Mechanism of NADPH Oxidase Activation by the Rac/Rho-GDI Complex†

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ABSTRACT: The low molecular weight GTP binding protein Rac is essential to the activation of the NADPH oxidase complex, involved in pathogen killing during phagocytosis. In resting cells, Rac exists as a heterodimeric complex with Rho GDP dissociation inhibitor (Rho-GDI). Two types of interactions exist between Rac and Rho-GDI: a protein−lipid interaction, implicating the polyisoprene of the GTPase, as well as protein−protein interactions. Using the two-hybrid system, we show that nonprenylated Rac1 interacts very weakly with Rho-GDI, pointing to the predominant role of protein−isoprene interaction in complex formation. In the absence of this strong interaction, we demonstrate that three sites of protein−protein interaction, $Arg66_{Rac}$-$Leu67_{Rac}$, $His103_{Rac}$, and the C-terminal polybasic region $Arg183_{Rac}$−$Lys188_{Rac}$, are involved and cooperate in complex formation. When Rac1 mutants are prenylated by expression in insect cells, they all interact with Rho-GDI. Rho-GDI is able to exert an inhibitory effect on the GDP/GTP exchange reaction except in the complex in which Rac1 has a deletion of the polybasic region ($Arg183_{Rac}$−$Lys188_{Rac}$). This complex is, most likely, held together through protein−lipid interaction only. Although able to function as GTPases, the mutants of Rac1 that failed to interact with Rho-GDI also failed to activate the NADPH oxidase in a cell-free assay after loading with GTP. Mutant $Leu119_{Rac}$-Gln could both interact with Rho-GDI and activate the NADPH oxidase. The Rac1/Rho-GDI and Rac1-(Leu119Gln)/Rho-GDI complexes, in which the GTPases were bound to GDP, were found to activate the oxidase efficiently. These data suggest that Rho-GDI stabilizes Rac in an active conformation, even in the GDP-bound state, and presents it to its effector, the p67phox component of the NADPH oxidase.

Professional phagocytes, monocytes, macrophages, and circulating neutrophils, play a key role in the defense against invading microorganisms. They recognize foreign particles and ingest them in a phagocytic vacuole, the phagosome. This process is greatly facilitated when the invading particles are opsonized upon cooperation with the specific immune system. In the phagosome two processes take place that generally result in destruction of the pathogen: degranulation of proteases and bactericidal proteins and production of reactive oxygen species by the NADPH oxidase complex (for review, see refs *1* and *2*). The NADPH oxidase is activated at the onset of phagocytosis and located to the phagosomal membrane. It produces superoxide anions, precursors of highly toxic hydroxyl radicals, and hypochlorite.

The NADPH oxidase complex comprises several Phox[1] proteins (for review, see ref *2*). The gp91phox and p22phox constitute with heme and flavin cofactors cytochrome b_{558} responsible for electron transfer from NADPH to oxygen. Two components of cytosolic origin p67phox and p47phox associate with the membrane-bound cytochrome b_{558} during activation of the enzyme. Defects in any of these Phox proteins result in a severe disease, chronic granulomatous disease (CGD), characterized by an increased susceptibility to infection. In addition to the Phox proteins, a low molecular weight GTP binding protein, Rac, in its GTP-bound form, is a minimal requirement to reconstitute an active NADPH oxidase in a cell-free activation system (*3*). In this system, an anionic amphiphile such as arachidonic acid, sodium dodecyl sulfate, or phosphatidic acid is also required. The effect of Rac on superoxide production is likely to be exerted

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[1] Abbreviations: Phox, phagocyte oxidase; ONPG, *o*-nitrophenyl β-D-galactopyranoside; X-Gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; PBS, phosphate-buffered saline; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; DTT, dithiothreitol; Rho-GDI, Rho guanine−nucleotide dissociation inhibitor; BSA, bovine serum albumin; GAP, GTPase-activating protein; BCR, breakpoint cluster region; GST, glutathione *S*-transferase; GEF, guanine−nucleotide exchange factor; TPR, tetratricopeptide repeat.

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through its GTP-dependent binding to the cytosolic factor p67phox (*4*).

Evidence for a participation of the Rho family GTPase Rac in the NADPH oxidase complex stems from the observation that antisense oligonucleotides directed against Rac1 inhibited superoxide production in B lymphocytes, a cell line with minimal NADPH oxidase activity (*5*). Several effector regions of Rac were shown to be involved in NADPH oxidase activation. First, replacement of amino acids 27 and 30 in CDC42 by their Rac counterparts rendered CDC42 able to activate the NADPH oxidase (*6*). Second, a series of point mutations in the effector loop of Rac1 (residues 26−45) abolished both NADPH oxidase activation and interaction with p67phox (*7*, *8*). Third, using Rac/Rho chimera, a second effector domain localized between amino acids 143 and 175 of Rac was found to be required for NADPH oxidase activation (*9*). Fourth, in a systematic peptide walking approach, peptides spanning the entire Rac1 sequence were tested in a cell-free activation system for their ability to inhibit the NADPH oxidase and five regions termed a−e, were defined (*10*). Critical amino acids in regions b and d were identified as H103 and K166 (*11*). Domain c overlaps with the insert region, an helical motif present in Rho GTPases and not in the Ras prototype of the family. This insert region was found to bind phosphoinositides (*12*) through its charged amino acids and may thereby be important for membrane localization of Rac1. It was also shown to control mitogenesis through superoxide production (*13*). As to domain e, it corresponds to a polybasic region of six amino acids that differs between Rac1 and Rac2 and renders Rac1 more effective in NADPH oxidase activation (*14*). Purification of an oxidase activating factor from the cytosol of macrophages or neutrophils yielded a fraction containing the Rac protein (Rac1 or Rac2) associated with Rho GDP dissociation inhibitor (Rho-GDI) (*15*−*18*). Rho-GDI is known to inhibit small GTPases of the Rho family by blocking dissociation of GDP and subsequent loading with GTP of their active site (for review, see ref *19*). Small G proteins of the Rho family are believed to exert their effects by a GTP-dependent activation of effector proteins after dissociation from Rho-GDI. However, the isolated Rac/Rho-GDI complexes containing Rac bound to GDP (*20*) were fully efficient in NADPH oxidase activation. Interactions between Rho GTPases and Rho-GDI have been recently confirmed by structural data (*21*−*23*). The GTPase is modified on its C-terminal CAAX sequence by addition of a geranylgeranyl moiety (for review, see ref *24*) that inserts into a hydrophobic pocket in the C-terminal core of Rho-GDI. Besides this lipid−protein interaction, protein−protein interactions are likely to be required for the specificity of recognition. Moreover, interaction of the N-terminus of Rho-GDI with Thr35 of the GTPase is responsible for GDP/GTP exchange inhibition. We have recently demonstrated the occurrence of an open complex between RhoA and Rho-GDI in which the lipid−protein interaction was released but the two proteins were still held together by protein−protein interactions (*25*). We have confirmed the strong protein−protein interactions between RhoA and Rho-GDI using CAAX box mutants that are no longer prenylated (*26*).

