>, Glu<sup>164</sup> and a number of main chain carbonyl groups (43). Based on this information, we hypothesized that liposomes of negatively charged phospholipid, comprising an acidic patch created by Gln<sup>130</sup>, Thr<sup>142</sup>, Tyr<sup>144</sup>, Glu<sup>163</sup>, Glu<sup>164</sup> and a number of main chain carbonyl groups (43). Based on this information, we hypothesized that liposomes of negatively charged phospholipid may compete with the acidic patch in RhoGDI for binding the C-terminal geranylgeranylated polybasic domain of Rac1. To test this assumption, we utilized FPLC gel filtration combined with in line fluorescence detection of mant-guanine nucleotide-labeled
Rac1, which allows accurate and easy quantification of both intact [Rac1-RhoGDI] complexes and of Rac1 dissociated from RhoGDI and bound to the anionic liposomes.

We generated complexes of [Rac1(mant-GDP)-RhoGDI] and incubated these with phospholipid vesicles, containing 100% PC or 66% PC and 33% of either PS, PI, PG, or PA. In control experiments, complexes of [Rac1(mant-GDP)-RhoGDI] were incubated with an equal volume of the buffer in which the phospholipid liposomes were suspended. As apparent in Figs. 2, C, D, E, and F, exposure of [Rac1-RhoGDI] complexes to liposomes containing anionic phospholipids resulted in the dissociation of the complexes and binding of 45 to 49% of Rac1(mant-GDP), present in the complexes, to the negatively charged liposomes. There was no significant difference in the ability of various anionic phospholipids to promote the disruption of [Rac1-RhoGDI] complexes. Exposure of [Rac1-RhoGDI] complexes to neutral liposomes, consisting of PC only, did not lead to complex dissociation; only about 3% of Rac1(mant-GDP) was bound to the liposomes, which was not significantly different from the control sample consisting of [Rac1-RhoGDI] complex and buffer (Figs. 2, A and B). Dissociation of the [Rac1-RhoGDI] complex occurred spontaneously upon mixing with the anionic liposomes and did not require the presence of a soluble anionic amphiphile.

In order to confirm the fact that the fluorescent signal associated with material eluting in the exclusion volume represented liposome-bound Rac1, no longer associated with RhoGDI and derived by dissociation from RhoGDI, we analyzed these fractions by SDS-PAGE and by immunoblotting with anti-Rac1 and anti-RhoGDI antibodies. As apparent in Fig. 3A, fractions corresponding to the peak of fluorescence eluting in the exclusion volume of the column (peak 1), contained only Rac1 and no RhoGDI. Fractions corresponding to the peak of fluorescence, detected at an elution volume corresponding to a molecular mass of 54.73 ± 0.75 kDa (means ± S.E. of 5 experiments), contained both Rac1 and RhoGDI (peak 2). It can be seen that the amount of RhoGDI present in the same lane is exceeding that of Rac1, suggesting, as expected, that these fractions contain both [Rac1(GDP)-RhoGDI] complexes and free RhoGDI, originating in the dissociation of the complex. The co-elution of [Rac1(GDP)-RhoGDI] complexes with free RhoGDI could be explained by the fact that RhoGDI was reported to form homodimers spontaneously, of a size overlapping the non-dissociated [Rac-RhoGDI] complexes (83). In order to ascertain that this is indeed the case and also to test the assumption that RhoGDI does not bind to anionic liposomes, we mixed free RhoGDI with liposomes composed of 66% PC and 33% PG and subjected the mixture to gel filtration on Superose 12. As seen in Fig. 3B (upper panel), RhoGDI eluted as a single peak, with a molecular mass of 54.8 kDa, which is compatible with the presence of a RhoGDI homodimer (peak 2). Indeed, SDS-PAGE analysis of fractions collected from this peak showed the presence of a single band corresponding in size to the RhoGDI monomer2 (Fig 3B, lower panel). No RhoGDI was found associated with the liposomes (Fig. 3B, upper panel, peak 1), as demonstrated by the inability to detect RhoGDI by immunoblotting of fractions containing the liposomes (Fig. 3B, middle panel).

Only the Prenylated Form of Rac1 Associates with Negatively Charged Phospholipid Liposomes - In these experiments we intended to define the conditions required for the association of Rac1 with phospholipid liposomes when Rac1 was in free form (not in a complex with RhoGDI). We compared the binding of free prenylated and non-prenylated Rac1(mant-GDP) to the five types of phospholipid liposomes described in the preceding section. Similar studies were done by us in the past (67) but these were performed with prenylated Rac1 produced in the baculovirus system and, thus, subject to more extensive posttranslational modifications, and did not include a comparison to non-prenylated Rac1. As apparent in TABLE ONE, there was no significant binding of non-prenylated Rac1 to either neutral or anionic liposomes but there was extensive binding of prenylated Rac1(mant-GDP) to neutral liposomes and some binding to anionic phospholipid liposomes described in the preceding section. Indeed, SDS-PAGE analysis of fractions collected from this peak showed the presence of a single band corresponding in size to the RhoGDI monomer2 (Fig 3B, lower panel). No RhoGDI was found associated with the liposomes (Fig. 3B, upper panel, peak 1), as demonstrated by the inability to detect RhoGDI by immunoblotting of fractions containing the liposomes (Fig. 3B, middle panel).
which there was no competition between liposomes and RhoGDI for prenylated Rac1.

Quantitative Parameters of the Dissociation of [Rac1(mant-GDP)-RhoGDI] Complexes by Anionic Phospholipid Liposomes -

We next examined the influence of increasing the proportion of anionic phospholipid in liposomes consisting of a constant amount of total phospholipid, on the dissociation of a model complex represented by [Rac1(mant-GDP)-RhoGDI]. Because no difference was found in the extent of dissociation with various negatively charged phospholipids (Fig. 2), we used liposomes consisting of PC and 16, 33, or 66% PG, with the amount of PC reduced accordingly, or 100% PG. As apparent in Fig. 4A, dissociation increased proportionally with the concentration of PG, with almost complete complex dissociation being achieved with liposomes containing 66% PG.

