Membrane Association of Rac Is Required for High Activity of the Respiratory Burst Oxidase†

Mary Linda Kreck,‡ Jennifer L. Freeman,‡ Arie Abo,§ and J. David Lambeth*,‡

Department of Biochemistry, Emory University School of Medicine, Atlanta, Georgia 30322, and Onyx Pharmaceuticals, 3031 Research Drive, Building A, Richmond, California 94806

Received August 16, 1996⊗

ABSTRACT: NADPH-dependent superoxide generation can be reconstituted in a cell-free system using recombinant cytosolic factors (p47-*phox*, p67-*phox*, and Rac) plus flavocytochrome b_{558}. Rac1 and Rac2 are closely related small GTPases, differing primarily in the C-terminal 10 residues where Rac1 but not Rac2 contains a polybasic sequence. In their nonisoprenylated forms, Rac1 was highly effective in reconstituting NADPH oxidase activity (low EC_{50}, high V_{max}), whereas Rac2 was only minimally effective (high EC_{50}, low V_{max}). In contrast, low concentrations of isoprenylated Rac1 and Rac2 both supported high rates of superoxide generation. Like full length Rac2, truncated forms of both Rac1 and Rac2 in which the C-terminal 10 residues were eliminated were poorly activating, pointing to the C terminus of Rac1 as a determinant of activity. Mutation of single positively charged residues in the C terminus of nonisoprenylated Rac1 markedly reduced its ability to support superoxide generation, affecting both its EC_{50} and the V_{max}. In contrast, mutation or truncation of the C terminus failed to affect the activation of PAK, a Rac-regulated protein kinase. The EC_{50} for Rac1 increased with increasing salt concentrations, whereas that of Rac2 was independent of salt, implicating the involvement of electrostatic forces for the former. Using flavocytochrome b_{558} reconstituted into phosphatidylcholine vesicles, the EC_{50} for Rac1 but not Rac2 decreased (increased binding) when an acidic phospholipid (phosphatidylinositol) was present, supporting a role for the Rac1 polybasic C terminus in binding to the membrane. A model in which Rac must associate simultaneously both with p67-*phox* and with the membrane to activate the NADPH oxidase can account for the above observations.

Activated phagocytic cells consume molecular oxygen in a process called the "respiratory burst", which participates in the killing of ingested bacteria. This reaction, which utilizes NADPH to reduce oxygen to superoxide, is catalyzed by a multicomponent enzyme, the NADPH oxidase. The oxidase is dormant in resting cells but is activated by a variety of agonists, including those which interact with cell surface receptors (e.g., opsonized particles and the chemotactic peptide f-Met-Leu-Phe). The NADPH oxidase is comprised of both membrane-associated and cytosolic proteins which assemble upon cell activation (Clark et al., 1990). The membrane-associated component is the heterodimer flavocytochrome b_{558}, which contains heme as well as putative NADPH- and FAD-binding sites (Rotrosen et al., 1992; Segal et al., 1992). The cytosolic proteins p47-*phox*[1] (Lomax et al., 1989) and p67-*phox* (Leto et al., 1990) and the small GTPase Rac (Knaus et al., 1991; Abo et al., 1992) translocate to the plasma membrane, where they associate directly or indirectly with cytochrome b_{558} (Heyworth et al., 1991; Uhlinger et al., 1993).

Rac is a member of the Rho subfamily of Ras-related small GTPases, which are involved in the regulation of diverse cellular processes such as growth, differentiation, organization of the cytoskeleton, and intracellular transport. Rac exists as two closely related isoforms, Rac1 and Rac2, which are 92% identical, differing in only 14 out of 190 residues (Didsbury et al., 1989). The highest density of divergent residues is in the carboxyl terminus, where 6 out of the last 10 residues differ (see below, bold letters). In Rac1, this region is highly basic, similar to a polybasic region seen in several other small GTPases, including K-Ras and Rap1a. In the former, this polybasic region is thought to participate in membrane interactions and is essential for function (Hancock et al., 1990).

Chart 1

```
            170          180          190
Rac1    DEAIRAVL CP  PPVKKRKRKC LLL
Rac2    DEAIRAVL CP  QPTRQQKRAC SLL
```

The mRNA for Rac1 is expressed ubiquitously, whereas Rac2 is found primarily in myeloid cells (Didsbury et al., 1989). Rac1 was isolated and identified as an oxidase-related factor from cytosol from guinea pig peritoneal macrophages (Abo et al., 1991), while Rac2 was implicated as an oxidase-related factor in human neutrophil cytosol (Knaus et al., 1991).

In their native forms, the Rho and Ras family small molecular weight GTP-binding proteins are modified with an isoprenyl group. These proteins initially contain the CAAX amino acid motif at their carboxyl termini, where C

† Supported by NIH Grant AI22809.

* To whom correspondence should be addressed. Phone: (404) 727-5962. Fax: (404) 727-2738. E-mail: dlambe@bimcore.emory.edu.

‡ Emory University School of Medicine.

§ Onyx Pharmaceuticals.

⊗ Abstract published in *Advance ACS Abstracts,* November 15, 1996.

[1] Abbreviations: *phox*, phagocytic oxidase; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); PAK, p21-activated kinase.

S0006-2960(96)02064-8 CCC: $12.00

is cysteine, A is any aliphatic residue, and X is any residue (see Chart 1, double-underlined region). This sequence signals for a series of post-translational modifications, which involves S isoprenylation of the cysteine [using the farnesyl (C15) or geranylgeranyl (C20) group for Ras and Rho subfamily members, respectively], followed by proteolytic cleavage of the AAX and carboxylmethylation of the cysteine (Clarke et al., 1988; Gutierrez et al., 1989; Philips et al., 1993). While these and other lipid modifications are critical for membrane association and function of Ras (Hancock et al., 1990, 1991), unmodified recombinant Rac1−GTPγS stimulates superoxide generation to a rate similar to that seen using neutrophil cytosol (Heyworth et al., 1993; Kreck et al., 1994), the latter containing isoprenylated Rac. The unmodified Rac fails to undergo rapid guanine nucleotide exchange but can be "preloaded" with GTPγS at low magnesium concentrations. Isoprenylation appears to promote the interaction with a guanine nucleotide exchange protein permitting rapid GTP binding (Ando et al., 1992; Heyworth et al., 1993). These findings have been interpreted as indicating that binding to the membrane is needed for guanine nucleotide exchange but is not important for activation of the NADPH oxidase.

Superoxide generation can be reconstituted in a cell-free system consisting of cytosol plus plasma membranes along with anionic amphiphiles such as arachidonate (Curnutte, 1985; McPhail et al., 1985; Bromberg & Pick, 1984) plus GTPγS (Uhlinger et al., 1991; Seifert & Schultz, 1987; Ligeti et al., 1989; Gabig et al., 1987). We have utilized plasma membranes plus recombinant p47-*phox*, p67-*phox*, and Rac1 in place of cytosol and refer to this as the semirecombinant system. In kinetic studies using this system, a peptide which corresponds to the carboxyl terminus of Rac1 [Rac1(178−188)] inhibited superoxide generation. Inhibition was competitive with respect to the Rac (Kreck et al., 1994) but noncompetitive with respect to p67-*phox* and p47-*phox*. The peptide was ineffective once activation had taken place, indicating that it blocked assembly but not the catalytic function of the oxidase complex. "Peptide walking" studies (Joseph & Pick, 1995) confirmed inhibition of oxidase activation by peptides containing the polybasic motif. These data have been interpreted as pointing to a role for the Rac C terminus in protein−protein interactions within the NADPH oxidase. However, in an earlier study, this group found that inhibition by polybasic peptides was independent of sequence (Joseph et al., 1994), suggesting that the peptide might be inhibiting by mechanisms other than by disruption of specific protein−protein interactions. In the present study, we have investigated the functional role of the carboxyl terminal region of Rac in interactions with components of the respiratory burst oxidase. We provide evidence that the polybasic C terminus of Rac1 mediates the association not with the oxidase itself but with the membrane and that membrane association (whether via a polybasic region or via isoprenylation) is essential for optimal activation of the NADPH oxidase.

