Rac Binding to p67phox

STRUCTURAL BASIS FOR INTERACTIONS OF THE Rac1 EFFECTOR REGION AND INSERT REGION WITH COMPONENTS OF THE RESPIRATORY BURST OXIDASE

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Activation of the respiratory burst oxidase involves the assembly of the membrane-associated flavocytochrome b558 with the cytosolic components p47phox, p67phox, and the small GTPase Rac. Herein, the interaction between Rac and p67phox is explored using functional and physical methods. Mutually facilitated binding (EC50) of Rac1 and p67phox within the NADPH oxidase complex was demonstrated using steady state kinetic methods measuring NADPH-dependent superoxide generation. Direct binding of Rac1 and Rac2 to p67phox was shown using a fluorescent analog of GTP (methylanthraniloyl guanosine-5’-[β,γ-imido]triphosphate) bound to Rac as a reporter group. An increase in the methylanthraniloyl fluorescence was seen with added p67phox but not p47phox, and the emission maximum shifted from 445 to 440 nm. Rac1 and Rac2 bound to p67phox with a 1:1 stoichiometry and with KD values of 120 and 60 nM, respectively. Mutational studies (Freeman, J., Kreck, M., Uhlinger, D. J., and Lambeth, J. D. (1994) Biochemistry 33, 13431–13435; Freeman, J. L., Abo, A., and Lambeth, J. D. (1996) J. Biol. Chem. 271, 19794–19801) previously identified two regions in Rac1 that are important for activity: the “effector region” (residues 26–45) and the “insert region” (residues 124–135). Proteins mutated in the effector region (Rac1(N26H), Rac1(I33N), and Rac1(D38N)) showed a marked increase in both the KD and the EC50, indicating that mutations in this region affect activity by inhibiting Rac binding to p67phox. Insert region mutations (Rac1(K132E) and L134R), while showing markedly elevated EC50 values, bound with normal affinity to p67phox. The structure of Rac1 determined by x-ray crystallography reveals that the effector region and the insert region are located in defined sectors on the surface of Rac1. A model is discussed in which the Rac1 effector region binds to p67phox, the C terminus binds to the membrane, and the insert region interacts with a different protein component, possibly cytochrome b558.

Neutrophils and macrophages reduce molecular oxygen with NADPH to produce superoxide (O2•−) and secondarily derived reactive oxygen species (H2O2, HOCl, OH•), which function to kill phagocytosed microorganisms (1–4). Superoxide generation is catalyzed by an NADPH oxidase (also called the respiratory burst oxidase), which is dormant in resting cells but becomes active upon exposure to bacteria or to a variety of soluble stimuli. The enzyme consists of both cytosolic and plasma membrane-associated protein factors. Flavocytochrome b558 is a membrane-associated heterodimer (5–7) that contains putative binding sites for NADPH, FAD, and heme (8–11) and putative binding sites for NADPH, FAD, and heme (8–11) and the respiratory burst oxidase involves the assembly of the membrane-associated flavocytochrome b558 with the cytosolic components p47phox, p67phox, and the small GTPase Rac. Herein, the interaction between Rac and p67phox is explored using functional and physical methods. Mutually facilitated binding (EC50) of Rac1 and p67phox within the NADPH oxidase complex was demonstrated using steady state kinetic methods measuring NADPH-dependent superoxide generation. Direct binding of Rac1 and Rac2 to p67phox was shown using a fluorescent analog of GTP (methylanthraniloyl guanosine-5’-[β,γ-imido]triphosphate) bound to Rac as a reporter group. An increase in the methylanthraniloyl fluorescence was seen with added p67phox but not p47phox, and the emission maximum shifted from 445 to 440 nm. Rac1 and Rac2 bound to p67phox with a 1:1 stoichiometry and with KD values of 120 and 60 nM, respectively. Mutational studies (Freeman, J., Kreck, M., Uhlinger, D. J., and Lambeth, J. D. (1994) Biochemistry 33, 13431–13435; Freeman, J. L., Abo, A., and Lambeth, J. D. (1996) J. Biol. Chem. 271, 19794–19801) previously identified two regions in Rac1 that are important for activity: the “effector region” (residues 26–45) and the “insert region” (residues 124–135). Proteins mutated in the effector region (Rac1(N26H), Rac1(I33N), and Rac1(D38N)) showed a marked increase in both the KD and the EC50, indicating that mutations in this region affect activity by inhibiting Rac binding to p67phox. Insert region mutations (Rac1(K132E) and L134R), while showing markedly elevated EC50 values, bound with normal affinity to p67phox. The structure of Rac1 determined by x-ray crystallography reveals that the effector region and the insert region are located in defined sectors on the surface of Rac1. A model is discussed in which the Rac1 effector region binds to p67phox, the C terminus binds to the membrane, and the insert region interacts with a different protein component, possibly cytochrome b558.