To better understand the mechanism of NADPH oxidase activation by the Rac/Rho-GDI complex, we produced previously characterized (*11*) as well as new mutants of Rac1, prenylated or not, and studied both their interactions with Rho-GDI and their ability to activate the NADPH oxidase in a cell-free system. We demonstrate here the predominant role of protein−lipid over protein−protein interaction in the affinity of the Rac1/Rho-GDI complex. We confirm the implication of several protein−protein interaction sites predicted by structural analysis, Arg66 $_{rac}$-Leu67 $_{rac}$, His103$_{rac}$, and the C-terminal polybasic region, in complex formation with Rho-GDI. Mutations affecting these residues result in Rac proteins devoid of their capacity to activate the NADPH oxidase. The Rac/Rho-GDI complex in which Rac was GDP bound was found to activate the oxidase nearly as efficiently as Rac-GTP. These data are discussed in light of the structure of the Rac1/Rho-GDI complex (*23*) and the p67phox/Rac1 complex (*27*).

EXPERIMENTAL PROCEDURES

Cloning and Mutagenesis. Rac1 cDNA was a gift from Prof. A. Hall, University College, London. It presented the polymorphism Phe78Ser previously noted (*28*) compared to the published sequence (*29*). The deltaI (previously deltaC, deletion of peptide walking region c), H103A, and K166E mutants of Rac1 (*11*) were tranferred in pBacPAKHis1 (Clontech) vector using the *Bam*HI and *Eco*RI sites of pGEX-2T. Other mutants of Rac1 were made by sequential PCR mutagenesis (*30*) introducing *Bam*HI and *Eco*RI sites at the extremities of the cDNA. The mutants were cloned in pBacPAKHis1 using classical protocols. The latter vector allows expression of the proteins with a short N-terminal His_6 tag. The correct sequence was checked. For two-hybrid analysis, to study the protein−protein interactions only, the CAAX box was mutated by changing Cys189 to Gly, thus impairing prenylation, and an *Xba*I site was introduced in the PCR reverse primer. The PCR product was digested by *Bam*HI and *Xba*I and ligated into the pGAD3S2X plasmid (a gift from Dr. J. Camonis, Institut Curie, Paris). The two-hybrid test was performed as described (*31*). Interaction was monitored by prototrophy for histidine on selective medium, by a filter β-galactosidase test (*32*), and by quantitative assay of β-galactosidase activity using ONPG as substrate (*26*).

Baculoviruses Production and Amplification. The Baculogold kit (Pharmingen) was used to generate recombinant baculoviruses using the manufacturer's protocol on monolayers of Sf9 insect cells (Clontech). After 5 days, the cell supernatant was harvested and used to infect fresh cells in successive amplification cycles of 5 days. The viral stocks recovered after four cycles of amplification were found to produce high yields of proteins and were used for protein production. The absence of wild-type baculoviruses in the initial stocks was assessed by the absence of a blue coloration in the presence of X-Gal and the absence of polyhedrin by visual inspection of the cells.

Protein Expression and Purification. For expression of prenylated Rac proteins, Sf9 cells were resuspended in fresh medium at a concentration of 2×10^6/mL and infected with 0.2 mL of viral stock/mL of cells. For production of Rac/Rho-GDI complexes, co-infection was performed using 0.2 mL of Rac virus stock and 0.2 mL of His-Rho-GDI virus stock (*25*). These conditions were found to result in expression of an excess of Rac compared to Rho-GDI. After 3 days, cells were harvested by centrifugation at 10000*g* for 15 min.

This relatively high speed was used to spin down not only the cells but also membrane particles that were rich in Rac proteins. The cell pellet was washed with PBS, pH 7.4, consisting of 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, and 1.5 mM KH_2PO_4, and frozen at −20 °C until use. For protein purification the pellet was suspended in 5 volumes of buffer A (20 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 100 μM DTT) supplemented with Complete without EDTA (Roche) as protease inhibitor cocktail. The cells were disrupted by sonication at 40 W for 2 min with 1 s bursts. A centrifugation at 5000*g* for 10 min was used to remove unbroken cells and cell debris. The supernatant was brought to final 17% glycerol and 0.4 M NaCl concentrations in buffer A (buffer B). The resulting homogenate was centrifuged at 200000*g* for 45 min. The high-speed supernatant was used as a source of Rac/Rho-GDI complexes after coinfection with two baculoviruses. The membrane pellet was a source of prenylated Rac (*33*). It was extracted by 1% CHAPS with short bursts of sonication, and the extract was recovered after a 15 min centrifugation at 300000*g*.