In a second series of experiments, we investigated the effect of the time of incubation on the extent of dissociation. [Rac1(mant-GDP)-RhoGDI] complexes were incubated with liposomes consisting of 66% PC and 33% PS for 2 min at room temperature or for 2 min at room temperature followed by further incubation for 3 h at 4 °C. The proportion of dissociated Rac1 was assessed by gel filtration on Superose 12, as described earlier in this section. As shown in Fig. 4B, extending the length of time of exposure of the complexes to the liposomes did not augment the extent of dissociation, suggesting that the effect of anionic liposomes was quite rapid. The precise length of time required for dissociation cannot be determined by using this methodology because about 40 min elapsed from the time of injection of the mixture of liposomes and [Rac-RhoGDI] complex until the elution of the liposome-bound Rac from the column. Thus, although it seems unlikely that the process of complex dissociation continues after the injection of the mixture of liposomes and complex into the column, we can only conclude with certainty that the process of complex dissociation is completed within a time interval of 2 to 40 min.

We next explored the possibility that [Rac1-RhoGDI] complexes may comprise subpopulations with varying susceptibilities to dissociation by anionic liposomes. In order to test this possibility, we prepared mixtures of 3 nmol of [Rac1(mant-GDP)-RhoGDI] and 500 nmol liposomes, consisting of 66% PC and 33% PS, and separated the dissociated and non-dissociated complexes by gel filtration. 17% of Rac1(mant-GDP) was found associated with the PC/PS liposomes. The lower level of complex dissociation, in comparison to the data appearing in Fig. 2C, is explained by the three-fold higher complex to liposome ratio used in the present experiment. 1 nmol of non-dissociated complex from the first stage of the experiment was mixed with 500 nmol of a fresh batch of PC/PS liposomes and subjected to a second round of gel filtration. Now, 38% of Rac was bound by the PC/PS liposomes, a value similar to that found in the experiments summarized in Fig. 2, in which binding of [Rac-RhoGDI] complexes, not subjected to a first round of binding and gel filtration, to PC/PS liposomes was measured. This indicates that dissociation of [Rac1-RhoGDI] complexes is random and governed exclusively by the quantitative relationship between the amount of complex and the amount of anionic liposomes, as expected from a homogenous population of complexes.

RhoGDI Prefers Binding the GDP-Bound Form of Rac1 -
The interaction of RhoGDI with the GTP-bound forms of Rho GTPases has been reported to exhibit an affinity inferior (32, 33) or equal to (34, 35) that of the GDP-bound forms. The development of a reproducible method to generate highly purified [Rac1(GDP)-RhoGDI] complexes in vitro from recombinant proteins led us to apply the same technique to the generation of a [Rac1(GMPPNP)-RhoGDI] complex. A stable complex was obtained by mixing prenylated Rac1(GMPPNP) with a four-fold excess of RhoGDI. This was separated on Mono Q and exhibited an elution profile (Fig. 5A) similar to that of the [Rac1(GDP)-RhoGDI] complex illustrated in Fig. 1A. The purity of the complex was confirmed by SDS-PAGE analysis (Fig. 5B).

The [Rac1(GMPPNP)-RhoGDI] complex differed from the [Rac1(GDP)-RhoGDI] complex by two characteristics: a lower recovery of complex from equal amounts of Rac and RhoGDI, which is evident by even a perfunctory look at the areas of the complex peaks in Figs. 5A and 1A, and the elution of the [Rac1(GMPPNP)-RhoGDI] complex from Mono Q at a higher conductivity (13 mS/cm in comparison to 11 mS/cm, for the [Rac1(GDP)-RhoGDI] complex), a shift also...
apparent in the SDS-PAGE analysis of the collected fractions from the two separations (Figs. 5B and 1B). As expected, no shift in buffer conductivity was observed for the time of elution of RhoGDI (19 mS/cm), proving that the difference between the experiments described in Figs. 5A and 1A was not due to technical reasons. The most likely explanation for this result is that the higher negative charge of GMPPNP, compared to GDP, results in the [Rac1(GMPPNP)-RhoGDI] complex having a lower pI than the [Rac1(GDP)-RhoGDI] complex. In order to make certain that apparent difference in pI is due to the nature of the nucleotide bound to Rac1, we identified and quantified the Rac-bound nucleotides present in highly purified [Rac1(GMPPNP)-RhoGDI] complex, by anion exchange chromatography on a Partisil 10 SAX column, and found that these consisted of 92.3% GMPPNP and only 7.7% GDP.

A quantitative expression of the difference in the ability of Rac1(GDP) versus Rac1(GMPPNP) to form a complex with RhoGDI is summarized in TABLE TWO. It is apparent that the mean amount of [Rac1(GDP)-RhoGDI] complex recovered by anion exchange chromatography on Mono Q, as expressed by the peak area, is 1.55 times larger than the mean amount of [Rac1(GMPPNP)-RhoGDI] complex, when both species of complexes were generated and isolated under strictly identical conditions. The peak areas of excess free RhoGDI, are, as expected, in inverse proportion to the areas of the [Rac1-RhoGDI] complexes. In order to make sure that one can compare peak areas, representing different complexes, we determined the extinction coefficients for purified complexes of [Rac1(GDP)-RhoGDI] and [Rac1(GMPPNP)-RhoGDI], freed of unbound nucleotides, based on absorbance at 280 nm, and found these to be 23.77 mM⁻¹ cm⁻¹ and 26.96 mM⁻¹ cm⁻¹, respectively. This means that peak areas are representative of the amounts of complex; the lower extinction coefficient for the [Rac1(GDP)-RhoGDI] complex (by 12%) means that the difference in favor of the [Rac1(GDP)-RhoGDI] complex is even larger than apparent from the table.

We also obtained a stable [Rac1-RhoGDI] complex using the prenylated GTPase deficient Rac1 mutant G12V (results not shown). This mutation is in the switch I domain and leads to Rac1 being predominantly in the GTP-bound form (see Ref. 84). On the other hand, no complex was obtained when using the prenylated GTPase deficient Rac1 mutant Q61L (results not shown). This latter mutation is in the switch II domain and also leads to Rac1 being predominantly in the GTP-bound form (see Refs. 84 and 73). These results confirm earlier reports that an intact switch II in Rac1 is crucial for interaction with RhoGDI (44, 64).