The small molecular weight GTP-binding protein, Rac, occurs as two isoforms (Rac1 and Rac2) that are 92% identical in amino acid sequence (23). The two isoforms differ primarily in their C termini; Rac1 but not Rac2 contains a polybasic C terminus. In resting cells, Rac is located in a cytosolic complex with an inhibitory protein, RhoGDI (24–27). Upon cell activation, Rac2, the more abundant isoform in neutrophils (28–30), becomes associated with the plasma membrane (28), and translocation correlates with NADPH oxidase activity (28, 30, 31). Translocation of Rac requires neither p47phox nor p67phox and occurs with different kinetics than these other cytosolic components (34–37), suggesting that the binding of Rac to the plasma membrane is regulated by mechanisms that are distinct from those that regulate p47phox/p67phox assembly. In their isoprenylated forms, both Rac1 and Rac2 can activate superoxide generation in a cell-free system (38–40). Kinetic characterization and binding studies are complicated by the presence of the isoprenyl group, which limits the solubility and requires the presence of a detergent. The proteins in their nonisoprenylated forms can be expressed in and purified from bacteria. However, in their nonisoprenylated forms, only Rac1 activates efficiently. This is because membrane association is essential for optimal activation of Rac, and nonisoprenylated

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1 However, two studies (32, 33) have concluded that activation fails to correlate with translocation.
Rac1 can interact with the membrane via its polybasic C terminus (41). Bacterially expressed versions of Rac1 have therefore been used for most of the studies described herein.

We have previously characterized two regions on Rac1 that are important for its ability to activate the NADPH oxidase. These are the effector region (within the range of residues 26–45) and the insert region (residues 124–135). The effector region shows homology to a region on Ras that has been characterized as mediating the GTP-dependent binding to the Ras effector Raf-1, a member of the MAP kinase cascade (42, 43). The effector region of Ras includes residues the conformation of which changes significantly depending on whether GTP or GDP is bound. The insert region on Rac has no counterpart in Ras. Mutation of residues in both the effector region and the insert region of Rac1 results in a marked decrease in the ability to support cell-free superoxide generation (44–46), and the primary effect was decreased affinity of Rac1, based upon an increase in the EC$_{50}$ (44, 47).

Rac1 has been shown to bind to immobilized p67phox (48, 49), and the interaction has been demonstrated using yeast two-hybrid analysis, but it is not clear whether Rac also interacts with other targets. In addition, no quantitative information on Rac binding to p67phox is available (e.g. affinity, stoichiometry). Herein, steady state kinetic analysis was used to demonstrate the functional linkage between Rac and p67phox. In addition, Rac binding to p67phox was measured directly, making use of a fluorescent GTP analog that binds tightly to Rac as a reporter and binds with a 5-fold molar excess of GTP$_{S}$, and 1 mM cytochrome b$_{558}$ reconstituted with FAD and cytosolic factors were incubated at 20 °C in 0.5 ml of 20 mM Tris acetate buffer, pH 7.45, containing 3 mM NaCl, 50 mM KCl, and 0.1 mM MgCl$_{2}$. Preloading of Rac with mant-GppNHp was carried out for 15–20 min, at which point the fluorescence change due to the guanine nucleotide binding was stable. Low MgCl$_{2}$ concentration was essential to facilitate complete guanine nucleotide exchange. Titrations were carried out by adding p67phox recording fluorescence readings until three successive stable readings, at least 45 s apart, were obtained. Fluorescence changes induced by p67phox occurred rapidly (within 1–2 min) and did not change further with prolonged incubation. Spectral resolution was 5 nm for both the excitation and emission paths, respectively.