EXPERIMENTAL PROCEDURES

Materials. Hespan (6.2% hetastarch in 0.9% NaCl) was obtained from American Hospital Supply Corp. Lymphocyte separation medium (6.2% Ficoll, 9.4% sodium diatrizoate) was purchased from Bionetics Laboratory Products. NADPH, cytochrome *c* (type IV, horse heart), GTPγS, diisopropyl fluorophosphate, glutathione (insolubilized on cross-linked 4% beaded agarose), thrombin (human), and isopropyl β-D-thiogalactopyranoside were obtained from Sigma. *N*-Methylisatoic anhydride (Mant) was obtained from Molecular Probes.

Isolation of Human Neutrophils and Preparation of Plasma Membranes. Human neutrophils were isolated from peripheral blood from healthy adult donors as described (Pember et al., 1983). Informed consent was obtained from all donors. Plasma membranes were prepared and stored as described (Kreck et al., 1994) and contained approximately 0.25 nmol of cytochrome b_{558}/(mg of protein), on the basis of the assumption of two hemes per cytochrome (Quinn et al., 1992; Nishimoto et al., 1995).

Expression and Purification of Proteins. Rac1 proteins (native and mutant) were expressed in *Escherichia coli* as fusion proteins with an N-terminal glutathione *S*-transferase using the PGEX-2T fusion vector and were purified to 99% homogeneity using thrombin cleavage from a glutathione affinity matrix (Kreck et al., 1994). The Rac1 gene was previously engineered with flanking *Bam*H1 and *Eco*R1 restriction enzyme sites, and the sequence was mutated to replace Cys 189 with Ser and thus eliminate the possibility of isoprenylation. Cytochrome b_{558} was purified as described previously (Segal et al., 1992; Nisimoto et al., 1995). Recombinant p47-*phox* and p67-*phox* were expressed in and purified from Sf9 and Hi5 cells, respectively, that had been infected with recombinant baculovirus encoding the respective proteins (Uhlinger et al., 1992). Isoprenylated Rac1 and Rac2 were expressed in Hi5 cells using the BlueBacHis vector (Invitrogen Corp., San Diego, CA) as polyhistidine fusion proteins. Cells were harvested 72 h after infection with virus, resuspended in buffer A [100 mM KCl, 3 mm NaCl, 4 mM $MgCl_2$, and 1 mM EGTA in 10 mM PIPES (pH 7.0)] containing 2.5 μM leupeptin, 2.5 μM pepstatin, and 2.5 μM aprotinin and 0.37 μM phenylmethanesulfonyl fluoride, and incubated with 4 mM diisopropyl fluorophosphate for 20 min on ice. Cells were disrupted by nitrogen cavitation at 500 psi and centrifuged for 1 h at 346000*g* to pellet the plasma membranes. The soluble fraction (containing nonisoprenylated Rac) was removed, and the pellets were briefly washed with buffer A before being resuspended in 20 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 1 mM β-mercaptoethanol, 10% glycerol, and 0.9% cholate. The suspension was gently agitated on ice for 2−8 h to solubilize Rac from membranes and centrifuged for 30 min at 346000*g*, and the supernatant was applied to a 3 mL Probond (Invitrogen) nickel column. The column was washed with at least six 10 mL volumes of wash buffer [20 mM NaPi, 500 mM NaCl (pH 6.0), 5 mM $MgCl_2$, 10% glycerol, and 1 mM β-mercaptoethanol]. The column was washed with an imidazole step gradient (5 mL each of 50, 80, and 100 mM imidazole in wash buffer), and the protein was eluted with 4−5 mL of 350 mM imidazole in wash buffer containing 0.05% octyl glucoside. The protein was dialyzed into 25 mM Tris-HCl (pH 8.0), 1 mM EDTA, 5 mM $MgCl_2$, 0.15 mM DTT, 0.05% octyl glucoside, and 20% glycerol.

Construction of Truncation and Site-Specific Mutations. Rac1(C189S), initially constructed to eliminate the possibility of isoprenylation, was used as the parent for further mutations and is referred to simply as Rac1. Truncated Rac1 and Rac2 were made using PCR primers by substituting a stop codon for the codon specifying residue 179. The other mutant Rac

proteins were made using oligonucleotide-directed mutagenesis in M13mp19 (Kunkel et al., 1987). Sequencing was carried out by the dideoxy method to confirm the mutation before subcloning into the PGEX-2T plasmid for protein expression.

Assay for Cell-Free NADPH Oxidase Activity. Superoxide generation was measured by superoxide dismutase-inhibitable reduction of cytochrome *c* (Burnham et al., 1990), using a Thermomax kinetic microplate reader (Molecular Devices, Menlo Park, CA). Unless otherwise noted, the semirecombinant cell-free reaction mixtures included 10 μg of plasma membrane protein, 1.3 μM recombinant p47-*phox*, 0.65−1.3 μM recombinant p67-*phox*, 1 μM recombinant Rac1 protein which had been preloaded with 10 μM GTPγS for 15 min at 25 °C, 10 μM GTPγS, and 160−240 μM arachidonic acid in a total volume of 50 μL. The concentration of arachidonate yielding optimal activity was determined prior to the experiment by titration and differed slightly among different plasma membrane preparations. Three 10 μL aliquots of each reaction mixture were transferred to 96-well assay plates (Corning) which were preincubated for 5 min at 25 °C before initiation with 240 μL of a solution containing 200 μM NADPH and 80 μM cytochrome *c* in buffer A. The 5 min preincubation was determined to be sufficient under the variety of conditions used herein to permit complete assembly of the components of the NADPH oxidase. The preincubation eliminates a lag phase which reflects the assembly process and thus permits linear reduction of cytochrome *c*. Thus, the conditions used allow observation under steady state rather than pre-steady state conditions. An extinction coefficient of 21 mM^{-1} cm^{-1} at 550 nm was used to calculate the quantity of cytochrome *c* reduced (Lambeth et al., 1988).

Experiments using purified cytochrome b_{558} in place of plasma membranes included 0.213 μM p47-*phox*, 0.145 μM p67-*phox*, varying amounts of GTPγS-preloaded Rac as indicated, and 5.8 nM cytochrome b_{558}. Components were incubated in buffer B [50 mM NaCl, 4 mM $MgCl_2$, and 1.25 mM EGTA in 20 mM Tris-HCl (pH 7.0)] containing 10 μM FAD and 10−30 μM arachidonic acid. Cytochrome b_{558} was reconstituted with FAD into phospholipid vesicles (1.8 mg/mL) composed of the following weight percent ratio of phospholipids: 31:15:8:23:23 phosphatidylcholine:phosphatidylethanolamine:phosphatidylinositol:sphingomyelin:cholesterol. Phospholipid vesicles lacking phosphatidylinositol were comprised of the following weight percent ratio of the above phospholipids: 33:17:0:25:25. Each reaction mixture (100 μL) was preincubated for 4 min at 25 °C before addition of 200 μM NADPH and 80 μM cytochrome *c*. The final concentration of phospholipids was 0.45 mg/mL, corresponding to a concentration of approximately 550 μM, and the concentration of phosphatidylinositol when present was 44 μM (8 mol %).

V_{max} and EC_{50} (effective concentration at 50% of the maximal velocity) were determined using a nonlinear least-squares regression fit of the data to the Michaelis−Menten equation, calculated and plotted using Sigma Plot (Jandel). The EC_{50} is formally equivalent to the binding constant if it is assumed that the cytosolic proteins are functioning as regulatory factors and that they are not altered (e.g., by post-translational modification) following binding to the oxidase.