The [Rac1(GMPPNP)-RhoGDI] Complex is More Susceptible to Dissociation by Anionic Phospholipid Liposomes than the [Rac1(GDP)-RhoGDI] Complex - The availability of both stable [Rac1(GDP)-RhoGDI] and [Rac1(GMPPNP)-RhoGDI] complexes enabled us to investigate the susceptibility of the two types of complexes to dissociation by anionic liposomes. [Rac1(mant-GDP)-RhoGDI] and [Rac1(mant-GMPPNP)-RhoGDI] were generated as described under "Experimental Procedures" and each type of complex was incubated with either neutral liposomes, consisting of 100% PC, or anionic liposomes, consisting of a mixture of 66% PC and 33% PS. As apparent in Figs. 6, A and C, both types of [Rac1-RhoGDI] complexes were unaffected by exposure to neutral liposomes. Incubation with anionic liposomes resulted in a much more pronounced (almost complete) dissociation of [Rac1(mant-GMPPNP)-RhoGDI] complexes compared to that of [Rac1(mant-GDP)-RhoGDI] complexes (Figs. 6, B and D). These results indicate that the guanine nucleotide bound to the Rac1 moiety of the complex affects both the affinity of Rac1 for RhoGDI and the stability of the resulting complex.

[Rac2(GDP)-RhoGDI] Complexes Are Less Susceptible to Dissociation than [Rac1(GDP)-RhoGDI] Complexes - The [Rac2(GDP)-RhoGDI] complex was found to elute from the Mono Q column at a higher conductivity than that at which the [Rac1(GDP)-RhoGDI] complex was eluted. This appears to be the consequence of the lesser positive charge of the C-terminus of Rac2 (3 basic residues) compared to Rac1 (6 basic residues). We were interested in examining the effect of this difference in positive charge on the ability of anionic liposomes to cause dissociation of the two types of complexes. As apparent in Fig. 7, A and B, neither [Rac1(mant-GDP)-RhoGDI] nor [Rac2(mant-GDP)-RhoGDI] were dissociated by neutral liposomes.
liposomes, consisting of PC, only. The presence in the liposomes of increasing amounts of the anionic phospholipid PG (33% and 66%), led to the dissociation of both Rac1- and Rac2-containing complexes, proportional to the concentration of anionic phospholipid in the liposomes, as already shown for [Rac1(mant-GDP)-RhoGDI] (see Fig. 4A). However, in the presence of the same concentration of PG, the dissociation of the [Rac2(mant-GDP)-RhoGDI] complex was half of that of the [Rac1(mant-GDP)-RhoGDI] complex, in a surprisingly direct relationship to the ratio of the number of basic residues in the C-terminus of Rac2 and Rac1 (compare Fig. 7, C and D, and E and F, respectively).

Native Macrophage Membranes Contain Sufficient Anionic Phospholipids to Cause Dissociation of [Rac1(GMPPNP)-RhoGDI] Complexes - The finding that [Rac-RhoGDI] complexes dissociate upon encountering liposomes containing anionic phospholipids, above a certain concentration threshold, raised the possibility that such conditions might be present in membranes of resting or stimulated phagocytes. It is well established that the repartition of phospholipids in plasma membranes is asymmetric, with the outer leaflet of the membrane containing most of the neutral phospholipids PC and sphingomyelin, whereas the cytosolic face of the membrane is enriched in the anionic phospholipids PS and PI (85). Membranes of rabbit alveolar macrophages (86), bone-marrow-derived mouse macrophages (87) and human neutrophils (88) contain 11.5 to 15% anionic phospholipids, representing the sum of PS and PI. Since a concentration of anionic phospholipid above 16% caused dissociation of [Rac1(GDP)-RhoGDI] complexes (see Fig. 4A), we reasoned that native macrophage membrane liposomes might also induce a low level of complex dissociation. Indeed, as seen in Fig. 8A, exposure of [Rac1(mant-GDP)-RhoGDI] to macrophage membrane liposomes containing an amount of total phospholipid identical to that present in the liposomes made from pure phospholipids caused a modest level of complex dissociation. Based on our finding that complexes of Rac1(GMPPNP) with RhoGDI are more susceptible to dissociation by PC/PS liposomes than complexes of Rac1(GDP) with RhoGDI (see Fig. 6, B and D), we looked at the ability of native macrophage membrane liposomes to cause dissociation of [Rac1(mant0-GMPPNP)-RhoGDI] complexes. As seen in Fig. 8B, a large proportion of the complex was indeed dissociated, indicating that membranes of resting phagocytes possess the potential of destabilizing low affinity but not high affinity [Rac-RhoGDI] complexes. This potential is likely to be augmented considerably when phagocytes are stimulated (see "Discussion").

The Phospholipid Composition of the Phagocyte Membrane Controls the Ability of [Rac1-RhoGDI] Complexes to Activate the NADPH Oxidase in the Absence of an Anionic Amphiphile - We have reported that oxidase activation can be achieved in vitro by mixtures of membrane liposomes, p67phox, and prenylated Rac1, in the absence of an amphiphilic activator and of p47phox (13, 67). We reasoned that modifying the phagocyte membrane by enrichment with anionic phospholipids will mimic the effect of anionic protein-free liposomes and cause dissociation of [Rac1-RhoGDI] complexes and subsequent translocation of prenylated Rac1 to the phagocyte membrane, which should serve as a preamble to oxidase activation (13). Phagocyte membranes were used either in the native, unmodified, form or after enrichment with either neutral (PC) or anionic (PS, PI, PG, and PA) phospholipids (as described under "Experimental Procedures"). A cell-free activation system was constructed consisting of: native or modified phagocyte membrane liposomes, purified [Rac1(GDP)-RhoGDI] or [Rac1(GMPPNP)-RhoGDI] complexes, and p67phox. Assays were performed in the absence of an anionic amphiphile activator, both in the presence and absence of p47phox, on the basis of our earlier finding that amphiphile-independent oxidase activation by prenylated Rac1 and p67phox was little influenced by the presence of p47phox (13). We found that the oxidase in native membrane liposomes was activated by [Rac1(GMPPNP)-RhoGDI] complexes at a moderate level, in the presence of p47phox, and to a lesser degree, in its absence (Fig. 9A). [Rac1(GDP)-RhoGDI] complexes were unable to activate. These results were consistent with the pattern of dissociation of the two types of complexes by native membrane liposomes (Fig. 8). Enrichment of membranes with neutral phospholipid (PC) eliminated totally the activation
of the oxidase by [Rac1(GMPPNP)-RhoGDI], even in the presence of p47phox (Fig. 9B).