Rac-p67phox Complex

Experimental Procedures

Materials—Cytochrome c (type VI), NADPH, N-octyl glucose, diisopropyl fluorophosphate, phenylmethylsulfonyl fluoride, and N-α-tosyl-l-lysine chloromethylethylene were from Sigma. GTP$_{S}$ was purchased from Boehringer Mannheim. HEPES (6% hexatartach in 0.9% NaCl) was from American Hospital Supply Corp., and lymyphocyte separation medium (6.2% Ficoll, 9.4% sodium diatrizoate) was obtained from Organon Tekniker. Superoxide dismutase and diithiothreitol were from Wako Pure Chemical Co. Heparin-Sepharose CL-6B, DEAE-Sepharose CL-6B, CM-Sepharose CL-6B, α-aminocetyl-agarose, and glutathione-Sepharose were purchased from Pharmacia LKB. L-α-phosphatidylcholine (bovine brain), L-α-phosphatidylethanolamine (bovine brain), L-α-phosphatidylinositol (bovine brain), and sphingomyelin (bovine erythrocyte) were from Sigma. All other reagents were of the highest grade available commercially. Methyl isocyanide was purchased from Molecular Probes (Eugene, OR), and mant-GppNHp was synthesized as described previously (51).

Preparation of Recombinant Proteins—Recombinant p67phox was expressed and purified from baculovirus-infected Hi5 insect cells as described (22, 31). Wild type and mutant Rac1 were expressed in E. coli as the glutathione S-transferase fusion proteins, purified using glutathione-Sepharose beads, and cleaved from the glutathione S-transferase domain with thrombin (52). All recombinant proteins were purified to greater than 95% homogeneity.

Isolation of Human Neutrophils and Plasma Membrane Preparation—Human neutrophils were obtained from peripheral blood of normal healthy donors after obtaining informed consent. Erythrocytes were sedimented with HESPAW, and the mononuclear cells were removed from the resulting supernatant by centrifugation through lymphocyte separation medium (53). The resulting cells were greater than 95% neutrophile granulocytes. Neutrophils were resuspended in cavi-
tation buffer (25 mM HEPES, pH 7.4, containing 100 mM KCl, 3 mM NaCl, 5 mM MgCl$_{2}$, 6 mM diisopropyl fluorophosphate, 0.5 mM phenyl-
methylsulfonyl fluoride, 2 μM each leupeptin, pepstatin, and aprotinin). Cells (6 × 10$^{8}$) in 20 ml of ice-cold buffer were disrupted by nitrogen cavitation after being pressurized at 500 p.s.i. for 20 min at 4 °C, and plasma membranes were prepared as described (54).

Purification and Reconstitution of Flavin-depleted Cytochrome b$_{558}$ with FAD—Plasma membrane was solubilized in the presence of 40 mM octyl glucoside and 0.5% sodium cholate (11). Detergent-solubilized cytochrome b$_{558}$ was purified as described previously (9) with some modifications (11). Purified FAD-depleted cytochrome b$_{558}$ (15.6 nmol of heme/mg of protein) was incubated in 50 mM Tris acetate buffer, pH 7.45, containing 5 mM KCl, 10% glycerol, 1 mM diithiothreitol, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 2 μM each leupeptin, pepstatin, and aprotinin (buffer B), and phospholipids (L-$\alpha$-phosphatidylcholine/L-$\alpha$-phosphatidylethanolamine/L-$\alpha$-phosphatidylinositol/sphin-
gomyelin/cholesterol = 4:2:1:3:3 (w/w/w/w/w); lipid/protein = 100, w/w) were added along with a 10-fold excess of FAD over heme. After incubating at 4 °C for 2 h, the mixture was dialyzed against two changes of buffer B to remove free FAD. The FAD-reconstituted material typically contained a FAD/heme ratio of 0.4–0.5 (11).

Spectrophotometric Assay—The heme content of cytochrome b$_{558}$ reconstituted with FAD and cytosolic factors was determined by reduced minus oxidized difference spectroscopy at 424–440 nm using an extinction coefficient of 161 mM$^{-1}$ cm$^{-1}$ (55). The flavin content of FAD-reconstituted cytochrome b$_{558}$ was estimated by the fluorimetric method (11). Fluorescence spectra were recorded with a Hitachi model F-3000 spectrophotometer, and routine fluorescence measurements were made with a Perkin-Elmer LS-5B spectrophotometer. Samples (mant-guanosine 5’-O-(thio)triphosphate [mant-GppNHp], Rac1, cytochrome b$_{558}$, and cytosolic factors) were incubated at 20 °C in 0.5 ml of 20 mM Tris acetate buffer, pH 7.45, containing 3 mM NaCl, 50 mM KCl, and 0.1 mM MgCl$_{2}$. Preloading of Rac with mant-GppNHp was carried out for 15–20 min, at which point the fluorescence change due to the guanine nucleotide binding was stable. Low MgCl$_{2}$ concentration was essential to facilitate complete guanine nucleotide exchange. Titrations were carried out by adding p67phox recording fluorescence readings until three successive stable readings, at least 45 s apart, were obtained. Fluorescence changes induced by p67phox occurred rapidly (within 1–2 min) and did not change further with prolonged incubation. Spectral resolution was 5 nm for both the excitation and emission paths, respectively.