Both the soluble proteins and the membrane-bound Rac proteins were purified by affinity on nickel beads (Probond, Invitrogen) taking advantage of the N-terminal His_6 tag. The fractions were incubated for 1 h at 4 °C with 1 mL of Nickel beads with gentle rotation. The beads were then loaded into an FPLC column HR 5/5 (Pharmacia) and washed with buffer B. His-tagged proteins were then eluted with 200 mM imidazole in buffer B. In the case of the membrane extract, all buffers were supplemented with 1% CHAPS. The purified proteins were about 95% pure as determined by Coomassie blue staining of the eluates. Since both Rac proteins and Rho-GDI proteins bear a His tag, separation of the Rac/Rho-GDI complexes from the isolated proteins was required. This was done by gel filtration chromatography on a Superdex 75 column (HR 10/10, Pharmacia) by loading 0.5 mL of the nickel bead eluates to the column, equilibrated in PBS, 5 mM $MgCl_2$, and 100 μM DTT. The Rac/Rho-GDI complexes were eluted first with an apparent molecular mass of 50−60 kDa. An excess of Rac protein was recovered with an apparent molecular mass of 20−25 kDa. Both fractions, namely, the Rac/Rho-GDI complexes and the isolated Rac proteins, were used in functional assays described below. Wild-type Rac1 and Rac1 mutants were processed the same way.

GDP/GTP Exchange and Hydrolysis by the Rac Mutants and the Rac/Rho-GDI Complexes. The prenylated Rac proteins were tested in both GDP/GTP exchange reaction (*34*) using [γ-^{35}S]GTP and GTPase activity using [γ-^{32}P]-GTP. Since both tests were in good agreement concerning the binding capacity, we later used only [γ-^{32}P]GTP for the functional assay of the Rac/Rho-GDI complexes and the Rac proteins isolated from the cytosol. In a first step, the exchange reaction, was initiated by addition of 10 mM EDTA to the Rac sample in binding buffer (20 mM Tris-HCl, pH 7.5, 25 mM NaCl, 0.1 mM DTT) containing 10 μM GTP (a ratio of GTP/Rac >10) and 2 μCi of [γ-^{32}P]GTP (6000 Ci/mmol). After 5 min of incubation at 30 °C, GTP hydrolysis was initiated by adding $MgCl_2$ to 20 mM and an excess of BSA (0.7 mg/mL final concentration). The hydrolysis reaction was followed for 15 min at 30 °C by filtration of aliquots on nitrocellulose filters (Millipore HA 0.45 μM). The filters were washed five times with 50 mM Tris-HCl, pH 7.5, and 5 mM $MgCl_2$, and the bound radioactivity was measured by liquid scintillation counting. The amount of bound GTP was quantified. In some experiments, 1 μg of the GAP domain of BCR was added during GTP hydrolysis. This domain (residues 871−1271) was prepared as GST fusion protein using classical protocols.

Assay of the NADPH Oxidase Activating Potency of the Rac Proteins and the Rac/Rho-GDI Complexes. The NADPH oxidase activating potency was assessed in a semirecombinant cell-free system (*33*). First, Rac proteins and mutants were loaded with GTP-γ-S or GDP-β-S by incubation for 10 min at 25 °C in the presence of 4 mM EDTA (after dilution of the protein leading to a Mg concentration of 2 mM) and 10 μM GTP-γ-S or GDP-β-S. This was followed by addition of $MgCl_2$ to 20 mM in order to stabilize the bound nucleotide (*34*). Second, a membrane fraction from bovine neutrophils was deprived of endogenous Rac protein by washing with 2 M KCl and resuspension at the initial protein concentration (*33*). Oxidase activation was achieved in the microplate by a 10 min preincubation of the membrane fraction (6 μg of protein), 8 pmol of recombinant p47phox (*35*), 200 pmol of a N-terminal fragment of p67phox, and 1−10 pmol of Rac proteins or 4−20 pmol of Rac combined with Rho-GDI. The $MgCl_2$ concentration ranged from 0 to 2 mM according to the volume of the Rac sample. In the case of the Rac/Rho-GDI complexes, it was 2 mM, and there was no addition of nucleotide to the preincubation medium. The volume was brought to 200 μL with PBS, and an optimal amount of arachidonic acid (10−20 nmol) was added with strong agitation. After this activation step, the elicited oxidase activity was assessed using the superoxide dismutase inhibitable cytochrome *c* reduction, followed at 550 nm using a Labsystem IEMS microplate reader. In brief, 25 μL of an assay medium containing 1 mM cytochrome C and 2 mM NADPH was added to the wells of the plate, and the rate of superoxide production was recorded at 550 nm for 2 min.

RESULTS

Mutagenesis of Rac1. The mutants used in the present study are described in Table 1. The choice of some mutants was dictated by the followup of the peptide walking analysis (*10*). In this experiment, peptides spanning the Rac sequence were found to be inhibitory in a cell-free system containing the Rac1/Rho-GDI complex as a source of Rac protein. The mutants corresponding to domains b and d (H103A and K166E, respectively) defined by peptide walking were used. Domain c corresponding to the Rho insert region (residues 124−135), which is characteristic of the Rho subfamily of GTPases, was deleted in delta insert mutant. The polybasic region at the C-terminus corresponding to domain e was deleted without removing the CAAX box responsible for prenylation. This mutant was termed deltaKR. Our findings on the RhoA/Rho-GDI complex (*26*) were also used. First, on RhoA, we had found that mutation of conserved $L121_{RhoA}$ to $P121_{RhoA}$ suppressed interaction with Rho-GDI tested in the two-hybrid system. In fact, this mutation was deleterious to RhoA stability, and the role of this residue could not be interpreted; a corresponding less drastic mutation L119Q was made on Rac1. Second, the RL66/67 motif conserved within the Rho subfamily but not present in RhoH, a member of the Rho family found to interact only weakly with Rho-GDI, was mutated to SI (corresponding sequence in RhoH).