Exposure of membranes enriched in any of the four anionic phospholipids (PS, PI, PG, and PA) to [Rac1(GMPPNP)-RhoGDI], in the presence of p47phox, resulted in high levels of oxidase activation (Fig. 9, B). Complexes of [Rac1(GDP)-RhoGDI] activated the oxidase in membranes enriched in PA and PG, and, to a limited degree, in membranes enriched in PS, when p47phox was present (Fig. 9, D, E, and F). In the absence of p47phox, only membranes modified with PA and PG, exposed to [Rac1(GMPPNP)-RhoGDI], produced significant levels of O2− (Fig. 9, E and F). These results indicate that: (a) native macrophage membranes contain sufficient anionic phospholipids to support oxidase activation by [Rac1(RhoGDI)] complexes and that artificially reducing the relative amount of anionic phospholipids makes the membrane non-responsive; (b) increasing the amount of anionic phospholipids markedly augments the ability of the membranes to support oxidase activation, and (c) whereas various anionic phospholipids have an equal capacity to dissociate [Rac1-RhoGDI] complexes, the nature of the anionic phospholipid incorporated into the phagocyte membrane influences the level of oxidase activation.

**DISCUSSION**

The mechanisms promoting the release of Rac from the [Rac-RhoGDI] complex and its subsequent accumulation at the plasma membrane in the course of oxidase activation are only partly understood. In order to approach these questions, we developed a novel methodology for generating highly purified and stable [Rac-RhoGDI] complexes. The essence of this was to mix recombinant Rac produced in E. coli, prenylated enzymatically in vitro, with an excess of recombinant RhoGDI, also produced in E. coli, and purify the resulting [Rac-RhoGDI] complex by anion exchange chromatography. To the best of our knowledge, this is the first description of the construction of [Rac1-RhoGDI] and [Rac2-RhoGDI] complexes from recombinant Rac1 and Rac2, geranylgeranylated in vitro, and recombinant RhoGDI. Rac1 and Rac2 employed for the generation of the complexes were subject to only partial post-translational modification since they were not carboxyl-methylated and the C-terminal LLL (Rac1)/SLL (Rac2) sequences were not removed. In preliminary experiments, we found that complexes prepared using bacterially expressed Rac1 prenylated in vitro by recombinant geranylgeranyl transferase I were undistinguishable from those prepared using prenylated Rac1 expressed in the baculovirus system (13, 67), as judged by chromatographic behavior on Mono Q and the ability to be dissociated upon exposure to liposomes containing anionic phospholipids (results not shown).

The generation of [Rho GTPase-RhoGDI] complexes from recombinant proteins was reported in the past by coinfection of Sf9 cells with baculoviruses encoding Rac and RhoGDI (44, 45, 64); by co-expression of RhoA (89) or Rac1 (48) with RhoGDI in yeast, and by mixing RhoA (57) or Cdc42Hs (43), expressed in Sf9 cells, with bacterially expressed RhoGDI. We believe that the methodology described in this report offers the advantages of simplicity and reproducibility and the ability of expansion to larger quantities.

In this study we show that [Rac(1 and 2)-RhoGDI] complexes are destabilized by contact with protein-free liposomes containing anionic phospholipids, serving as "membrane models". Four types of anionic phospholipid liposomes exhibited an equal potential to release Rac1 from [Rac1-RhoGDI] complexes and although no such comparative studies were performed with [Rac2-RhoGDI] complexes, it is most likely that the non-specificity of the charge effect also applies to the latter. Dissociation of [Rac(1 and 2)-RhoGDI] complexes by anionic phospholipid liposomes was found to be phospholipid dose-dependent and fast. Liposomes consisting of neutral phospholipid had no effect on the stability of [Rac1-RhoGDI] complexes. [Rac2-RhoGDI] complexes were more resistant to dissociation than [Rac1-RhoGDI] complexes, reflecting the lower affinity of Rac2, which possesses only half the number of positively charged residues at its C-terminus, for negatively charged phospholipids. The biological significance of the lesser ability of [Rac2-RhoGDI] complexes to be dissociated by anionic liposomes remains an open question. It is conceivable that this points toward subtle differences in the mechanism of dissociation of [Rac-RhoGDI] complexes in neutrophils, in which the predominant isoform is...
Rac2 (25), and monocytes/macrophages, in which the predominant isoform is Rac1 (23, 24).

In accordance with earlier reports, we also found that RhoGDI exhibits a higher affinity towards the GDP bound form of prenylated Rac1. Nevertheless, a [Rac1(GMPPNP)-RhoGDI] complex could be generated with ease from recombinant components and isolated in stable form by anion exchange chromatography. This complex was more susceptible to dissociation by anionic liposomes than complexes generated with Rac1(GDP).

Dissociation of [Rho GTPase-RhoGDI] complexes by phospholipid liposomes was described in the past by two groups of investigators. Read et al. (89) reported that [RhoA-RhoGDI] complexes generated by coexpression of the two components of the complex in yeast were dissociated by liposomes prepared from E. coli lipids, provided that RhoA in the complex was first converted to the GTPγS-bound form. [RhoA(GDP)-RhoGDI] complexes could not be dissociated but no information is provided on the nature and ionic characteristics of the E. coli lipids which served for the preparation of the liposomes. Antonny and coworkers (48) also found that [Rac1(GDP)-RhoGDI] complexes, prepared by coexpression in yeast, showed minimal dissociation when incubated with liposomes prepared from unpurified soybean PC, known to contain anionic phospholipids, but exchanging GDP to GTPγS on Rac resulted in enhanced dissociation. In a later report (90), the same group showed that a [Rac1(GDP)-RhoGDI] complex was dissociated by liposomes enriched in well defined acidic phospholipids (PA, PG, or PS), conditional on it being first converted to a [Rac1(GTP)-RhoGDI] complex.