Assay of Cell-free Superoxide Generation—Superoxide generation was measured by superoxide dismutase-inhibitable reduction of cytochrome c (described previously (54) using a assay. The theoretical curve was calculated by use of a nonlinear least squares fit of the data using the Michaelis-Menten equation and were plotted using Sigma Plot. Kinetic constants are reported as V$_{max}$ and E$_{cat}$ (effective concentration at 50% of V$_{max}$). Fluorescence titrations were fit to a single site binding equation as described previously (58) as follows, 

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\Delta F = \Delta F_{max}(K_{D} + L_{e} + R_{p}) - (K_{D} + L_{e} + R_{p})^{2}/4\Delta F_{max}$$

(1)

where $\Delta F$ is the fluorescence change after each addition of p67phox, $\Delta F_{max}$ is the maximal fluorescence change at infinite (extrapolated) p67phox, $K_{D}$ is the dissociation constant, $L_{e}$ is the concentration of p67phox, and $R_{p}$ is the total concentration of Rac(mant-GppNHp). Sigma plot was used to generate a nonlinear least squares fit of the data, solving for $K_{D}$ and $\Delta F_{max}$, constraining the fit to the actual concentration of Rac(mant-GppNHp) used in the experiment. For the Rac(D38N) mutation, it was necessary to assume that $\Delta F_{max}$ to be the same as that of the wild type, since binding was weak and it was not feasible to approach saturation.

The abbreviations used are: mant-GppNHp, methylnlanthraniloyl guanosine-5’-[3-(5, 7-imido)triposphate; GTP$_{S}$, guanosine 5’-O-(thio)triposphate; mant, methylnlanthraniloyl.
RESULTS

Mutually Facilitated Binding of Rac and p67-phox in the NADPH Oxidase Complex, Demonstrated by Steady State Kinetics—We previously showed that a functional interaction between p67-phox and Rac1, which are known to form a complex, can be demonstrated using steady state kinetics assaying superoxide generation; in the presence of the other required components, the EC\textsubscript{50} for each protein was found to vary inversely with the concentration of the partner protein (59). This result indicates a thermodynamic linkage between the binding of the two proteins within the NADPH oxidase complex and is consistent with a complex between the partner proteins. This approach was tried with p67-phox and Rac1 under conventional assay conditions (in the presence of p47-phox), but effects of varying the concentration of one protein on the EC\textsubscript{50} of the other protein were not seen. We attribute this to the already high affinity of both components in the oxidase complex, which makes it difficult to observe further decreases in their EC\textsubscript{50} values, and to the presence of multiple binding interactions for each of the proteins, which may obscure observation of such interactions. However, the system can be simplified, since p47-phox need not be present to observe high activity when high concentrations of p67-phox and Rac1 are used (12). The omission of p47-phox simplifies the kinetic analysis; the 50–100-fold weaker binding of Rac1 and p67-phox makes any decreases in EC\textsubscript{50} values easy to detect, and p47-phox is ruled out as a mediator of any observed kinetic linkage that must therefore be due to direct interactions among the remaining components (Rac1, p67-phox, cytochrome b\textsubscript{558}). Under these conditions, the EC\textsubscript{50} for Rac1 decreased at increasing concentrations of p67-phox (Fig. 1A). Likewise, at increasing concentrations of Rac1, the EC\textsubscript{50} for p67-phox decreased (Fig. 1B). A 6-fold increase in the concentration of p47-phox resulted in a roughly 5-fold decrease in the EC\textsubscript{50} for Rac1, while a 10-fold increase in Rac concentration resulted in a 2.5-fold decrease in the EC\textsubscript{50} for p67-phox. These data imply a functional interaction between p67-phox and Rac1, consistent with a complex between these two proteins.