Binding of GTPγS to Rac Using Gel Filtration Chromatography. Normal or mutant proteins were incubated with a 4-fold molar excess of [^{35}S]GTPγS (464 Ci/mol) in 100 mM KCl, 3 mM NaCl, 10 mM PIPES, and 1 mM EDTA at pH 7.0 and 25 °C for 4 min. The mixture was then diluted with 4 volumes of this buffer without EDTA but containing 10 mM $MgCl_2$, and protein was separated from free GTPγS by chromatography on a 25 × 1 cm Sephadex G-50/80 column. Protein-containing fractions were pooled, and the [^{35}S]GTPγS was quantitated by scintillation counting. The molar ratio of GTPγS molecules per Rac protein was then calculated.

Binding of Mant-GppNHp to Rac Using Fluorescence. Mant-GppNHp was synthesized as described previously (Hiratsuka, 1983). To quantify the apparent dissociation constant for Mant-GppNHp binding to Rac and to the Rac mutant proteins, increasing concentrations of Mant-GppNHp were added to the fluorescence cuvette in the presence and absence of the Rac proteins, and the fluorescence emission at 445 nm was measured using an excitation wavelength of 355 mm. Fluorescence measurements were made using a Perkin-Elmer model LS-5B spectrofluorimeter. The fluorescent increase due to the binding of Mant-GppNHp to Rac was calculated by subtracting the fluorescence of the Mant-GppNHp alone. The K_d using each of the proteins was calculated using the equation $K_d = [Mant_{free}][Rac_{free}]/[complex]$, where [complex] was calculated for values off the tangent lines according to the equation $(F_0/F_{max})[Rac_{total}]$. The [complex] was subtracted from the total concentration of Mant-GppNHp added and the total concentration of Rac added to get $[Rac_{free}]$ and $[Mant_{free}]$, respectively. Two to four K_d values were determined in this manner for each titration, and averages of these values are reported. The apparent stoichiometry of binding was determined from the intersection of the tangents with the initial slope and the horizontal slope representing saturation of binding. Examples of titrations and more detailed methodology are provided in Freeman et al. (1996).

Assay for PAK Activity. Recombinant hPAK65 (1−2 μg) (bound to protein G Sepharose conjugated with monoclonal Myc antibody) was washed once and incubated in 40 μL of kinase buffer: 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 10 mM $MgCl_2$, and 1 mM $MnCl_2$ with 1−2 μg of either Rac1 or the indicated Rac mutant, previously loaded with GTPγS or with buffer alone. The reaction was initiated by adding 10 μL of kinase buffer containing 50 μM ATP and 5 μCi [γ-^{32}P]ATP, and the incubation was continued for 20 min at 30 °C. The reaction was terminated by adding 10 μL of 5X SDS−PAGE sample buffer and heating in boiling water for 5 min. In the presence of Rac under these conditions, autophosphorylation of PAK is linear up to several hours. Samples were applied to a 14% SDS−PAGE, and the gel was stained with Coomassie Blue, destained, dried, and exposed to a film for 1−2 h. Phosphorylated bands were excised, and the incorporated ^{32}P was counted. Autophosphorylation in the absence of Rac was subtracted from that in its presence.

RESULTS

Comparison of Full Length and Truncated Rac1 and Rac2 in Supporting NADPH Oxidase Activity. Full length Rac1 and Rac2 were expressed in *E. coli* containing a C189S mutation so that no isoprenylation was possible after addition to cell fractions. Truncated Rac proteins were designed to

Table 1: Characterization of Rac Mutations with Respect to Superoxide Generation and Kinetic Parameters[a]

mutation	superoxide generation: relative rate at 1 μM Rac (%)	superoxide generation: relative V_{max} ([Rac] $\rightarrow \infty$) (%)	EC_{50} for Rac (nM)
Rac1[c]	100	100[b]	≤ 60[d]
Rac2[c]	11	23	1300 ± 200
Rac1(1−178)	5	12	1200 ± 145
Rac2(1−178)	19	28	900 ± 210
Rac2(S190L)[c]	26	38	1000 ± 243
Rac1(K183E)[c]	18	35	1600 ± 120
Rac1(K183Q)[c]	27	51	1500 ± 100
Rac1(K186E)[c]	33	52	600 ± 50
Rac1(K186Q)	33	42	400 ± 60
Rac1(R187E)[c]	24	40	800 ± 30
Rac1(R187Q)	29	47	800 ± 30
Rac1(K188E)[c]	78	83	300 ± 120
Rac1(K188Q)[c]	74	82	300 ± 50

[a] The indicated mutations were constructed in Rac1 and expressed as described in Experimental Procedures. Superoxide generation was determined as described in Experimental Procedures. Data shown are representative of at least four determinations. [b] $V_{max} = 6480$ (± 739) nmol of O_2^- min^{-1} (mg of PM protein)$^{-1}$. Because of small variations in the activity of various plasma membrane preparations, V_{max} values for all mutants were normalized to the rate obtained using 1 μM (a saturating level) of native Rac1. [c] Also contains the C189S mutation. [d] Because of the tight binding of native Rac1 and inaccuracies inherent in estimating very low EC_{50} values, this number should be considered as an upper limit.

Table 2: Binding of GTPγS to Rac and Rac Mutants[a]

protein	GTPγS bound (mol/mol)
Rac1	0.73 ± 0.4
Rac2	0.76 ± 0.2
Rac1(1−178)	0.69 ± 0.1
Rac2(1−178)	0.63 ± 0.1
Rac1(K183Q)	0.53 ± 0.1
Rac1(K186E)	1.10
Rac1(K186Q)	0.80
Rac1(R187E)	0.74 ± 0.1
Rac1(R187Q)	0.60 ± 0.2
Rac1(K188E)	0.81
Rac1(K188Q)	0.73 ± 0.2

[a] Proteins were incubated with a 4-fold molar excess of [^{35}S]GTPγS (464 Ci/mol) as described in Experimental Procedures. The protein was separated from free GTPγS by chromatography on a Sephadex G-50/80 column, and [^{35}S]GTPγS bound to protein was quantified. For each protein, the binding assay was carried out between one and six times, and averages are reported.

terminate after residue 178 (Chart 1). As shown in Table 1 (column 1), at 1 μM, Rac2 was consistently about 10-fold less potent than Rac1 in supporting oxidase activity. Truncated Rac1 and truncated Rac2 also had relatively low activities, similar to that seen with full length Rac2.

Activity differences could not be accounted for on the basis of differences in guanine nucleotide binding among the various forms of Rac. Binding of guanine nucleotide was measured in two ways. In the first method, excess radiolabeled GTPγS was added to the GTPase, as detailed in Experimental Procedures. Free GTPγS was separated from Rac-bound GTPγS by gel exclusion chromatography, and the molar ratio of GTPγS to Rac was determined. Using this method, the binding stoichiometry ranged from 0.63 to 0.76 mol of guanine nucleotide/(mol Rac) (see Table 2), indicating that all preparations showed significant guanine nucleotide binding and that binding was essentially the same for wild type and truncated forms of the protein. Binding

Table 3: Characteristics of Mant-GppNHp Binding of Rac1, Rac1(1−178), Rac2, and Rac2(1−178)[a]

GTPase	apparent K_d (nM)	apparent stoichiometry (mol:mol)
Rac1	14 ± 9	1.0 ± 0.10
Rac1(1−178)	17 ± 6	0.9 ± 0.03
Rac2	13 ± 5	1.0 ± 0.10
Rac2 (1−178)	10 ± 2	0.5 ± 0.02

[a] The Rac form shown (120 nM) was titrated with Mant-GppNHp, and the fluorescence change was recorded. The binding constant (K_d) and apparent stoichiometry of Mant-GppNHp binding to Rac were calculated as described in Experimental Procedures. Data shown are the averages of two or three experiments and the standard error or range.