Table 1: Description of the Rac1 Mutants Used in the Two-Hybrid Assay[a]

description	name	reference	localization of the mutation in the structural domains	name in the peptide walking nomenclature
Rac1 wild-type Phe78Ser polymorphism	Rac1	Hirschberg et al. (*28*)		
Rac1 Cys189Gly	Rac1Gly		CAAX box	
Rac1 Gly189 His103Ala	H103A	Toporik et al. (*11*)	A3 helix	domain b
Rac1 Gly189 Lys166Glu	K166E	Toporik et al. (*11*)		domain d
Rac1 Gly189 delta insert (124−135)	deltaI	Toporik et al. (*11*)	insert region	domain c
Rac1 Gly189 Leu119Gln	L119Q			
Rac1 Gly189 Arg66Ser-Leu67Ile	RL/SI	Fauré and Dagher (*26*)	switch II region	upstream of domain a
Rac1 Gly189Met190 delta polybasic (183−188)	deltaKR	Joseph et al. (*10*), Kreck et al. (*14*)	upstream of the CAAX box	domain e

[a] The corresponding mutants without Gly189 mutation were produced in insect cells.

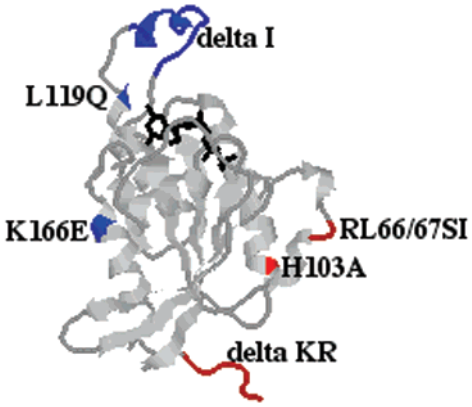

FIGURE 1: Localization of the Rac mutants on the Rac1 structure. The coordinates of the Rac1 protein are derived from the Rac1/Rho-GDI complex [Grizot et al. (*23*), PDB accession number 1hh4]. Mutations that prevent interaction with Rho-GDI are in red. Mutations that do not affect interaction with Rho-GDI are in blue.

Figure 1 shows the localization of the mutations on the Rac1 structure (*23*). For study of protein−protein interactions, the mutants were modified in the CAAX box (C189G), thus impairing prenylation. For expression in insect cells, Rac mutants had an intact CAAX box, therefore being capable of both protein−lipid and protein−protein interactions.

Protein−Protein Interactions in the Rac1/Rho-GDI Complex. To investigate the relative role of protein−lipid and protein−protein interaction sites in a cellular environment, the two-hybrid system in yeast was used. The yeast L40 strain was first transformed with a pLex plasmid coding a fusion between Rho-GDI and the DNA binding protein Lex A. In a second step, a series of yeast transformations were made using the Rac mutants in pGAD3S2X vector, coding fusions with the activation domain of Gal4. Interaction was monitored by prototrophy for histidine and by the appearance of a blue coloration in the presence of X-Gal on a filter-based assay. As shown by the quantitative β-galactosidase test of Figure 2C, prenylated Rac interacts much more strongly with Rho-GDI than unprocessed Rac generated by C189G mutation, pointing to the preponderant role of protein−lipid interaction in complex formation. The protein−protein interactions are rather weak, which explains the failure of our previous attempts to form a stable complex between unprocessed Rac produced in *Escherichia coli* and Rho-GDI (data not shown). The two-hybrid test appears to be sufficiently sensitive to investigate the protein−protein interaction sites. Nonprenylated Rac mutants generated by C189G mutation (see Table 1) were therefore tested for their interaction with Rho-GDI.

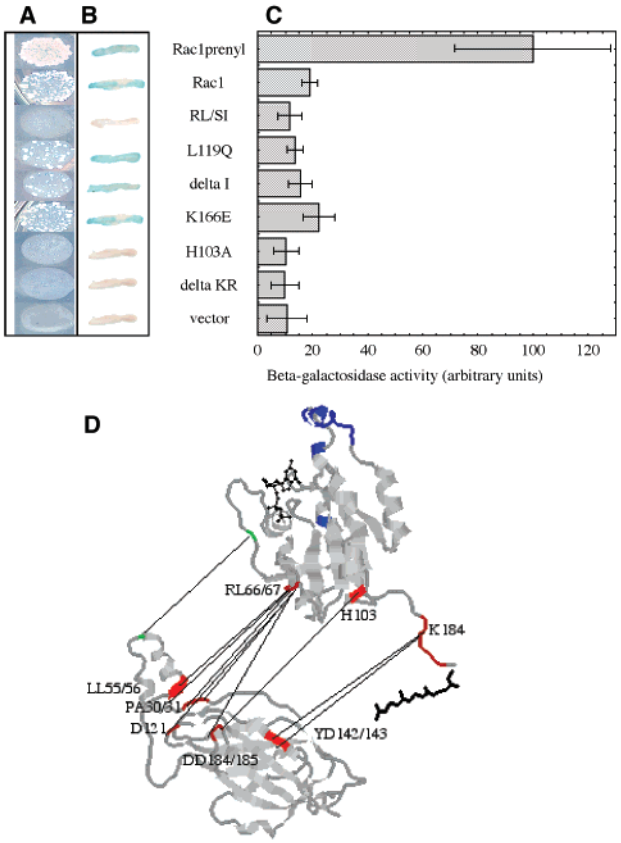

FIGURE 2: Two-hybrid analysis of the protein−protein interactions between Rac1 and Rho-GDI. Prenylated Rac1 and nonprenylated Rac1 mutants were produced from the pGAD3S2X vector together with Rho-GDI from the pLex vector. (A) The interaction was monitored by growth of the L40 strain on selective medium lacking Trp, Leu, and His (the latter being the reporter gene). (B) The second reporter gene LacZ was tested on a filter after 16 h of incubation. (C) Quantitative β-galactosidase activity. Results are given as means ± SD of at least three independent experiments. (D) Interactions of the mutated amino acids of Rac1, H103, RL66/67, and K184 from the polybasic region with Rho-GDI. Residues implicated in GDP/GTP exchange inhibition, $T35_{Rac}$ and $D45_{GDI}$, are in green.