Our studies demonstrate the ability of anionic liposomes to dissociate both [Rac(GDP)-RhoGDI] and [Rac(GMPPNP)-RhoGDI] complexes and establish the experimental conditions and quantitative parameters governing this dissociation (charge of phospholipid, isoform of Rac, and nature of bound nucleotide). The ability to cause dissociation of [Rac-RhoGDI] complexes is correlated exclusively with the negative charge of the phospholipids making up the liposomes; the nature of the polar moiety and that of the fatty acid residues were of no relevance. As shown by the inability of neutral liposomes to induce complex dissociation, the competition between the hydrophobic pocket of RhoGDI and the large hydrophobic surface of the liposomes is, by itself, not sufficient for causing detachment of Rac from the former and its attachment to the latter, in spite of the very large molar excess of liposomes over RhoGDI. Clearly, dissociation requires the cooperation between electrostatic and hydrophobic forces, as also apparent from the data in TABLE ONE, showing the lack of binding of non-prenylated Rac1 to anionic liposomes and the low binding of prenylated Rac1 to neutral liposomes. Such synergy between electrostatic and hydrophobic interactions is common in the binding of prenylated or myristoylated proteins to membranes (reviewed in Ref. 91). It has, indeed, been shown that Rac1 requires two membrane localization motifs: a C-terminal polybasic domain and the isoprenyl group (80-82, 92). Structural studies demonstrated a significant contribution of both the isoprenyl group and the polybasic domain to binding to the immunoglobulin-like pocket of RhoGDI (42, 43). Furthermore, a prenylated Rac1 mutant, lacking the C-terminal polybasic region, was found defective in its interaction with RhoGDI (64). Contrary to the initial assumption (79), the role of the C-terminal polybasic domain of Rac1, responsible for membrane association, appears not to be sequence specific and depends only on the net positive charge.

It should be noted that our conclusions are based on work with Rac prenylated in vitro and, thus, lacking the additional processing occurring in vivo. Although we do not have any indication for it, we cannot exclude the possibility that post-prenylation processing might lead to a finer tuning of the selectivity of Rac for specific anionic phospholipids.

The cellular location of the dissociation of [Rho GTPase-RhoGDI] complexes is yet another open question. Do complexes reach the plasma membrane intact and are dissociated at the level of the membrane or does dissociation take place in the cytosol? Recently, Dransart et al. (93) reported mutations in RhoGDI-1, which caused a loss of its ability to inhibit the cellular effects of CDC42Hs (formation of microspikes), but did not affect the ability of RhoGDI-1 to form a cytosolic complex with CDC42Hs and to serve as a shuttle leading to the co-localization of RhoGDI-1 with Cdc42Hs, at the cell membrane. This argues for the movement of...
of an intact complex to the membrane, where dissociation is to take place. Such a sequence of events is compatible with the mechanism suggested by us in which the molecule responsible for complex dissociation is located on the cytosolic face of the plasma membrane.

A significant outcome of these studies was the finding that [Rac1-RhoGDI] complexes, together with p67phox, were capable of eliciting NADPH oxidase activation in vitro in the absence of an anionic amphiphilic activator, provided that the phospholipid environment of cytochrome b559 comprised a certain amount of anionic phospholipids. Native phagocyte membranes indeed contain up to 15% anionic phospholipids, the bulk of which are exposed on the cytosolic face of the plasma membrane (85-88). This explains the ability of native macrophage membranes to cause dissociation of [Rac(GMPPNP)-RhoGDI] complexes and to support oxidase activation by such complexes. This ground-level activity can be markedly enhanced by artificial enrichment of membranes with exogenous anionic phospholipids (PI, PS, PG, and PA) and totally suppressed by raising the relative amount of neutral phospholipid (PC).

Activation was enhanced by but not conditional upon the presence of p47phox, indicating that the actual effectors of activation were free prenylated Rac1, derived by dissociation of the [Rac1-RhoGDI] complex, and p67phox. An enhancing effect of p47phox on cell-free activation systems, which can also function in the absence of p47phox, was observed in a number of situations (13, 94, 95) and is, probably, explained by an increased stability of the assembled oxidase complex when it includes p47phox (95).

Another characteristic of the amphiphile-independent oxidase activation by [Rac-RhoGDI] was that complexes containing Rac(GDP) were also active, provided that membranes were enriched with certain anionic phospholipids (principally, PA and PG). This raises the questions of whether [Rac(GDP)-RhoGDI] complexes can activate the oxidase in vivo, and whether nucleotide exchange on Rac, from GDP to GTP, takes place before or after the dissociation of the [Rac(GDP)-RhoGDI] complex. In the past, arguments were put forward in support of oxidase activation by [Rac(GDP)-RhoGDI] complexes, as such, or following prior dissociation of the complex but without the need for GDP to GTP exchange (29, 63, 64). Alternatively, it was suggested that nucleotide exchange to GTP on Rac(GDP) in complex with RhoGDI, by a membrane-localized GEF, precedes and causes the dissociation of the complex (61).

The anionic phospholipid specificity of oxidase activation by [Rac-RhoGDI] complexes was found to be different from that governing the process of complex dissociation. Thus, whereas all anionic phospholipids induced the same level of dissociation, oxidase activation by [Rac-RhoGDI] complexes was more pronounced with membranes enriched in PA or PG and of lesser intensity, with membranes enriched in PS or PI. On the other hand, oxidase activation in membranes enriched in PS or PI was characterized by the superiority of complexes containing Rac(GMPPNP) whereas the oxidase in membranes enriched in PA or PG was activated almost equally by complexes comprising Rac(GMPPNP) or Rac(GDP). A possible explanation for this is that PS and PI might act exclusively by causing the dissociation of Rac from RhoGDI whereas PA and PG have an additional effect, down-stream to the dissociation of the [Rac-RhoGDI] complexes. The nature of the post-dissociation event is unknown but it is conceivable that anionic phospholipids provide a micro-environment for gp91phox in which the catalytic performance of gp91phox varies with the nature of the phospholipid. This hypothesis is supported by the findings by the Jesaitis group that anionic phospholipids induce a conformational change in cytochrome b559 (96). It is possible that a more pronounced direct effect of PA and PG on cytochrome b559 is explained by the smaller size of the polar head of these phospholipids (97).

Earlier descriptions of the activation of the oxidase by [Rac-RhoGDI] complexes indicated a requirement for the presence of a soluble anionic amphiphile, serving as activator (29, 50, 64) and it was generally assumed that the role of the amphiphile was to dissociate Rac from RhoGDI. Experimental evidence for complex dissociation by what was grouped under the general term of "biologically active lipids" was presented; all active compounds were found to be negatively charged (51). The activation system described here is the first not to require a soluble anionic activator. Instead, the anionic phospholipid responsible for the dissociation of the complex is a
structural component of the membrane. For such a model to have an in vivo equivalent, we have to hypothesize that: (a) the anionic phospholipid is to be exposed on the cytosolic aspect of the plasma membrane; (b) the [Rac-RhoGDI] complex must make contact with such regions, and (c) a mechanism must exist for the rapid generation of anionic phospholipid domains and this must be connected to a signal transduction pathway starting at membrane receptors participating in oxidase activation.