Table 4: PAK Activation by Rac1, Rac2, and Truncated Rac1 and Rac2

GTPase	PAK activity (% cpm normalized[a])
Rac1	1.0 ± 0.1
Rac2	0.9 ± 0.2
Rac1(1−178)	1.5 ± 0.8
Rac2(1−178)	0.7 ± 0.2

[a] Autophosphorylation of PAK was measured as described in Experimental Procedures. Results from two experiments were normalized to the fraction of PAK which became autophosphorylated using Rac1. Basal autophosphorylation in the absence of Rac was typically around 25% of the stimulated level and was subtracted from the values reported here. The average and range of two experiments are shown.

stoichiometry and affinity were also determined fluorometrically, using a stable fluorescent derivative of GTP (Mant-GppNHp), as described in Experimental Procedures. Using this method, Rac1, Rac2, and truncated Rac proteins all bound the guanine nucleotide with the same affinity, showing apparent binding constants of 10−17 nM (see Table 3). The Mant-GppNHp:Rac binding stoichiometry was also estimated by this method and was found to be about 1:1 for Rac1, Rac2, and truncated Rac1 but was about 0.5 for truncated Rac2. Thus, these expressed forms of Rac appear to bind guanine nucleotide with normal affinity and near the expected stoichiometry. This indicates that the folding of native and truncated forms of Rac is essentially identical with respect to the guanine nucleotide binding site.

Comparison of Full Length and Truncated Rac1 and Rac2 in Activating PAK. To further confirm whether the expressed forms of Rac were functionally intact, their ability to activate PAK was investigated. PAK is a soluble protein kinase that is activated by Rac and CDC42 and which becomes autophosphorylated upon activation. The assay conditions used reflect initial rate conditions, since autophosphorylation is well below stoichiometric. Rac1, Rac2, and their truncated forms were all nearly equally active under these conditions, within experimental error (Table 4). Importantly, Rac1 and Rac2 were equally active, indicating that the differences in activity seen with the NADPH oxidase assay were not due to expression artifacts (e.g., a partially denatured form of Rac2). The NADPH oxidase and PAK utilize the same effector site on Rac, which includes amino acids within the range of 26−45 (Freeman et al., 1996). These data indicate that this effector region is functionally intact in Rac1, Rac2, and truncated forms of Rac.

Kinetic Analysis of the Activation of the NADPH Oxidase by Rac1, Rac2, and Truncated Racs. Kinetic analysis was carried out (Figure 1) to determine the origin of the activity differences seen between Rac1 and Rac2, and kinetic parameters are summarized in Table 1. Rac1 consistently

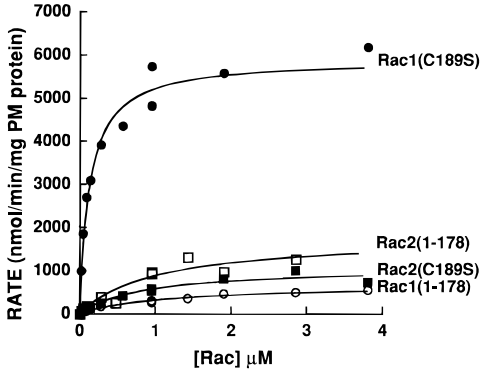

FIGURE 1: Concentration dependence for Rac1(C189S), Rac2-(C189S), and truncated Rac in supporting NADPH oxidase activity. Reaction mixtures included 10 μg of plasma membrane protein, 1.3 μM recombinant p47-*phox*, 1.3 μM p67-*phox*, and varying amounts of GTPγS-preloaded, recombinant Rac [Rac1(C189S), filled circles; Rac1(1−178), open circles; Rac2(C189S), filled squares; and Rac2(1−178), open squares] as indicated on the abscissa. GTPγS (10 μM) and an optimal concentration of arachidonic acid (160−240 μM, depending on the preparation) were added 5 min prior to initiating the assay with NADPH and cytochrome *c* in a total volume of 50 μL. Cytochrome *c* reduction was measured as described in Experimental Procedures. Michaelis−Menten kinetic parameters were determined using a nonlinear least-squares regression fit of the data. Data points represent the mean of three determinations obtained in a single experiment. Similar results were obtained in at least four separate experiments using three separate plasma membrane preparations and three individual preparations of Rac proteins.

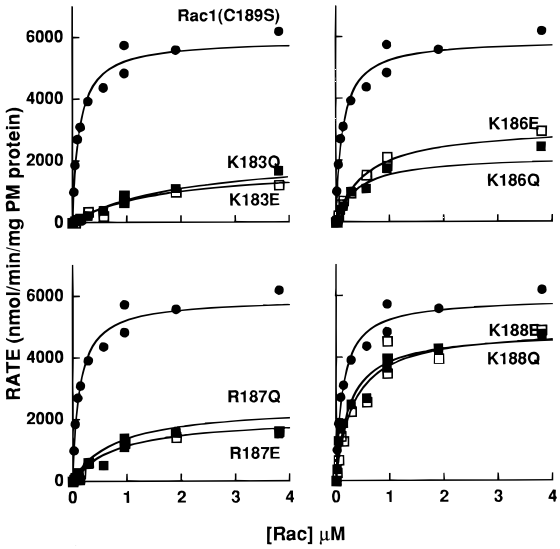

FIGURE 2: Concentration dependence of C-terminal point mutant Rac1 in supporting NADPH oxidase activity. The reaction mixture contained plasma membrane (10 μg), 1.3 μM p47-*phox*, 1.3 μM p67-*phox*, and varying concentrations of GTPγS-preloaded, recombinant Rac1(C189S) or Rac mutants, as indicated on the abscissa. Data using Rac1(C189S) are indicated in each panel for comparison (filled circles), and data for the various mutant Rac proteins are labeled in each panel. Incubation conditions were as in Figure 1. Similar results were obtained in at least five different experiments using three separate plasma membrane preparations and at least two preparations of Rac proteins.

showed a low EC_{50} (<60 nM) and produced a high V_{max} (6480 ± 739). Rac2, Rac1(1−178), and Rac2(1−178), which were all weakly activating, showed EC_{50} values which were increased by 15−20-fold compared with that of Rac1, indicating a significantly weaker association with the oxidase complex. V_{max} values (extrapolating to infinite Rac concentration) were also significantly reduced, ranging between 12 and 28% of that seen with full length Rac1(C189S). As Rac1 and Rac2 differ mainly in the carboxyl terminus, their truncated versions are 96% identical, and differences for the most part are conservative changes. Thus, in the cell-free system, the sequence differences between Rac1 and Rac2 in the regions other than the C terminus do not affect Rac function significantly, whereas differences in carboxyl termini account for the large differences in activity seen between nonisoprenylated Rac1 and Rac2.

Effect of Point Mutations in the Carboxyl Terminus of Rac on NADPH Oxidase Activity. To ensure that activity differences between Rac1 and Rac2 were not due to the single amino acid sequence differences in the CAAX box (see Chart 1), point mutation was first constructed to convert serine 190 of Rac2 to a leucine, which is seen in Rac1. There was no significant effect of this mutation on the kinetic constants (Table 1). Thus, activity differences between Rac1 and Rac2 are due to differences in the region preceding residue 189 (i.e., the polybasic region of Rac1) and are not due to the amino acid difference at position 190.

Mutations at positions 183 and 186−188 converted some of the basic residues (lysines and arginines) in the polybasic region to either a neutral (glutamine) or an acidic (glutamate) residue. Figure 2 shows representative experiments in which positively charged residues were converted to glutamine (Q) or glutamate (E). As summarized in column 1 of Table 1, at 1 μM, all mutants supported NADPH oxidase activity to a significantly lower degree than did Rac1, although activities were typically higher than with the truncation mutants. As shown in Table 2, the binding of GTPγS to all of the mutated forms was similar to that seen for native forms of Rac, indicating that activity differences cannot be attributed to differences in guanine nucleotide binding. Single amino acid substitutions at position 183, 186, or 188 all caused an increase in the apparent dissociation constant compared with that of Rac1 and caused a decrease in the V_{max} (Table 1). These effects were similar in magnitude, regardless of whether the residue was changed to a glutamine or to a glutamate. Mutations at positions 183 and 187 produced the largest effects, and these forms resembled the truncated Racs in their activation properties.