As shown in Figure 2, absence of growth on a medium lacking histidine (2A) and absence of blue coloration (2B) was found with three Rac mutants, the RL66/67SI, the H103A, and the deltaKR mutants. In contrast, deletion of the insert region (deltaI mutant) specific of the Rho family of GTPases or a mutation close to the insert region (L119Q) did not impair interaction between Rho-GDI and Rac. The K166E mutant that was not able to activate the NADPH oxidase interacted normally with Rho-GDI. The three sites

of interaction found in our study, namely, H103, RL66/67, and the polybasic region, are consistent with the structural data on the CDC42/Rho-GDI complex, the Rac2/GDI-2 complex, and the Rac1/Rho-GDI complex (*21*−*23*). They are shown in Figure 2D. In the RL/SI mutant six bonds were suppressed. $Arg66_{Rac}$ makes four contacts with not only the C-terminal core ($Glu121_{GDI}$, $Asp185_{GDI}$) but also the N-terminal region of Rho-GDI ($Pro30_{GDI}$, $Ala31_{GDI}$). $Leu67_{Rac}$ makes hydrophobic contacts with $Leu55_{GDI}$ and $Leu56_{GDI}$. In contrast, $His103_{Rac}$ makes only one hydrogen bond with $Asp184_{GDI}$ in the C-terminal core of Rho-GDI. It is noteworthy that $His103_{Rac}$ is localized in close proximity to the switch II region of Rac1 and may be involved in stabilization of this region (*11*, *23*). The C-terminal polybasic region is poorly defined in the Rac1/Rho-GDI complex structure, but it is likely to behave like the corresponding region of CDC42 (*21*): $Lys184_{CDC42}$ and $Arg186_{CDC42}$ interact with amino acids $Asp143_{GDI}$ and $Tyr142_{GDI}$ and with $Tyr144_{GDI}$ and $Gln130_{GDI}$, respectively, residues located close to the isoprene binding pocket. $Lys184_{Rac}$ is also present in Rac1, and other basic residues, for example, $Arg187_{Rac}$, are likely to interact with Rho-GDI. The mutant proteins allow us to investigate two main sites of interaction, one related to switch II conformation, the second close to the isoprene. Our data also suggest that each individual protein−protein interaction is weak and that they all synergize for the complex formation. In intact cells, however, the presence of the isoprene strengthens the interactions between the two proteins.

Production of Complexes between Prenylated Rac Proteins and Rho-GDI. To check whether protein−lipid and protein−protein interactions synergize for the production of in vivo complexes, we produced the Rac proteins and mutants in a baculovirus/insect cell expression system, with a short N-terminal His_6 tag that could not be cleaved. We have previously shown by metabolic labeling with an isoprene precursor that Rac proteins associated to the membrane (*33*) or RhoA protein associated with Rho-GDI in the cytosol (*25*) were prenylated. Prenylated wild-type or mutant Rac proteins were purified from membranes of insect cells. For in vivo complex production between Rho-GDI and Rac proteins, coexpression of the two His-tagged proteins was done by co-infection with two baculoviruses (*25*). Both proteins were recovered from the cytosol after nickel bead purification. The nickel bead eluate could therefore contain not only Rac/Rho-GDI complexes but also isolated Rac and Rho-GDI proteins. Upon gel filtration chromatography on a Superdex 75 column, a complex containing both proteins was eluted with an apparent molecular mass of 50−60 kDa, followed by an excess of Rac protein (Figure 3). Our conditions for infection of insect cells were such that an excess of Rho-GDI was never found, probably due to a lower level of expression compared to Rac. The same experiment was done for all the mutants of Rac coexpressed with Rho-GDI that repeatedly eluted with an elution volume compatible with a complex. These data together with data from the two-hybrid assay indicate that the protein−lipid interaction makes a large contribution to complex formation, even when some protein−protein interaction sites are mutated, the remaining sites providing the specificity of recognition. They confirm our initial proposal that protein−protein interactions could be studied only in the absence of the stronger protein−lipid interaction.

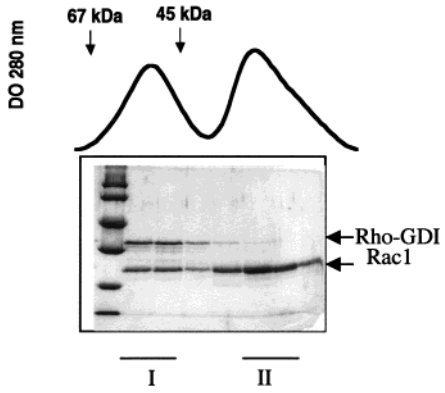

FIGURE 3: Purification of the Rac/Rho-GDI complexes by gel filtration on a Superdex 75 column. The upper part of the figure shows the elution profile of the column. The lower part shows SDS−PAGE on aliquots of the eluted fractions. Rho-GDI has a molecular mass of 33 kDa due to a long N-terminal His tag. Two fractions of elution corresponding to the complex (peak I) and to isolated Rac protein (peak II) were pooled. The profile shown corresponds to the RL66/67SI mutant.

Table 2: Assay of GDP/GTP Exchange and Its Inhibition by Rho-GDI of the Rac1 Mutants[a]

GTPase	GTP binding (mol of GTP/mol of Rac)	complex	inhibition of GDP/GTP exchange (%)
Rac1	0.4	Rac1 + GDI	76
K166E	0.46	K166E + GDI	67
deltaKR	0.5	delta KR + GDI	0
deltaI	0.88	delta I + GDI	84
L119Q	0.86	L119Q + GDI	85
RL/SI	0.79	RL/SI + GDI	51
H103A	0.76	H103A + GDI	82

[a] Optimal [γ-^{32}P]GTP binding capacities of the Rac proteins and the Rac/Rho-GDI complexes used in this study. The values were taken from a representative experiment from three separate experiments.