Characterization of the Binding of Mant-GppNHp, a Fluorescent Derivative of GTP, to Rac1—We previously showed that mant-GppNHp binding to Rac1 is accompanied by an increase in fluorescence at 445 nm, the emission peak of the mant moiety, and we have used this increased fluorescence to quantify the binding of mant-GppNHp to Rac1 and point-mutated types of Rac1 (47). We used this fluorescence increase in the present studies to verify that Rac1, Rac2, Rac1(N26H), Rac1(D38N), Rac1(M54T), Rac1(K132E), and Rac1(L134R) all bind mant-GppNHp tightly and achieve maximal binding within the same time period. Quantifying binding as described previously, all of these recombinant forms of Rac bound the guanine nucleotide with an approximate 1:1 stoichiometry and with apparent K\textsubscript{d} values in the 1–20 nM range. In addition, all had achieved maximal fluorescence change by about 5 min, well within the 15–20 min incubation period used for preloading of Rac1 with the nucleotide. Examination of the Rac1 structure (50) shows that the side chains of these mutants do not directly interact with the nucleotide, are relatively exposed, and can accommodate the respective mutations without altering the overall fold of the protein. As a control, Rac1(T71N), a mutation that in Ras renders this GTPase incapable of binding guanine nucleotide, did not produce any fluorescence change when added to mant-GppNHp. Thus, by preincubating mant-GppNHp in the presence of a slight excess of Rac or Rac mutants, a fully associated complex between the fluorescent nucleotide and Rac was formed. The ability of mant-GppNHp to produce an active conformation of representative versions of Rac1 was also investigated. Mant-GppNHp was compared with GTP\textsubscript{Y}S (0.5 μM each) in their abilities to support NADPH-dependent superoxide generation, using purified oxidase components in the cell-free system. Using wild type Rac1 (Fig. 2), the mant-GppNHp supported superoxide generation to about 60% of the level seen with GTP\textsubscript{Y}S. For representative point-mutated forms (Rac1(N26H) and Rac1(D38N)) that have a reduced ability to stimulate superoxide generation (44), the mant-GppNHp worked nearly as well as GTP\textsubscript{Y}S.

Effects of NADPH Oxidase Component on the Fluorescence of Mant-GppNHp—Fig. 3A (solid line) shows the emission spectrum for mant-GppNHp free in solution. Upon addition of Rac1, there was a small increase in fluorescence (about 5%), as was described previously (dotted line). When p67-phox was added, there was a further increase in the mant fluorescence, and the emission maximum was blue-shifted about 5 nm (Fig. 3A, dashed line). This was not due to any direct effect of p67-phox on the free mant-GppNHp, since p67-phox had no effect on its flu-
The increase in fluorescence of Rac(mant-GppNHp) was used to quantify the strength of binding of Rac to p67phox. Fig. 4 shows the titration of Rac(mant-GppNHp) with p67phox and p47phox. As above, p47phox had no effect on the fluorescence of Rac(mant-GppNHp), while p67phox produced a saturable increase in the fluorescence. The increase in fluorescence fit a theoretical curve (Fig. 4, solid line) for single site binding of p67phox to Rac (i.e. no cooperativity was detected). In the experiment shown, the apparent binding constant was 170 nM, somewhat weaker than published values for the EC50 (≈50 nM) for Rac in cell-free superoxide generation assays (47). The average of 11 such titrations gave an value of 124 ± 15 nM, as

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**TABLE I**

Comparison of binding affinity of native and point-mutated forms of Rac(mant-GppNHp) for p67phox with kinetically determined EC50 values for Rac activation of superoxide generation

| Rac isoform or mutant | Kd (nM) | EC50 (nM) |
|-----------------------|---------|-----------|
| Rac1                  | 124 ± 15 (10) | <500* |
| Rac2                  | 61 ± 26 (2) | 1,300 |
| Rac1 (N26H)           | 800 ± 230 (4) | 2,700 ± 1,000 (3) |
| Rac1 (I33N)           | 3,900 ± 700 (5) | 2,400 ± 1,600 (2) |
| Rac1 (D38N)           | 5,100 ± 920 (6) | 4,600 ± 400 (3) |
| Rac1 (M45T)           | 230 ± 20 (3) | 2,500 ± 1,400 (3) |
| Rac1 (K132E)          | 90 ± 20 (4) | 1,500 ± 500 (3) |
| Rac1 (L134R)          | 100 ± 10 (4) | 1,350 ± 850 (3) |
| Rac/Rho chimera       | >20,000 | No activity |
| Rac1 + p47            | 140 ± 30 (2) | |