Effect of the C Terminus of Rac on the Binding of p67-phox and p47-phox to the NADPH Oxidase Complex. To investigate whether the carboxyl terminus of Rac affected the association of p47-*phox* and p67-*phox* within the NADPH oxidase complex, the concentration dependencies of these proteins for superoxide generation were determined in the presence of Rac1 and C-terminally truncated Rac1. As shown in Figure 3 (upper panel), when truncated Rac1 was used in place of full length Rac1 and the concentration of p47-*phox* was varied, the EC_{50} for p47-*phox* increased by a relatively small amount (from 450 to 1500 nM), whereas the V_{max} was reduced by about 75%. As determined from Figure 3 (lower panel), the kinetic constants for p67-*phox* were altered to a similar degree (the EC_{50} increased from 50 to 200 nM) when truncated Rac1 was used in place of full length Rac1. A near-saturating level of Rac or truncated Rac was used in all cases. Because truncation changes the EC_{50} for p67-*phox* and for p67-*phox* by a relatively small amount (3−4-fold compared with 20-fold for the EC_{50} for Rac1), the effects on the binding of either p47-*phox* or p67-*phox* do not appear to account for the activity differences seen between Rac1 and Rac2.

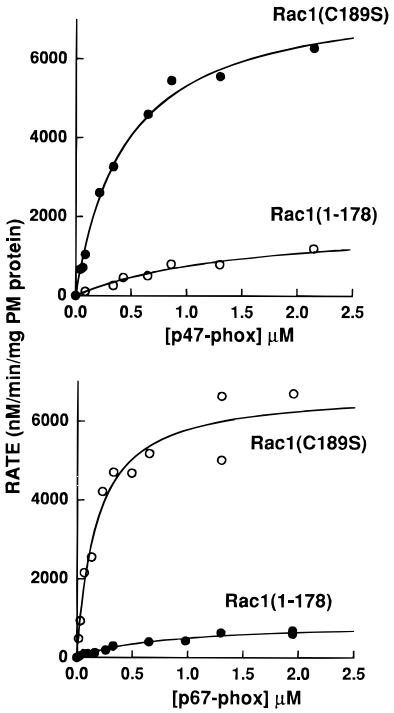

FIGURE 3: Effect of Rac truncation on the concentration dependence of p47-*phox* and p67-*phox* for superoxide generation. Each incubation contained plasma membrane (10 μg), GTPγS-preloaded Rac1(C189S) (1 μM) or Rac1(1−178) (2 μM), plus either 1.3 μM p47-*phox* and varying amounts of p67-*phox* (lower panel) or 1.3 μM p67-*phox* and varying amounts of p47-*phox* (upper panel). Similar results were obtained in six separate experiments using three plasma membrane preparations and three individual preparations of Rac proteins.

Effect of Ionic Strength on Rac Function. The role of electrostatic interactions in binding of Rac to NADPH oxidase was investigated by comparing the effects of salt concentration on the EC_{50} for Rac1(Q61H) and Rac2. Rac1(Q61H) rather than Rac1 was used in these studies in order to more accurately access the effects of salt on the EC_{50}.[2] As shown in Figure 4, increasing the ionic strength had no effect on the EC_{50} for Rac2. However, the EC_{50} for Rac1(Q61H) increased 3.5-fold, from 30 to 54 to 105 nM at respective NaCl concentrations of 3, 50, and 200 mM NaCl [corresponding to ionic strengths (Γ/2) of 33.5, 80.5, and 230.5 mM, correcting for the ionic strength of the buffer]. These data indicate that the binding of nonisoprenylated forms of Rac1 but not Rac2 is mediated in part by electrostatic interactions.

Effect of Lipid Composition on Rac Function. To investigate whether the origin of the electrostatic component of binding of Rac1 might be due to interaction of its polybasic region with negatively charged phospholipids, cytochrome b_{558} was first reconstituted into phospholipid vesicles which varied in their content of phosphatidylinositol (0 *versus* 8 mol %). Concentration dependencies for Rac1 and Rac2 were then carried out, and EC_{50} values were calculated. As shown in Figure 5, the EC_{50} of Rac1(Q61H) decreased (increased binding) nearly 4-fold from a value of 140 ± 30 nM in the absence of phosphatidylinositol to a value of 40 ± 6 nM when 8 wt % phosphatidylinositol was included. In contrast, phosphatidylinositol weakened the binding of Rac2 (EC_{50} values were 100 ± 20 *versus* 310 ± 100 nM in the absence and presence of phosphatidylinositol, respectively), although because of the low rates, EC_{50} values with Rac2 are less accurate than those for Rac1. The presence of 25 μM arachidonate in the assay mixture complicates the quantitative analysis of the effects of membrane charge. Under our conditions, there is a total of 550 μM phospholipid, of which there is 44 μM phosphatidylinositol, corresponding to 8 wt %. Although it is not clear how much arachidonate partitions into the membrane, the worst case scenario assuming all the arachidonate partitions into the membrane is that the membrane charge increases by including PI by around 3-fold. Thus, the above data indicate that the presence of negatively charged lipids significantly enhances the binding of nonisoprenylated Rac1 but not Rac2 to the NADPH oxidase complex.

Effect of Isoprenylation on the Function of Rac1 and Rac2. To investigate the effects of isoprenylation on Rac function, the concentration dependencies for recombinant Rac1 and Rac2 expressed in insect cells were determined and compared with that for Rac. As shown in Figure 6, isoprenylation permitted both Rac1 and Rac2 to support high activity at concentrations as low or lower than effective concentrations of nonisoprenylated Rac1. For unknown reasons, isoprenylated versions of both Rac1 and Rac2 showed inhibition of superoxide generation at higher concentrations, as has been reported previously (Heyworth et al., 1993). The inhibition was probably not due to a contaminating inhibitor as it was not seen when excess GDP-bound Rac1 or Rac2 (isoprenylated) was added to a maximally stimulating concentration of GTPγS-bound Rac (data not shown). In addition, when isoprenylated Rac was added to the cell-free oxidase as a complex with RhoGDI, superoxide generation was maximally stimulated and no inhibition was seen. This suggests that some artifact which is dependent upon the mode of delivery of isoprenylated Rac accounts for the inhibitory phase. We have experimented with various solvents and other methods of delivery of the isoprenylated Rac but have not found a way to eliminate the inhibitory phase. Despite these technical problems, these data indicate that isoprenylation renders Rac2 highly potent in supporting superoxide generation in the cell-free system.

DISCUSSION

The role of membrane association of Rac in the activation of the NADPH oxidase has been a matter of debate in recent years. Ras requires both the farnesylation and a second membrane-binding region (either palmitoylation or a polybasic domain) for complete plasma membrane localization (Hancock et al., 1990; Cadwallader et al., 1994). Both the farnesylation and the polybasic domain are required for the transforming activity of K-Ras (Symons et al., 1996). By analogy with Ras, it had been predicted that the function of Rac would depend upon its association with the membrane.

[2] The data-fitting method used becomes less accurate under conditions of very tight binding (the assumption that the free concentration of Rac equals the total Rac concentration is no longer valid), such as is the case with Rac1, and it becomes difficult or impossible to distinguish changes in in binding constants on the order of 3-fold. Thus, it was not possible to demonstrate reliably an effect of ionic strength on the EC_{50} for native Rac1. For this reason, we utilized a mutant form of Rac, Rac(Q61H), which shows a slightly weaker binding (higher EC_{50}). This mutation, while weakening binding, is in a region far removed from the C terminus, as judged by molecular modeling of the Rac structure (J. D. Lambeth, unpublished observations). The weaker binding permits relatively small perturbations in binding affinity to be quantified very accurately.