A role for membrane-localized anionic phospholipids in oxidase activation in vitro was recently described by two groups (98, 99) and by us3. In all cases, it was found that membrane enrichment with anionic phospholipids enabled oxidase activation in the absence of a soluble anionic amphiphile. One example of rapid generation of anionic phospholipids in the membrane is the formation of phosphatidylinositol (3,4,5)-trisphosphate (PtdIns(3,4,5)P3) from the constitutive membrane component phosphatidylinositol (4,5)-diphosphate by type I phosphoinositide 3-kinase, activated by trimeric G protein-coupled receptors (reviewed in Ref. 100). It was, indeed, reported that PtdIns(3,4,5)P3 is generated at the phagosomal cup during phagocytosis (101). Generation of polyphosphoinositides in the cytosolic leaflet of the plasma membrane signifies a major increase in negative charge compared to the resting state, in which phospholipids of modest negative charge (PS, PI) are exposed. Polyphosphoinositides were found to bind the polybasic region of Rac1 and, to a lesser degree, that of Rac2 (92). In addition, PtdIns(3,4,5)P3 affects Rac function by two other pathways: stimulation of several Rac GEFs and a direct GDP-dissociating effect on Rac (reviewed in Ref. 100). It might be significant that the latter two effects could work in synergy with the direct, purely charge-related effect of PtdIns(3,4,5)P3. Yet another transductional pathway, which might lead to an increase in the exposure of anionic phospholipids, is the generation of PA by phospholipase D. This pathway links up with other effects of PA, namely its ability to activate the oxidase in a cell-free system (102) and to bind the polybasic region of Rac1 (92). Obviously, a major future challenge is to investigate the effect of polyphosphoinositides on [Rac-RhoGDI] complexes in vitro and connect the results to events taking place in the intact cell.

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1 The abbreviations used are: \( \text{O}_2^- \), superoxide; RhoGDI, Rho GDP dissociation inhibitor; GEF, guanine nucleotide exchange factor; GAP, GTPase activating protein; GMPPNP, guanylyl-imidodiphosphate; mant-GMPPNP, 2'-(or 3')-O-(N-methylanthraniloyl)-guanylyl-imidodiphosphate; mant-GDP, 2'-(or 3')-O-(N-methylanthraniloyl)-guanosine 5'-diphosphate; PC, L-\( \alpha \)-phosphatidylcholine; PA, L-\( \alpha \)-phosphatidic acid; PG, L-\( \alpha \)-phosphatidyl-DL-glycerol; PI, L-\( \alpha \)-phosphatidylinositol; PS, L-\( \alpha \)-phosphatidyl-L-serine; GGT I, geranylgeranyltransferase type I; FPLC, fast protein liquid chromatography; HPLC, high pressure liquid chromatography; GST, glutathione S-transferase.

2 The size of the RhoGDI monomer is larger than expected because of the presence of a remaining fusion linker in recombinant RhoGDI, prepared by thrombin digestion of the RhoGDI-GST fusion protein.

3 Mizrahi, A., Berdichevsky, Y., Ugolev, Y., Molshanski-Mor, S., and Pick, E., manuscript in preparation.

FIGURE LEGENDS

FIGURE 1. Isolation of the [Rac1(GDP)-RhoGDI] complex by anion exchange chromatography on Mono Q. The [Rac1(GDP)-RhoGDI] complex was generated from 10 nmol of prenylated Rac1(GDP) and 40 nmol RhoGDI and purified on a Mono Q FPLC column, as described under "Experimental Procedures". A, absorbance at 280 nm profile in relation to the conductivity gradient curve and elution volume (ml). B, SDS-PAGE analysis of column fractions (0.5 ml per fraction). 20 \( \mu \)l amounts of each fraction eluting in the conductivity range of 9-20 mS/cm were applied per lane. The figure illustrates the results of one representative experiment, out of more than 30 experiments performed under identical conditions.

FIGURE 2. Dissociation of [Rac1(mant-GDP)-RhoGDI] complex by anionic liposomes. 1 nmol of \textit{in vitro} generated [Rac1(mant-GDP)-RhoGDI] complex was mixed with buffer (A); or with 500 nmol of total phospholipid in the form of liposomes, consisting of 100% phosphatidylcholine (B); 66.6% phosphatidylycerine and 33.3% phosphatidylserine (C); 66.6% phosphatidylcholine and 33.3% phosphatidylglycerol (D); 66.6% phosphatidylcholine and 33.3% phosphatidylglycerol (E), or 66.6% phosphatidylcholine and 33.3% phosphatidic acid (F). In all experiments the [Rac1(mant-GDP)-RhoGDI] complex was preincubated with phospholipid liposomes for 2 min at room temperature. The mixtures were subjected to gel filtration on a Superose 12 FPLC column and the eluates were monitored in line for the fluorescent signal of Rac1(mant-GDP), as described under "Experimental Procedures". In each \textit{panel}, \textit{peak 1} represents Rac1(mant-GDP) dissociated from the [Rac1(mant-GDP)-RhoGDI] complex and bound to the liposomes eluting in the exclusion volume. \textit{Peak 2} represents the non-dissociated [Rac1(mant-GDP)-RhoGDI] complex. The numbers next to \textit{peaks 1} and \textit{2} represent the amount of Rac1(mant-GDP) dissociated from the complex and left in the complex, respectively, expressed as percent of the total amount of Rac1(mant-GDP) present in the complex injected into the column. Calculations were based on the integration of the relevant peaks. The \textit{panels} illustrate representative individual experiments out of at least 3 experiments performed for each combination of components.

FIGURE 3. Assessment of liposome-induced dissociation of [Rac-RhoGDI] complexes by gel filtration - Analysis of fractions by SDS-PAGE and immunoblotting. 1 nmol of [Rac1(mant-GDP)-RhoGDI] complex, generated \textit{in vitro}, was mixed with 750 nmol of phospholipid liposomes, consisting
of 66.6% phosphatidylcholine and 33.3% phosphatidylglycerol, and incubated for 2 min at room temperature. Following incubation, the mixture was subjected to gel filtration on a Superose 12 FPLC column and the eluate was monitored in line for the fluorescent signal of Rac1(mant-GDP) (upper panel).

For a description of what peaks 1 and 2 and the % values next to the peaks, in this panel, represent, see the legend of Fig. 2. 35 µl of each fraction (0.6ml) corresponding to peak 1 (fractions 12 to 15) were subjected to SDS-PAGE followed by immunoblotting with anti-Rac1 and anti-RhoGDI antibodies, as described under “Experimental Procedures” (2nd and 3rd panels from the top). [Rac1-RhoGDI] complex, applied to the last lane at the right, served as a control for the ability of the two antibodies to detect Rac1 and RhoGDI, respectively. 35 µl of each fraction (0.6ml) corresponding to peak 2 (fractions 21 to 24) were analyzed by SDS-PAGE (lower panel).