* Reported by Freeman et al. (47) as an upper limit value.
* Reported by Kreck et al. (41).
Comparison of the Binding of Rac1, Rac2, and Mutants of Rac1 to p67phox—Titrations of mant-GppNHp complexes of Rac1 and Rac2 with p67phox are shown in Fig. 5. Binding of p67phox to Rac2(mant-GppNHp) gave a somewhat smaller maximal fluorescence yield, but the calculated $K_d$ value was 2-fold lower than that using Rac1(mant-GppNHp), as summarized in Table I. This slightly higher affinity of Rac2 is consistent with studies using the yeast two-hybrid system to detect the interaction of Rac1 and Rac2 with p67phox (49). While quantitation is not possible using this method, the use of the Rac2 hybrid yielded a more intense blue color in the two-hybrid assay, suggesting enhanced binding. In contrast to the increased binding of Rac2 to p67phox, the EC$_{50}$ for Rac2 was markedly elevated compared with Rac1 in the cell-free NADPH-superoxide generation assay (Table I).

Point mutations of Rac1 were previously characterized (44) and shown to produce large changes in the EC$_{50}$ for Rac1 in the cell-free NADPH-superoxide generation assay. Previous published values as well as unpublished experiments were averaged to obtain the EC$_{50}$ values (Table I). The binding of these mutated Rac1 forms was quantified using mant fluorescence as above. Results are shown in Fig. 5, and averages of $K_d$ values obtained in several independent experiments are summarized in Table I. Representative mutations in both the effector region and the insert region were investigated. While native Rac1 bound tightly to p67phox, mutations in the effector region weakened the binding. In particular, the mutations at the 33- and 38-positions showed large effects on binding, while those at the more extreme ends of the effector region (positions 26 and 45) produced smaller but reproducible effects. Thus, decreased binding to p67phox accounts for the decreased ability of effector region mutants to support superoxide generation. In contrast, mutations in the insert region of Rac1 produced large effects on the EC$_{50}$ for superoxide generation in the cell-free system but showed no effect on the $K_d$ for Rac1 binding to p67phox. Thus, Rac1 does not appear to utilize its insert region to bind to p67phox.

Finally, an additional region on Rac C-terminal to residue 143 for interaction with p67phox has been postulated, based on the inability of a Rac143Rho chimera (N-terminal 143 residues from Rac1, with the rest from RhoA) to bind to p67phox using overlay blotting methods (60). Using this chimera, we confirmed that the chimera was inactive in supporting superoxide generation and that it failed to bind significantly to p67phox (Table I).

**DISCUSSION**

**Binding of Rac1 to p67phox**—Direct demonstration of a stoichiometric complex between Rac1 and p67phox has proven to be difficult, presumably due to the kinetic and or thermodynamic lability of the complex. We previously attempted to demonstrate interaction using gel filtration but were unable to detect a stable complex. Binding of Rac isoforms to p67phox has been demonstrated using p67phox-GST fusion protein immobilized on glutathione beads (46) or on a nitrocellulose filter (48, 60) and also by yeast two-hybrid analysis (49). However, a large excess of Rac was used in the first two cases, and the percentage of binding was low compared with that predicted for a 1:1 complex. Using both methods, effector region

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3 D. J. Uhlinger and J. D. Lambeth, unpublished data.
mutations disrupted the interactions, suggesting that the Rac-p67phox binding is relevant to regulation of the NADPH oxidase. While informative, neither of these methods is quantitative, and neither can be used to obtain direct measures of binding affinity or stoichiometry. In the present studies, a fluorescent derivative of GDP has proven to be effective for this purpose. The rant is bound through the 3'-hydroxyl group of the ribose ring. O3' is exposed to solvent, making only a weak hydrogen bond with E31 (50). The mant-GppNHp should bind to Rac without altering the basic interactions between the nucleotide and the protein, thus serving as a good reporter for binding. The quantum yields of mant guanine nucleotides shows high sensitivity to solvent polarity and to small changes in protein environment (51), and mant-modified nucleotides have been used to demonstrate both binding of nucleotides to guanine nucleotide-protein complex with a second protein (58). In the latter study, the complex between RhoGDI and Cdc42Hs has been demonstrated, based on quenching of the fluorescence of the mant moiety. As was shown previously, this fluorescence change was used to quantify the binding of mant-GppNHp to RhoGDI.