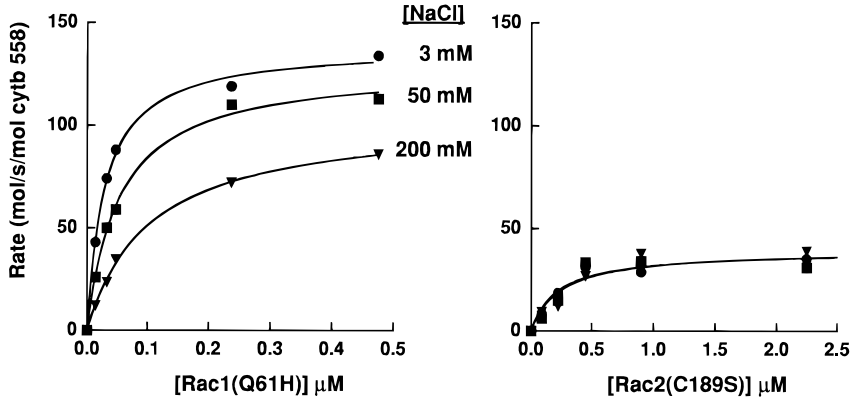

FIGURE 4: Effect of salt on the concentration dependence of Rac1 and Rac2 for superoxide generation. As detailed in Experimental Procedures, purified cytochrome b_{558} was reconstituted with FAD and incorporated into phospholipid vesicles comprised of the following weight percent ratio of phospholipids: 31:15:8:23:23 phosphatidylcholine:phosphatidylethanolamine:phosphatidylinositol:sphingomyelin:cholesterol. The cytochrome (5.8 nM) in a volume of 100 μL was preincubated with the indicated concentrations of GTPγS-preloaded Rac1(Q61H) or Rac2(C189S), 0.213 μM p47-*phox*, 0.145 μM p67-*phox*, and 10−50 μM arachidonate for 4 min at 25 °C before activation with NADPH and assay of cytochrome *c* reduction, as described in Experimental Procedures. The final concentration of lipid in the assay was 0.55 mM. The Rac1(Q61H) mutation also contained the C189S mutation. The reaction was carried out in buffer B with either 3 mM (circles), 50 mM (squares), or 200 mM (triangles) NaCl. Similar results were obtained using two separate cytochrome b_{558} preparations.

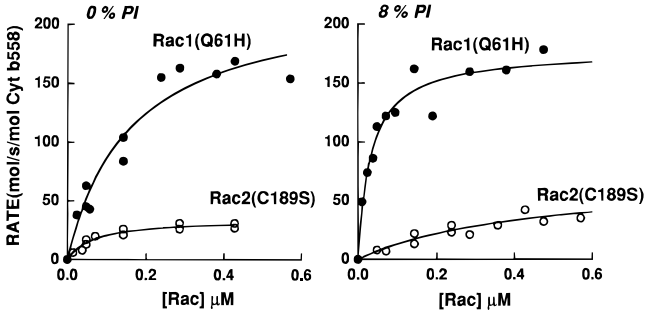

FIGURE 5: Effect of membrane charge on the concentration dependence for Rac activation of superoxide generation. Purified cytochrome b_{558} was reconstituted with FAD into phospholipid vesicles comprised of a mixture of phospholipids (see Experimental Procedures) containing either 0 (left panel) or 8 (right panel) wt % phosphatidylinositol. The final concentration of lipid in the assay was 0.55 mM, and the final concentration of phosphatidylinositol was 44 μM. The concentration of GTPγS-preloaded Rac protein [Rac1(Q61H), filled circles; or Rac2(C189S), open circles] was varied, and the rate of superoxide generation was measured as described in Figure 1. Incubation conditions were as in Figure 4, except that 20 μM arachidonate was used. Similar results were obtained using three individual membrane-reconstituted preparations of cytochrome b_{558}.

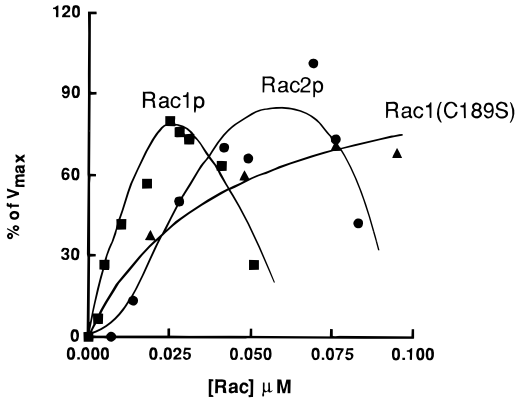

FIGURE 6: Comparison of the ability of isoprenylated and nonisoprenylated Rac to activate superoxide generation. Concentrations of Rac(C189S) (filled triangles) or prenylated Rac1 (filled squares, indicated by Rac1p) or Rac2 (filled circles, indicated by Rac2p) were varied, and superoxide generation was measured as in Figure 1. Incubation conditions were as in Figure 1. Similar results were obtained in at least five experiments using three individual plasma membrane preparations and four separate preparations of Rac1p and Rac2p.

Indeed, early reports seemed to confirm this prediction (Ando et al., 1992). In these studies, isoprenylated forms of both Rac1 and Rac2 but not their nonisoprenylated forms were effective in supporting cell-free superoxide generation. Notably, these studies used isolated plasma membranes as the source of flavocytochrome b_{558}. It was subsequently discovered (Heyworth et al., 1993) that isoprenylation was needed to promote rapid guanine nucleotide exchange, presumably by permitting the interaction with an exchange factor in the plasma membrane. However, guanine nucleotide exchange occurred readily when magnesium was depleted, permitting preloading of Rac with GTPγS. The earlier failure to observe Rac1 activation of the oxidase was found to be due to its failure to exchange guanine nucleotides, since the Rac1−GTPγS complex was highly effective in reconstituting superoxide-generating activity (Kreck et al., 1994; Kwong et al., 1993). The finding that nonisoprenylated Rac1−GTPγS supported high levels of superoxide generation was taken as evidence that membrane association was not critical for the function of Rac.

A second issue is the role of the C-terminal region of Rac in activation of the NADPH oxidase. On the basis of peptide inhibition studies using a polybasic peptide from the C terminus of Rac1 (Kreck et al., 1994), and upon subsequent peptide scanning studies (Joseph & Pick, 1995), it was suggested that the C terminus is an important determinant of protein−protein interactions. The peptide inhibited competitively with respect to Rac but noncompetitively with respect to p67-*phox* and p47-*phox*, suggesting a specific interaction of the peptide with a Rac binding site. However, because of the highly charged nature of the peptide, it was possible that it was inhibiting by other mechanisms (Joseph et al., 1994). We now believe that the polybasic peptide inhibits Rac1 function by binding at or near the membrane surface where it neutralizes negatively charged phospholipids, thus interfering with the docking of Rac with its membrane interaction site. Such a mechanism could give rise to the observed competitive inhibition kinetics, and it seems unnecessary to postulate protein−protein interactions mediated by the C terminus of Rac.

The present studies support the idea that membrane interaction is indeed necessary for optimal activation of the respiratory burst oxidase. On the basis of the present studies, nonisoprenylated forms of Rac1 but not Rac2 activate the NADPH oxidase. The ability of nonisoprenylated Rac1 to activate high levels of superoxide generation depends upon the presence of a polybasic region at the C terminus. Mutation or removal of basic residues in this region renders Rac1 poorly effective in activating the oxidase, similar to nonisoprenylated Rac2. This region is involved in electrostatic interactions with its target, since Rac1 but not Rac2 shows significant effects of salt on its EC_{50} for the oxidase complex. That the C-terminal region interacts with the membrane is supported by the finding that the binding of Rac1 but not Rac2 is enhanced when a negatively charged phospholipid is included along with the neutral phosphatidylcholine in the reconstituted cytochrome preparation. This indicates that the Rac either binds directly to the membrane or is in close enough proximity to "sense" the membrane charge. The importance of membrane binding is further substantiated by the finding that, when Rac2 is isoprenylated, it becomes as effective as Rac1(C189S) in supporting NADPH oxidase activity.