Assay of GTP Binding and Hydrolysis by Rac Mutants and Rac/Rho-GDI Complexes. The Rac mutants used in this study were expressed at about the same level in insect cells, except the delta insert mutant that showed lower recovery. For control of correct expression, GTP binding and GTPase activity of the proteins were tested. The two tests were performed using [γ-^{32}P]GTP. First, GTP binding was followed for 5 min at 30 °C. Optimal binding was obtained at 2.5 min and ranged from 0.4 to 1 mol of GTP/mol of Rac (Table 2). Assay of GTPase activity was initiated at 2.5 min by addition of an excess of magnesium to stabilize the bound nucleotide. All of the mutants were able to hydrolyze GTP with a $t_{1/2}$ of about 10 min, similarly to wild-type Rac. Moreover, the GTPase activity of all mutants was stimulated by addition of the GTPase-activating domain of BCR (Figure 4). This functional assay also allowed us to assess Rho-GDI function, i.e., inhibition of GDP/GTP exchange. This was determined on the first peak of the gel filtration, which contained the Rac/Rho-GDI complex using as control the isolated Rac protein eluted later in the same column. As shown in Table 2, interaction of Rac with Rho-GDI was confirmed by the inhibition of GDP/GTP exchange observed on the complex. This was observed for all of the mutants except the deltaKR mutant that was found to exchange GDP for GTP at a high rate even when coeluted in a complex with Rho-GDI (Figure 4B). In the deltaKR mutant, suppression of the six amino acid arm between the N-terminal

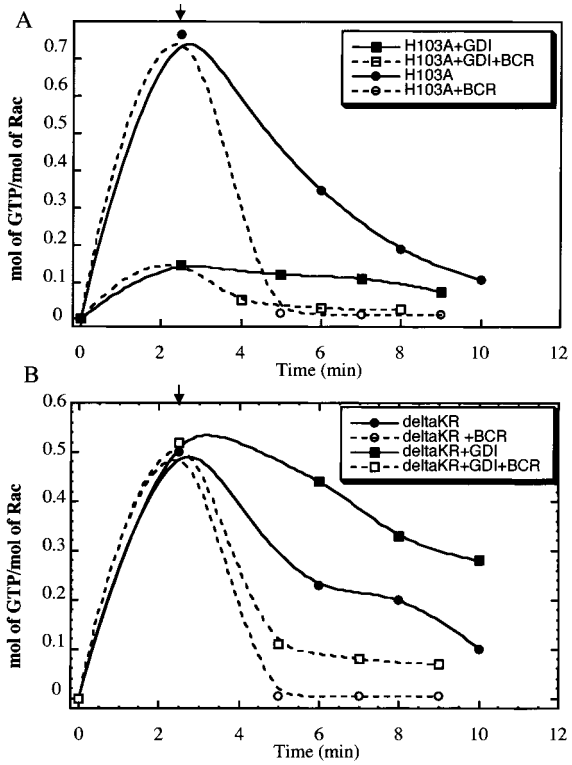

FIGURE 4: Assay of GTP binding and hydrolysis of Rac mutants and Rac/Rho-GDI complexes. (A) Assay of GTP binding and hydrolysis of the Rac1H103A mutant (circles) and the Rac1H103A/Rho-GDI complex (squares) isolated from the two peaks of the gel filtration. At time 0, GDP/GTP exchange was initiated by addition of 10 mM EDTA to 2−5 pmol of Rac protein. At 2.5 min, GTP hydrolysis was initiated by an excess of $MgCl_2$ (indicated by an arrow) and followed for 10 min. A duplicate sample containing the GAP domain of BCR was tested in parallel (dashed lines). Aliquots were taken and Rac proteins were retained on nitrocellulose filters under vacuum. The filters were washed, and radioactivity bound to Rac was measured by liquid scintillation counting. (B) [γ-^{32}P]GTP binding and hydrolysis by the isolated deltaKR protein (circles) and the deltaKR/Rho-GDI complex (squares). Duplicate samples containing the GAP domain of BCR were analyzed (dashed lines).

domain and the C-terminal CAAX box of Rac allows either protein−protein interactions or protein−lipid interaction to occur, but not both. The fact that GDP/GTP exchange is no longer inhibited is in favor of protein−lipid interaction only. Of course, this mutant is not physiological, and the specificity of recognition brought by the protein−protein interaction sites in the cell is replaced in our experiments by the fact that the two proteins are concentrated together during the purification process. When combined with the protein−protein interaction mutants of Rac, RL66/67SI, or H103A, Rho-GDI was able to exert an inhibitory effect, indicating that protein−lipid interaction induces good positioning of the two partner proteins in the complex even in the absence of some protein−protein interactions. In particular, interaction of $Thr35_{Rac}$ with $Asp45_{GDI}$ has to take place for inhibition of GDP/GTP exchange to occur. In the RL66/67SI mutant, however, inhibition of GDP/GTP exchange by Rho-GDI was only 50%, consistent with the loss of multiple stabilizing interactions. In the other cases, inhibition by Rho-GDI was not complete, and about 10−15% of Rac protein was able to exchange its GDP to GTP. This was already observed in the case of the RhoA/Rho-GDI complex (*25*) and fits with a dynamic complex (*36*). The fraction of complexed Rac that could bind GTP also had its GTPase activity stimulated by the GAP domain of BCR (Figure 4A), in favor of an access of this GAP domain to the effector loop (including Thr35) of the GTPase. These data suggest that the nucleotide site is not tightly locked and is moderately accessible in the complexes.