A requirement for the use of this method is that the fluorescent group must bind tightly to one of the interacting proteins and that it should induce an active conformation of the protein. The complex between mant-GppNHp and Rac1 was therefore initially characterized. As is shown in Fig. 3, complex formation was accompanied by a small increase in the fluorescence of the mant moiety. As was shown previously, this fluorescence change was used to quantify the binding of mant-GppNHp to Rac1 (47). In agreement with earlier studies, the complexes show high affinity, with apparent \( K_d \) values ranging from 1 to 20 nM for native and point mutated forms of Rac. Mutant forms

| Rac   | Activity | Binding to p67phox | Reference |
|-------|----------|-------------------|-----------|
| Rac1 (WT) | ++++     | Normal            | 44        |
| Rac1 (N26H) | +        | Weak              | 44 and present study |
| Rac1 (A27K) | +        | ND             | 66        |
| Rac2 (F28L) | +        | ND              | 45        |
| Rac1 (G30S) | +        | ND              | 66        |
| Rac1 (I33N) | +        | Weak            | 44 and present study |
| Rac1 (I33V) | ++++     | ND              | 66        |
| Rac1 (T35A) | +        | Weak            | 46        |
| Rac2 (T35A) | +        | ND              | 49        |
| Rac2 (V36R) | +++     | ND              | 45        |
| Rac1 (D38N) | +        | Weak            | 44 and present study |
| Rac1 (D38A) | +        | Weak            | 46        |
| Rac2 (D38A) | +        | ND              | 45        |
| Rac1 (Y40K) | +        | Weak            | 46        |
| Rac1 (M45T) | +        | Weak            | 44 and present study |
| Rac1 (Q61H) | +        | ND              | 45        |
| Rac2 (A61L) | +++     | ND              | 45        |
| Rac1 (Y64F) | +++     | ND              | c         |
| Rac1 (D65N) | +++     | ND              | c         |
| Rac1 (V55E) | +++     | ND              | c         |
| Rac1 (K102E) | ++++    | ND              | d         |
| Rac1 (H104A) | ++++    | ND              | d         |
| Rac1 (H105A) | ++++    | ND              | d         |
| Rac1 (E127Q) | +       | ND              | 47        |
| Rac1 (K130N) | +       | ND              | 47        |
| Rac1 (K132E) | +       | Normal           | 47 and present study |
| Rac1 (L134R) | +       | Normal           | 47 and present study |
| Rac1 (T135N) | +       | ND              | 47        |

\( a \) ND, not determined.
\( b \) Inhibits GTPase activity.
\( c \) J. Freeman and J. Lambeth, unpublished results.
\( d \) M. Kreck and J. Lambeth, unpublished results.
of Rac showing elevated $K_d$ values for guanine nucleotide binding (including Rac1(A59T) and Rac1(T75K)) were eliminated from consideration in this study. The mant-GppNHp generates an active conformation of Rac1, albeit somewhat less active than that produced by GTPγS. We presume that the binding affinities generally reflect those that occur in the active complex with nonfluorescent nucleotide, but it is possible that these are perturbed in subtle ways due to the presence of the fluorescent group.

Using the fluorescence method we find that Rac(mant-GppNHp) binds to p67phox with a $K_d$ of 110 nM. While this is a moderately high affinity, it is considerably weaker than the binding of many signaling complexes, perhaps accounting for the instability of this complex to gel filtration chromatography and the low stoichiometry of binding found using other methods. The $EC_{50}$ for Rac1 in activating superoxide generation is $\leq 60$ nM (the actual $EC_{50}$ is probably less, since this represents an upper limit value; see Ref. 47). The most likely explanation for the lower value for the $EC_{50}$ is that other binding interactions in addition to p67phox participate in Rac binding within the NADPH oxidase complex.