The finding that some mutations in the C terminus of Rac1 (e.g., at positions 183 and 187) give relatively larger effects than others (e.g., 186 and 188, see Table 1) might be interpreted as evidence for protein−protein rather than protein−membrane interactions. However, it seems likely that these charged side chains will reside at different distances from the membrane surface and/or might be partially neutralized by negatively charged residues on other parts of the protein, either of which would give rise to the observed differences in the effects of the mutations. For example, if the C terminus is modeled as an α helix (as in Ras) lying along the plane of the membrane, then the side chains of residues 183 and 187 project on the same face of a helical wheel diagram, whereas the side chains of residues 186 and 188 would point away from this face. Thus, if the side chains of residues 183 and 187 lie close to the membrane, then those of residues 186 and 187 would be predicted to be several angstroms more distant from the membrane surface and would have less influence on the electrostatic interactions. Thus, positional effects of mutations do not distinguish between protein−membrane and protein−protein interactions.

Rac also interacts with protein components within the NADPH oxidase complex. Rac has an effector site, residues 26−45, which binds to p67-*phox* (Diekmann et al., 1994; Dorseuil et al., 1996; Nisimoto et al., unpublished studies), and this region is important for Rac activation of superoxide generation (Freeman et al., 1994). An additional effector region which we refer to as the "insert" region, residues 124−135, is also crucial for Rac activity in the respiratory burst (Freeman et al., 1996), but its binding target is unknown. The finding that truncation of Rac1 causes a modest change in the EC_{50} values for p47-*phox* and p67-*phox* suggests that Rac participates in the cooperative assembly of the NADPH oxidase complex. This does not imply that the C terminus of Rac1 participates directly in binding to other cytosolic factors. Indeed, Rac1 and truncated Rac1 bind with the same affinity to p67-*phox* (Nishimoto et al., unpublished studies), indicating that the C terminus does not participate in this interaction. Rather, we propose that Rac binds simultaneously to p67-*phox* via its effector region and to the membrane via its C terminus. Through interactions with the membrane, Rac may help to tether and orient one or more other components of the NADPH oxidase complex.

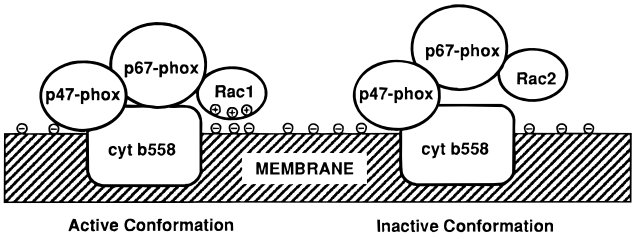

FIGURE 7: Model for simultaneous interactions of Rac with both p67-*phox* and membrane. The model diagrams the interaction of the polybasic region of nonisoprenylated Rac1 with the negative charges of the membrane, resulting in an active conformation of the NADPH oxidase (left). For Rac2 (as well as for C-terminally truncated forms of Rac1 and Rac2 and Rac1 mutants), there is a weaker interaction with the membrane, resulting in an inactive conformation of the oxidase complex (right).

One version of such a model is shown in Figure 7. According to this model, nonisoprenylated Rac1 interacts simultaneously with both p67-*phox* and the membrane to induce an active conformation of the complex. Nonisoprenylated Rac2 also binds efficiently to p67-*phox* but does not interact effectively with the membrane, resulting in an inactive (or weakly active) complex. This is not inconsistent with data using the yeast two-hybrid system which show that both Rac1 and Rac2 bind to p67-*phox* and that Rac2 may bind more tightly than Rac1 (Dorseuil et al., 1996). According to the proposed model, the binding of Rac to p67-*phox* is not the sole determinant of activity, and binding affinity with p67-*phox alone* would not be expected to correlate with activity. This model accounts for known protein−protein interactions as well as the observed functional differences between nonisoprenylated forms of Rac1 and Rac2 (also the effects of truncation and mutation) with regard to not only the EC_{50} but also the V_{max}. According to the model, even when the binding of Rac2 (or Rac1 mutants) is saturating, suboptimal activity will be seen since the weak interaction with the membrane results in an inactive (or weakly active) conformation. This model predicts that there should be an inverse relationship between the EC_{50} and the V_{max}, since interaction with the membrane affects not only the overall binding affinity to the oxidase complex but also an equilibrium between active and inactive conformations (Figure 7). This prediction is verified in Figure 8, in which an inverse correlation between EC_{50} and V_{max} is seen for 11 out of 13 forms of Rac. The possible exceptions are the two mutations at positions 183 (open symbols), which have higher EC_{50} values than are predicted from their V_{max} values. This may indicate that mutations at this position cause additional direct or indirect perturbations in binding to the oxidase. The model also accounts for the effects of ionic strength and membrane charge on EC_{50} values (Figures 4 and 5, respectively) and predicts that the isoprenylated forms of both Rac1 and RAc2 will be equally active (Figure 6).

The function of the polybasic region in the isoprenylated form of Rac1 remains an open question. Rac1 can function in a variety of cellular processes, including regulation of mitosis, of differentiation, and of the actin cytoskeleton. Direct targets include the protein kinase PAK. Rac2 is expressed exclusively in phagocytic cells where its mRNA is significantly more abundant than that of Rac1 (Didsbury

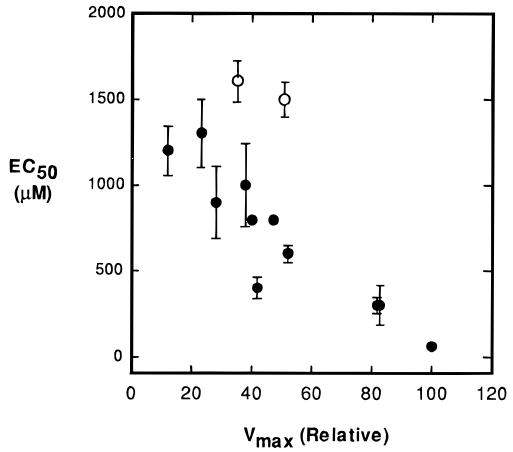

FIGURE 8: Correlation between Rac V_{max} and EC_{50}. For native, C-terminally mutated and C-terminally truncated forms of Rac, the V_{max} value for each was plotted against the respective EC_{50} values. The open symbols represent the two point mutations at position 183.

et al., 1989). This suggests that Rac2 is the physiologically relevant GTPase for regulating the NADPH oxidase. It is also possible that both Rac1 and Rac2 function *in vivo* but that the higher levels of Rac2 are needed to balance the high levels of other NADPH oxidase components in myeloid cells. *In vitro*, both Rac1 and Rac2 can function to support NADPH oxidase activity, provided Rac2 is isoprenylated. The polybasic region of Rac1 might provide a localization signal which would either enhance its membrane interactions or target Rac1 to regions of the cell where it colocalizes with its targets. On the basis of biophysical studies using artificial lipid vesicles, protein isoprenylation should cause the protein to have a strong but reversible membrane association (Silvius & l'Heureux, 1994; Peitzsch & McLaughlin, 1993). There is growing evidence for the importance of electrostatic interactions between polybasic amino acid residues and membrane lipids in the plasma membrane in lipid-anchored signaling proteins such as *src* and MARCKS (McLaughlin & Aderem, 1995; Kim et al., 1991; Buser et al., 1995), and such interactions may help to further anchor the protein to the membrane. Signaling proteins with polybasic regions may also localize within subregions of the plasma membrane enriched in acidic phospholipids (McLaughlin & Aderem, 1995), and this may play a role in colocalizing relevant interacting proteins within the same membrane domain.