Assay of a Rac Effector, the NADPH Oxidase Complex. We have shown that the Rac mutants were functional, able to shift from the GDP-bound form to the GTP-bound form, and that Rho-GDI inhibited this process in all of the complexes tested except the deltaKR/Rho-GDI complex. The following step was to check whether Rac mutants were active, using the NADPH oxidase complex as an effector of Rac. After being loaded with nucleotides, the Rac mutants were tested for their oxidase activating potency. Several features of our cell-free assay are noteworthy. First, we used a truncated form of p67phox that comprised the N-terminus of the molecule (AA 1−240). We found that the optimal amount of truncated p67phox was about 20 times higher than that of p47phox. Since activation was still dependent on p47phox, we concluded that the recombinant p67phox N-ter was able to interact with p47phox, although with lower affinity than the full-length molecule. Second, Rac proteins were loaded with 10 μM GTP-γ-S after treatment with EDTA. This resulted in a final free GTP-γ-S concentration of less than 1 μM. We found that GTP-γ-S alone at this concentration was able to elicit an oxidase activity of about 135 nmol of O_2^- min^{-1} (mg of membrane protein)$^{-1}$. This background was probably due to the effect of GTP on endogenous Rac protein, which was still present in the membrane fraction after treatment with KCl. Third, the optimal activating potency of the Rac proteins and mutants was found for amounts similar to those of p47phox protein (6−8 pmol). Fourth, an optimal amount of arachidonic acid was determined for each experimental condition. As shown in Figure 5 (white bars), only the wild-type Rac1 and the Rac1 mutant L119Q were found to stimulate the NADPH oxidase, the L119Q mutant showing an EC_{50} 6 times higher than that of Rac1. The H103A, K166E, RL66/67SI, deltaI, and deltaKR mutants displayed only background activating potency. Concerning the deltaI mutant, our data resemble those of Lambeth's group (*7*), who found a large reduction in superoxide generation with a very similar mutant and therefore identified the insert region as important for oxidase activation. On the contrary, deletion of the insert region was found by others not to be deleterious (*11*). The main difference between the two types of results resides in the cell-free system used. When arachidonic acid was used, as in the present case, the deltaI mutant failed to activate the NADPH oxidase whereas it was almost as efficient as wild-type Rac1 in a LiDS-based cell-free activation system.

In a second step, the Rac/Rho-GDI complexes were tested (Figure 5, black bars). As was previously shown (*20*), the Rac1/Rho-GDI complex, in which Rac1 was in the GDP-bound form, displayed a significant activating potency compared to the isolated Rac1 bound to GTP-γ-S, after deduction of the background. The V_{max} was obtained for amounts of Rho-GDI-bound Rac about 5 times higher than GTP-bound Rac. It is noteworthy that no nucleotide was added to the assay medium and that the background was therefore much lower (about 38 nmol of O_2^- min^{-1} (mg of membrane protein)$^{-1}$ than in the presence of GTP-γ-S. This

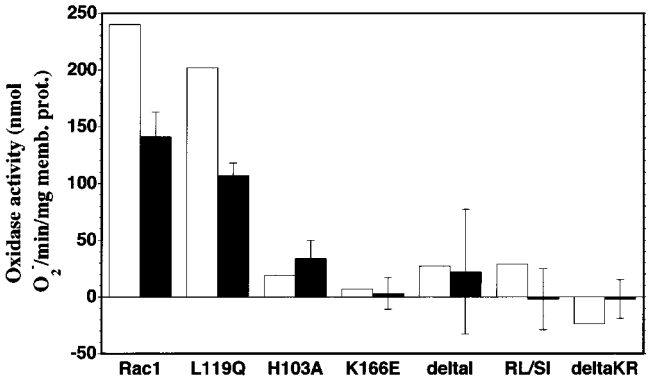

FIGURE 5: Assay of NADPH oxidase activation by Rac-GTP and Rac/Rho-GDI complexes. The maximal oxidase activating potency of Rac and Rac mutants loaded with GTP-γ-S is shown (white bars). Data from a representative experiment over three separate experiments are shown. For each Rac/Rho-GDI complex in the absence of added nucleotide, the elicited oxidase activity is represented by black bars. The activity of the Rac/Rho-GDI complexes was obtained for amounts of complexed Rac and Rho-GDI between 4 and 20 pmol each. Results are expressed as mean ± SD of at least three separate experiments. The background value was determined in the absence of Rac proteins, using a concentration of free GTP-γ-S or GDP-β-S similar to that present in the Rac sample after nucleotide exchange. The background activity was deduced [38 nmol of O_2^- min^{-1} (mg of membrane protein)$^{-1}$ for the Rac/Rho-GDI complexes or Rac-GDP or Rho-GDI alone and 135 nmol of O_2^- min^{-1} (mg of membrane protein)$^{-1}$ for Rac-GTP]. The activity of the GDP-bound GTPases and of Rho-GDI alone was equal to the background (not shown).

activation was reproduced with the L119Q/Rho-GDI complex. The possibility that, under cell-free conditions (arachidonic acid concentration inferior to 100 μM), the complex between Rac and Rho-GDI would be fully dissociated is very unlikely. This would result in a release of Rac in the GDP-bound form. Because of reported activity of GDP-bound Rac alone (*11*), controls were run with similar amounts of GDP-β-S-bound Rac proteins and mutants, and only background activating potency was observed (data not shown). The activation by the Rac/Rho-GDI complex could not be reproduced by addition of Rho-GDI alone over a wide concentration range (data not shown), indicating that it was not an effect of Rho-GDI on endogenous Rac after dissociation of the complex. The effective amounts of Rac in the Rac/Rho-GDI complexes were only 5-fold higher than amounts of isolated Rac protein, which makes it unlikely that a small proportion of GTP-bound Rac would have dissociated from Rho-GDI. In summary, the Rac/Rho-GDI complexes behave like the corresponding GTP-bound GTPases, yet at higher concentrations. It was recently shown that prenylated Rac had a special role in the assembly of the NADPH oxidase (*37*) in a nucleotide-independent manner, but this new activation system was inhibited by amphiphiles, by Rho-GDI, and was independent of p47phox. It is therefore very unlikely that some free prenylated Rac would be responsible for the activity of our Rac/Rho-GDI complex.

DISCUSSION

In the present work we have deciphered the two types of interactions between Rac and Rho-GDI and demonstrated the predominant role of protein−lipid over protein−protein interactions. We have shown that the two-hybrid system is a good means to study low-affinity protein−protein interactions. Using the baculovirus insect cell system, we have produced complexes in which Rac proteins are able to interact with Rho-GDI through the protein−lipid interaction mainly. We have shown, using an arachidonic acid based cell-free activation system, that the Rac/Rho-GDI complex is a potent activator of the NADPH oxidase in vitro. Rho-GDI therefore does not play an inhibitory role but rather behaves as an activator in the present case, probably by maintaining a conformation of Rac that is readily able to associate with its effector(s).