B, 2 nmol of RhoGDI was mixed with 750 nmol of phospholipid liposomes, consisting of 66.6% phosphatidylcholine and 33.3% phosphatidylglycerol and incubated for 2 min at room temperature. Following incubation, the mixture was subjected to gel filtration on a Superose 12 FPLC column and the eluate was monitored for absorbance at 280 nm (upper panel).

Peak 1 represents the liposomes eluted in the exclusion volume. Peak 2 consists of free RhoGDI and represents a homodimer of RhoGDI. 35 µl of each fraction (0.6ml) corresponding to peak 1 (fractions 10 to 15), were subjected to SDS-PAGE followed by immunoblotting with anti-RhoGDI antibody, as described under “Experimental Procedures” (middle panel). [Rac1-RhoGDI] complex, applied to the first lane at the left, served as a control for the ability of anti-RhoGDI antibody to detect RhoGDI. 35 µl of each fraction (0.6ml) corresponding to peak 2 (fractions 20 to 26) were analyzed by SDS-PAGE (lower panel). The figure illustrates representative individual experiments, out of 2 performed with [Rac1(GDP)-RhoGDI] complexes mixed with liposomes, and 2 performed with free RhoGDI mixed with liposomes.

**FIGURE 4. Quantitative parameters of the dissociation of [Rac1(mant-GDP)-RhoGDI] complexes by anionic liposomes.**

A, dose dependence of dissociation on the proportion of anionic phospholipid. 1 nmol of [Rac1(mant-GDP)-RhoGDI] complex was mixed with 500 nmol of phospholipid liposomes containing either no anionic phospholipid (100% phosphatidylcholine) or 16, 33, 66 and 100% of the anionic phospholipid, phosphatidylglycerol. Following incubation for 2 min at room temperature, the mixtures were subjected to gel filtration on a Superose 12 FPLC column. The dissociation of the [Rac1(mant-GDP)-RhoGDI] complex is expressed as % of liposome-bound Rac1(mant-GDP) out of the total Rac1(mant-GDP) present initially in the [Rac1(mant-GDP)-RhoGDI] complex injected into the column. The data were plotted from means ± S.E. of 3 experiments for each concentration of phosphatidylglycerol.

B, time dependence of dissociation. 1 nmol of [Rac1(mant-GDP)-RhoGDI] complex was incubated for 2 min at room temperature or 2 min at room temperature and subsequently 3 h, at 4 °C, with 300 nmol of phospholipid liposomes consisting of 66.6% phosphatidylcholine and 33.3% phosphatidylserine and subjected to gel filtration on a Superose 12 FPLC column. For a description of what peaks 1 and 2 and the % values next to the peaks, in these panels, represent, see the legend of Fig. 2. Results are those of a single experiment representative of 2 or 3 performed for each time interval.

**FIGURE 5. Isolation of the [Rac1(mant-GMPPNP)-RhoGDI] complex by anion exchange chromatography on Mono Q.** The [Rac1(GMPPNP)-RhoGDI] complex was generated from 10 nmol of prenylated Rac1(GMPPNP) and 40 nmol RhoGDI and purified on a Mono Q FPLC column, as described under "Experimental Procedures". A, absorbance at 280 nm profile in relation to the conductivity gradient curve and elution volume (ml). B, SDS-PAGE analysis of column fractions (0.5 ml). 20 µl amounts of each fraction eluting in the conductivity range of 9-20 mS/cm were applied per lane. The figure illustrates the results of one representative experiment, out of more than 20 experiments performed under identical conditions.

**FIGURE 6. [Rac1(mant-GMPPNP)-RhoGDI] complexes are more susceptible to dissociation by anionic liposomes than [Rac1(mant-GDP)-RhoGDI] complexes.** 1 nmol of [Rac1(mant-GDP)-RhoGDI], generated in vitro, was mixed with liposomes consisting of 300 nmol of phosphatidylcholine (A) or of a mixture of 66.6% phosphatidylcholine and 33.3% phosphatidylserine, amounting to a total of
300 nmol phospholipid (B). For comparison, 1 nmol of [Rac1(mant-GMPMP)-RhoGDI] complex, generated in vitro, was mixed with liposomes consisting of 300 nmol of phosphatidylcholine (C) or of a mixture of 66.6% phosphatidylcholine and 33.3% phosphatidylserine, amounting to a total of 300 nmol phospholipid (D). Following incubation for 2 min at room temperature, the mixtures were subjected to gel filtration on a Superose 12 FPLC column and in line recording of fluorescence. In each panel, peak 1 represents fluorescently labeled Rac1 dissociated from the [Rac1-RhoGDI] complex and bound to phospholipid liposomes. Peak 2 represents non-dissociated [Rac1(mant-GDP)-RhoGDI] or [Rac1(mant-GMPPNP)-RhoGDI] complexes. For a description of what the % values next to the peaks represent, see the legend of Fig. 2. The panels illustrate representative individual experiments out of 2 or 3 performed for each combination of components.

FIGURE 7. [Rac2(mant-GDP)-RhoGDI] complexes are less susceptible to dissociation by anionic liposomes than [Rac1(mant-GDP)-RhoGDI] complexes. 1 nmol of [Rac1(mant-GDP)-RhoGDI] or [Rac2(mant-GDP)-RhoGDI] complex, generated in vitro, was mixed with 500 nmol phospholipid liposomes, consisting of 100% phosphatidylcholine (A and B), or with a mixture of 66.6% phosphatidylcholine and 33.3% phosphatidylglycerol, amounting to a total of 500 nmol phospholipid (C and D), or with a mixture of 33.3% phosphatidylcholine and 66.6% phosphatidylglycerol, amounting to a total of 500 nmol phospholipid (E and F). Following incubation for 2 min at room temperature, the mixtures were subjected to gel filtration on a Superose 12 FPLC column and in line recording of fluorescence, as described under "Experimental Procedures". For a description of what peaks 1 and 2 and the % values next to the peaks, represent, see the legend of Fig. 2 (however note that panels A, C, and E refer to complexes of RhoGDI with Rac1, whereas panels B, D, and F refer to complexes of RhoGDI with Rac2). The panels illustrate representative individual experiments out of 2 or 3 performed for each combination of components.