In summary, the present studies imply that, as with Ras, membrane association of Rac is important for the activity of the NADPH oxidase. *In vitro*, the mechanism of membrane attachment is not critical for function, since membrane association may be provided either by isoprenylation or by electrostatic interactions through the polybasic region of Rac1. Rac also interacts with one or more NADPH oxidase protein components, and the membrane binding may help tether and orient p67-*phox* for optimal interaction with the flavocytochrome b_{558}. The proximity of Rac to the membrane in the active complex places geometric constraints on any structural model of the assembled NADPH oxidase complex.

REFERENCES

Abo, A., & Pick, E. (1991) *J. Biol. Chem. 266*, 23577−23585.

Abo, A., Pick, E., Hall, A., Totty, N., Teahan, C. G., & Segal, A. W. (1991) *Nature 353*, 668−670.

Abo, A., Boyhan, A., West, I., Thrasher, A. J., & Segal, A. W. (1992) *J. Biol. Chem. 267*, 16767−16770.

Ando, S., Kaibuchi, K., Sasaki, T., Hiraoka, K., Nishiyama, T., Mizuno, T., Asada, M., Nunoi, H., Matsuda, I., Matsuura, Y., Polakis, P., McCormick, F., & Takai, Y. (1992) *J. Biol. Chem. 267*, 25709−25713.

Bromberg, Y., & Pick, E. (1984) *Cell. Immunol. 88*, 213−221.

Burnham, D. N., Uhlinger, D. J., & Lambeth, J. D. (1990) *J. Biol. Chem. 265*, 17550−17559.

Buser, C., Kim, J., McLaughlin, S., & Peitzsch, R. (1995) *Mol. Membr. Biol. 12*, 69−75.

Cadwallader, K., Paterson, H., Macdonald, S., & Hancock, J. (1994) *Mol. Cell. Biol. 14*, 4722−4730.

Clark, R. A., Volpp, B. D., Leidal, K. G., & Nauseef, W. M. (1990) *J. Clin. Invest. 85*, 714−721.

Clarke, S., Yogel, J., Deschenes, R., & Stock, J. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 4643−4647.

Curnutte, J. T. (1985) *J. Clin. Invest. 75*, 1740−1743.

Didsbury, J., Weber, R. F., Bokoch, G. M., Evans, T., & Snyderman, R. (1989) *J. Biol. Chem. 264*, 16378−16382.

Diekmann, D., Abo, A., Johnson, C., Segal, A., & Hall, A. (1994a) *Science 265*, 531−533.

Dorseuil, O., Reibel, L., Bokoch, G., Camonis, J., & Gacon, G. (1996) *J. Biol. Chem. 271*, 83−88.

Freeman, J. L. R., Kreck, M. L., Uhlinger, D. J., & Lambeth, J. D. (1994) *Biochemistry 33*, 13431−13435.

Freeman, J. L. R., Abo, A., & Lambeth, J. D. (1996) *J. Biol. Chem. 271*, 19794−19801.

Gabig, T. G., English, D., Akard, L. P., & Schell, M. J. (1987) *J. Biol. Chem. 262*, 1685−1690.

Gutierrez, L., Magee, A., Marsall, C., & Hancock, J. (1989) *EMBO J. 8*, 4643−4647.

Hancock, J., Paterson, H., & Marshall, C. (1990) *Cell 63*, 133−139.

Hancock, J., Cadwallader, K., Paterson, H., & Marshall, C. (1991) *EMBO J. 10*, 4033−4039.

Heyworth, P. G., Curnutte, J. T., Nauseef, W. M., Volpp, B. D., Pearson, D. W., Rosen, H., & Clark, R. A. (1991) *J. Clin. Invest. 87*, 352−356.

Heyworth, P. G., Knaus, U. G., Xu, X., Uhlinger, D. J., Conroy, L., Bokoch, G. M., & Curnutte, J. T. (1993) *Mol. Biol. Cell 4*, 261−269.

Hiratsuka, T. (1991) *Biochim. Biophys. Acta 742*, 496−508.

Joseph, G., & Pick, E. (1995) *J. Biol. Chem. 270*, 29079−29082.

Joseph, G., Gorzlczany, Y., Koshkin, V., & Pick, E. (1994) *J. Biol. Chem. 269*, 29024−29031.

Kim, J., Mosior, M., Chung, L., Wu, H., & McLaughlin, S. (1991) *Biophys. J. 60*, 135−148.

Knaus, U. G., Heyworth, P. G., Evans, T., Curnutte, J. T., & Bokoch, G. M. (1991) *Science 254*, 1512−1515.

Kreck, M. L., Uhlinger, D. J., Tyagi, S. R., Inge, K. L., & Lambeth, J. D. (1994) *J. Biol. Chem. 269*, 4161−4168.

Kunkel, T. A., Roberts, J. D., & Zakour, R. A. (1987) *Methods Enzymol. 154*, 367−382.

Kwong, C. H., Malech, H. L., Rotrosen, D., & Leto, T. L. (1993) *Biochemistry 32*, 5711−5717.

Lambeth, J. D., Burnham, D. N., & Tyagi, S. R. (1988) *J. Biol. Chem. 263*, 3818−3822.

Leto, T. L., Lomax, K. J., Volpp, B. D., Nunoi, H., Sechler, J. M. G., Nauseef, W. M., Clark, R. A., Gallin, J. I., & Malech, H. L. (1990) *Science 248*, 727−730.

Ligeti, E., Tardif, M., & Vignais, P. V. (1989) *Biochemistry 28*, 7116−7123.

Lomax, K. J., Leto, T. L., Nunoi, H., Gallin, J. I., & Malech, H. L. (1989) *Science 245*, 409−412.

McLaughlin, S., & Aderem, A. (1995) *Trends Biochem. Sci. 20*, 272−276.

McPhail, L. C., Shirley, P. S., Clayton, C. C., & Snyderman, R. (1985) *J. Clin. Invest. 75*, 1735−1739.

Nisimoto, Y., Otsuka-Murakami, H., & Lambeth, D. (1995) *J. Biol. Chem. 270*, 16428−16434.

Peitzsch, R., & McLaughlin, S. (1993) *Biochemistry 32*, 10436−10443.

Pember, S. O., Shapira, R., & Kinkade, J. M., Jr. (1983) *Arch. Biochem. Biophys. 221*, 391−403.

Philips, M. R., Pillinger, M. H., Staud, R., Volker, C., Rosenfeld, M. G., Weissmann, G., & Stock, J. B. (1993) *Science 259*, 977−980.
Quinn, M. T., Mullen, M. L., & Jesaitis, A. J. (1992) *J. Biol. Chem. 267*, 7303−7309.
Rotrosen, D., Yeung, C. L., Leto, T. L., Malech, H. L., & Kwong, C. H. (1992) *Science 256*, 1459−1462.
Segal, A. W., West, I., Wientjes, F., Nugent, J. H. A., Chavan, A. J., Haley, B., Garcia, R. C., Rosen, H., & Scrace, G. (1992) *Biochem. J. 284*, 781−788.
Seifert, R., & Schultz, G. (1987) *Biochem. Biophys. Res. Commun. 146*, 1296−1302.
Silvius, J., & l'Heureux, F. (1994) *Biochemistry 33*, 3014−3022.
Symons, M., Derry, J., Karlak, B., Jiang, S., Lemahieu, V., McCormick, F., Francke, U., & Abo, A. (1996) *Cell 84*, 723−734.
Uhlinger, D. J., Burnham, D. N., & Lambeth, J. D. (1991) *J. Biol. Chem. 266*, 20990−20997.
Uhlinger, D. J., Inge, K. L., Kreck, M. L., Tyagi, S. R., Neckelmann, N., & Lambeth, J. D. (1992) *Biochem. Biophys. Res. Commun. 186*, 509−516.
Uhlinger, D. J., Tyagi, S. R., Inge, K. L., & Lambeth, J. D. (1993) *J. Biol. Chem. 268*, 8624−8631.

BI962064L