Structural Considerations and Rationale for the Effects of Point Mutations on Binding and Activity—Table II lists most of the point mutations that have been characterized either in our own laboratory or in those of other labs and depicts effects on activity and on binding to p67phox. Because the methodologies used and the expression of data are not directly comparable, the relative activity is indicated by addition symbols (+), with ++ representing normal activity, and binding is summarized as either “normal” or “weak.” Inspection of Table II reveals two regions, the effector region and the insert region, that have large effects on activity and reveals additional regions (e.g. residues in the range of residues 61–105) that have small or no effects. The effector region has been studied extensively by several groups, and a large number of mutations have been made. Except for conservative replacements (e.g. Rac1(I33V)), most changes in this region have large effects on the ability of Rac1 and Rac2 to activate cell-free superoxide generation. The structure of Rac1-GppNHp was recently determined by x-ray crystallography (50) and is shown in Fig. 6A, with the guanine nucleotide indicated in violet. The overall fold of the protein is very similar to that of Ras except for two regions. The insert region, which forms a well defined, exposed, helical domain, and residues 28–38, which are very flexible and are less defined in the x-ray structure (see Ref. 50 for a discussion of these two regions). Effector region mutations summarized in Table II are indicated in maroon, and insert region mutations are in green. As shown in Fig. 6B, the effector region and the insert region represent distinct regions on Rac1. Each can be envisioned as forming a surface for interaction of partner proteins within the NADPH oxidase complex. Except for position 45, which is slightly more distant, all of the effector region mutations cluster closely to form a single surface. Other possible exceptions within the effector region may include threonine 35, which coordinates the magnesium, which is involved in guanine nucleotide binding (50). Other residues within this region are exposed on the surface as part of the effector loop, and mutations of these residues are unlikely to produce a long range effect on the structure of Rac1. Representative mutations (indicated by stars in Fig. 6A) within this region were investigated for their effects on binding to p67phox. These mutations resulted in effects, some quite large, on binding to p67phox. All effector region mutations also affected the $EC_{50}$ for Rac1 in the cell-free assay. Quantitatively, the magnitude of the effects on binding paralleled the effects on the $EC_{50}$ for mutations at residues 33 and 38, but the effects on binding were smaller than the effects on EC50 for mutations at positions 26 and 45. This may indicate that the presence of other protein component within the NADPH oxidase complex perturbs the binding of Rac1 to p67phox, particularly at the periphery of the effector region, or that the different assay conditions perturbed the binding energies. The fact that all of the mutations produced effects on activity and binding argues strongly that the effector region is utilized within the NADPH oxidase complex for binding to p67phox.

In contrast to the effector region, representative activity-affecting mutations in the insert region (K132E and L134R in Fig. 6A) had no effect on the binding to p67phox. These residues are largely exposed on the surface of the insert region, and mutations are unlikely to produce structural perturbations outside of the effector region. In addition, the mutated residues do not directly contact the guanine nucleotide, consistent with a lack of effect on guanine nucleotide binding or hydrolysis. Thus, the most likely explanation for the effects of insert region mutations on the $EC_{50}$ for Rac1 is that these residues participate in binding to another component within the NADPH oxidase complex. We have previously shown that the association of the polycystic C terminus of (nonisoprenylated) Rac1 with the membrane is essential for optimal activity and that this does not occur with nonisoprenylated Rac2 (41). The insert region lies at the opposite pole from the C terminus (Fig. 6B), making unlikely that both regions interact with the membrane. Thus, the data are most consistent with the insert region interacting with a distinct protein component of the NADPH oxidase rather than with the membrane. Since insert region mutations reduce relative activity regardless of whether p47phox is present (data not shown), we speculate that the insert region binds directly to cytochrome b$_{558}$. We propose a model in which a minimum of three interaction regions on Rac are important for reconstituting cell-free NADPH oxidase activity. The C terminus anchors the Rac to the membrane, while the effector region and the insert region bind, respectively, to p67phox and to another component of the oxidase, possibly cytochrome b$_{558}$.

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It is not clear from the Rac structure whether the poor binding of p67phox by the Rac-Rho chimera reflects an additional binding surface for p67phox within the 143–175 range. A peptide within this range centered around 163–169 is inhibitory (65), but its mechanism of inhibition has not been reported. In addition, this region contains relatively buried $a$-helix, and it is difficult to envision this helix acting as a binding surface. Much of the 143–175 range also is buried and provides a structural foundation for part of the effector loop. This region differs in 20 of 33 residues between Rac1 and RhoA, and it seems possible that such changes may perturb binding indirectly via effects on the Rac effector loop. Thus, while it is conceivable that this region contains an additional binding site, an indirect effect cannot be ruled out.