FIGURE 8. Dissociation of [Rac1-RhoGDI] complexes by native membrane derived from resting phagocytes. Liposomes prepared from solubilized membranes of resting macrophages, in an amount corresponding to 500 nmol of intrinsic membrane phospholipid, were mixed with 1 nmol of [Rac1(mant-GDP)-RhoGDI] complex (A) or with 1 nmol of [Rac1(mant-GMPMP)-RhoGDI] complex (B). Following incubation for 2 min at room temperature, the mixtures were subjected to gel filtration on a Superose 12 FPLC column and in line recording of fluorescence. In each panel, peak 1 represents fluorescently labeled Rac1 dissociated from the [Rac1-RhoGDI] complex and bound to the membrane liposomes, and peak 2 represents non-dissociated [Rac1(mant-GDP)-RhoGDI] or [Rac1(mant-GMPPNP)-RhoGDI] complexes. For a description of what the % values next to the peaks represent, see the legend of Fig. 2. The panels illustrate single experiments for each combination of membrane liposomes and [Rac1-RhoGDI] complexes.

FIGURE 9. Amphiphile-independent activation of NADPH oxidase in native phagocyte membranes or membranes enriched in anionic phospholipids by [Rac1(GDP)-RhoGDI] and [Rac1(GMPPNP)-RhoGDI] complexes, in the absence of amphiphile. In these experiments, macrophage membrane liposomes were enriched with either neutral or anionic phospholipids. Thus, native (not supplemented) phagocyte membranes (A) or phagocyte membranes supplemented with the neutral phospholipid phosphatidylcholine (B), or with the anionic phospholipids phosphatidylinositol (C), phosphatidylserine (D), phosphatidylglycerol (E), or phosphatidic acid (F), were prepared. NADPH oxidase activation was assayed in a cell-free system consisting of either native or supplemented phagocyte membranes (equivalent to 5 nM cytochrome b559 heme), [Rac1(GDP)-RhoGDI] or [Rac1(GMPPNP)-RhoGDI] complexes (200 nM), and p67phox (300 nM), supplemented or not with 47phox (300 nM). The assay mixtures were incubated for 90 s at room temperature, in the absence of an anionic amphiphile, and O2· production was initiated by the addition of NADPH (240 μM). The results are means ± S.E. of three experiments.
TABLE ONE

Both hydrophobic and electrostatic interactions contribute to binding of prenylated Rac1 to anionic phospholipid liposomes

1 nmol of prenylated Rac1(mant-GDP) or non-prenylated Rac1(mant-GDP) was mixed with 500 nmol of either neutral or negatively charged phospholipid liposomes, preincubated for 2 min at room temperature and subjected to gel filtration on a Superose 12 FPLC column, as described under "Experimental Procedures". Results represent means ± S.E. of three experiments for each combination of Rac1 and phospholipid liposomes.

| Composition of phospholipid liposomes | % of acidic phospholipid | % of Rac bound to phospholipid liposomes |
|--------------------------------------|---------------------------|------------------------------------------|
|                                      |                           | Non-prenylated Rac1(mant-GDP) | Prenylated Rac1(mant-GDP) |
| Phosphatidylcholine                  | 0                         | 0                           | 18.33 ± 1.56              |
| Phosphatidylserine                   | 33                        | 4.60 ± 1.14                  | 62.00 ± 2.65              |
| Phosphatidylinositol                 | 33                        | 1.34 ± 0.20                  | 57.00 ± 3.51              |
| Phosphatidylglycerol                 | 33                        | 1.07 ± 0.07                  | 66.33 ± 2.60              |
| Phosphatidic acid                    | 33                        | 1.43 ± 0.09                  | 59.00 ± 4.93              |
TABLE TWO

Chromatographic evidence for higher stability of [Rac1(GDP)-RhoGDI] versus [Rac1(GMPPNP)-RhoGDI] complexes

[Rac1-RhoGDI] complexes were generated *in vitro* by mixing 10 nmol of prenylated Rac1 with 20 nmol of RhoGDI. The complexes were purified by anion exchange chromatography, as described under “Experimental Procedures” and illustrated in Figs. 1A and 5A. Integrated peak areas of [Rac1-RhoGDI] complexes and of excess free RhoGDI, based on absorbance at 280 nm, were measured. Results represent means ± S.E. of three experiments.

|                     | Peak area comparison ( mAUI*ml) |
|---------------------|---------------------------------|
|                     | [Rac1(GDP)-RhoGDI] complex      | [Rac1(GMPPNP)-RhoGDI] complex |
|                     | Excess free RhoGDI              | Excess free RhoGDI             |
| Prenylated Rac1(GDP) + RhoGDI | 94.67 ± 4.58                   | 60.96 ± 7.75                  |
| Prenylated Rac1(GMPPNP) + RhoGDI | 37.78 ± 8.77                   | 62.29 ± 4.23                  |
Figure 1

A

Free RhoGDI

Absorbance at 280 nm (mAU)

Conductivity (mS/cm)

Elution volume (ml)

[Rac1(GDP)-RhoGDI]

B

kDa

45

31

21.5

14.4

Fraction number

RhoGDI

Rac1
Figure 2

A

B

C

D

E

F

Relative fluorescence

Elution volume (ml)
Figure 4

A

% of dissociation

% of anionic phospholipid (PG) in PC/PG liposomes

B

2 minutes

3 hours

Relative fluorescence

Elution volume (ml)
Figure 6

A

B

C

D

Elution volume (ml)

Relative fluorescence

1 3%

2 97%

1 33%

2 66%

1 96.7%

2 93%

1 93%

2 7%
Figure 8

A

B

Relative fluorescence

Elution volume (ml)

1 8.9%

2 91.1%

1 74%

2 26%
Figure 9

A. Membrane

B. Membrane + PC

C. Membrane + PI

D. Membrane + PS

E. Membrane + PG

F. Membrane + PA

With p47phox

Without p47phox

1. [Rac1(GDP)-RhoGDI]

2. [Rac1(GMPPNP)-RhoGDI]

NADPH oxidase activity (mol O₂/s/mol cytochrome b₅₅₉ heme)
Liposomes comprising anionic but not neutral phospholipids cause dissociation of [Rac(1 or 2)-RhoGDI] complexes and support amphiphile-independent NADPH oxidase activation by such complexes

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