There are several examples in the literature in support of an activating role of Rho-GDI. FOAD, a factor involved in exocytosis in mast cells, was found to contain both Rac1 and Rho-GDI (*38*). More recently, a complex between CDC42 and Ly-GDI (also called GDI-2), a Rho-GDI homologue, has been purified and shown to be implicated in phospholipase C-β2 activation (*39*). In both cases, similarly to the case of NADPH oxidase activation, the complex in which the GTPase was bound to GDP, could be later replaced by the GTPase alone, bound to GTP.

A Role for Rac/RhoGDI in Signal Transduction Upstream of the NADPH Oxidase? Although the direct effect of the Rac/Rho-GDI complex on purified cytochrome b_{558} was previously shown (*40*), an effect on signal transduction enzymes upstream of the final steps of NADPH oxidase activation has to be considered in intact cells. From the above-mentioned data, the possibility that the Rac/Rho-GDI complex might act through phospholipase C activation or through an exocytic process cannot be ruled out. Another possibility is that the Rac/Rho-GDI complex might play its role through activation of lipid kinases. Superoxide production was indeed recently shown to be dependent on the presence of phosphatidic acid, after activation of a diacylglycerol kinase (*41*), and a multicomponent complex containing Rac, Rho-GDI, diacylglycerol kinase, and phosphatidylinositol 4-P-5-kinase was identified from bovine brain homogenate (*42*). NADPH oxidase activation is clearly a concerted mechanism, and some pathways may be independent of GEF proteins, using the bulk of Rac bound to Rho-GDI whereas other pathways would require Rac-GTP.

Direct Effect of Rac1 Mutations on the NADPH Oxidase Complex. In this study, the effect of three new Rac mutants and three previously characterized Rac mutants on NADPH oxidase activation and Rho-GDI binding is investigated. It is noteworthy that the Rac1 mutants unable to interact with Rho-GDI, RL66/67SI, deltaKR, and H103A were also unable to activate the NADPH oxidase. The L119Q mutant, which interacted normally, was active, although with higher EC_{50} values for oxidase activation than wild-type Rac1. These data suggest that the Rho-GDI bound conformation of Rac, except for the switch I region, is similar to that of GTP-bound Rac and that this conformation is required for NADPH oxidase activation. Other mutants, although able to interact with Rho-GDI, K166E, and deltaI, were inactive. These sites are located opposite of the Rho-GDI binding sites on the Rac/Rho-GDI structure. The K166E mutation may affect the folding of the second effector domain (159−162) that interacts with the inserted β-strands in the p67phox TPR domain (*27*). The absence of activity of the deltaKR proteins is likely to be explained by the lack of basic amino acids

required for membrane localization (*14*). This may also be true for the deltaI mutant, which displays reduced phosphoinositide binding (*12*), and may be unable to bind to the membrane or to another oxidase component, such as cytochrome b_{558} (*7*). Moreover, in the deltaKR mutant, the shorter distance between the isoprene and the globular domain of Rac would prevent simultaneous interaction, for example, with p67phox and with the membrane. In the RL66/67SI mutant, the conformation of the switch II region is probably severely affected, which would prevent interaction with another Rac partner within the NADPH oxidase complex. The reduced efficiency of the L119Q mutant compared to wild-type Rac1 indicates that the global fold of the protein has to be strictly conserved in order for the protein to be able to activate the NADPH oxidase.

Molecular Mechanisms of NADPH Oxidase Activation by the Rac/Rho-GDI Complex. The structure of the N-terminus of the p67phox protein in complex with Rac (*27*) highlighted the regions of Rac in contact with p67phox. This interaction involves the effector loop (switch I) of Rac as well as region 159−162 that probably corresponds to the second effector domain (*9*) and/or the peptide walking domain d (*10*). A number of amino acids from the activation loop responsible for interaction with p67phox are exposed at the surface of the Rac1/Rho-GDI complex with their side chain pointing toward the solvent (*23*). However, the conformation of this switch I region clearly differs between the GDP- and the GTP-bound forms of Rac. This may explain why we were not able to show a stable interaction of the Rac1/Rho-GDI complex with p67phox.

Examination of structural data shows few differences between the conformation of switch II of Rac in the GDP-bound form (in the Rac/Rho-GDI complex) and the GTP-bound forms. Moreover, Rho-GDI stabilizes the switch II region of Rac in the GTP-bound conformation. In other words, apart from switch I, the overall conformation of Rac-GDP/Rho-GDI would be only slightly different from Rac-GTP/Rho-GDI. We would like to propose that, since there are two regions of interaction between Rac and p67phox (*27*), a low-affinity ternary complex (Rac-GDP/Rho-GDI/p67phox) could arise, upon binding of region 159−162 of Rac, in complex with Rho-GDI, to p67phox. Such a complex would be a plausible intermediate in the activation of the NADPH oxidase and would be converted to a higher affinity complex after GDP/GTP exchange on Rac and modification of switch I conformation, given that a slow rate of GDP/GTP exchange exists within the Rac1/Rho-GDI complex.

We have shown that phosphoinositides induce the formation of an open complex between RhoA and Rho-GDI, probably by disrupting the protein−lipid interaction (*25*). In this complex, the Rho GTPase was able to exchange its GDP for GTP and to translocate to the membrane. Phosphoinositides (and other amphiphilic reagents known to activate the oxidase, such as phosphatidic acid) are likely to play a role in opening the lipid−protein interaction and therefore in membrane targeting of Rac since they interact with the polybasic region that contributes to the isoprene binding pocket. They would facilitate extraction of the isoprene from the hydrophobic pocket of Rho-GDI. This would be followed by translocation to the membrane.

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