Rac "Insert Region" Is a Novel Effector Region That Is Implicated in the Activation of NADPH Oxidase, but Not PAK65*

(Received for publication, March 7, 1996, and in revised form, May 20, 1996)

Jennifer L. Freeman‡§, Arie Abo†, and J. David Lambeth‡¶
From the ‡Department of Biochemistry, Emory University Medical School, Atlanta, Georgia 30322 and ¶Onyx Pharmaceuticals, Richmond, California 94806

The small GTPase Rac assembles with the cytosolic p47phox and p67phox and the membrane-associated flavocytochrome b558 to form the multicomponent respiratory burst oxidase. Mutation of amino acids in a region of Rac (residues 26–45), homologous to an effector region in Ras, was previously shown to interfere with Rac binding to the oxidase. Herein we have elucidated an additional region in Rac involved in regulating oxidase activity. Rho family small GTPases contain a 12-amino acid "insert" region (residues 124–135) that is not present in Ras. Point mutations in and deletion of this region were constructed and used for in vitro studies of the activation of PAK65 and NADPH oxidase. Apparent binding constants (based on EC50 values) of the mutant Rac proteins for the oxidase are at least 13-25-fold higher than for wild-type Rac. Mutations in the insert region versus the 26–45 effector region resulted in distinct kinetic consequences, pointing to different roles for these two protein regions: mutations in the insert region but not the 26–45 effector region resulted in an increase in the EC50 for p67phox. Although mutations in the 26–45 amino acid effector region showed markedly diminished activation of both PAK and the NADPH oxidase, insert region mutations did not affect activation of PAK. We propose that the combinatorial use of the 26–45 effector region and the insert region provides the Rho family GTPases with versatility in their specificity for several downstream targets.

During the respiratory burst, neutrophils and other phagocytic cells reduce molecular oxygen to generate superoxide anion, with subsequent production of secondary products such as hydrogen peroxide and hydroxyl radical, all of which participate in microbial killing. The enzyme that initiates the respiratory burst, the NADPH oxidase, is a multicomponent enzyme that utilizes reducing equivalents from NADPH to reduce oxygen to superoxide (reviewed in Ref. 1). The oxidase is dormant in resting cells but becomes activated in response to microorganisms or to a variety of soluble agonists. Activation involves the assembly of three cytosolic proteins, p47phox, p67phox, and the GTP-binding protein Rac (1 or 2), with the membrane-bound flavocytochrome b558. The flavocytochrome contains flavin, heme, and the NADPH binding site but catalyzes oxygen reduction only when the cytosolic components are associated.

In response to cell activation, p47phox, p67phox, and Rac translocate to the cytochrome (2–5). SH3 domains in p47phox bind directly to proline-rich sequences in the p22phox subunit of the cytochrome (6), and p67phox binds directly to p47phox (7), also utilizing SH3 domains in the former to bind to proline-rich regions in the latter. Kinetic evidence also supports a direct interaction between p67phox and cytochrome b558 (8). Based on experiments using cells from chronic granulomatous disease patients lacking either p47phox or p67phox, Rac translocation occurs independently of these components (9). NADPH oxidase activation (9–11) and partial assembly (12, 13) can be reconstituted in vitro using recombinant p47phox, p67phox, Rac (1 or 2), and either neutrophil plasma membrane or purified, lipid-reconstituted cytochrome b558 plus the activators GTP·γS1 and an anionic amphiphile such as arachidonate.

Rac is a small GTPase belonging to the Rho family of proteins. Rho family GTPases regulate cytoskeletal rearrangements (14–16) and participate in cell growth and transformation (17, 18). Direct targets of this family of GTPases include phospholipase D (Rho) (19–21) and the protein kinase PAK (22, 23) as well as the respiratory burst oxidase (Rac). The Rho type GTPases are approximately 30% homologous to Ras and 50% homologous to each other. As shown in Fig. 1, Ras contains a well studied region within residues 26–45, which participates in the binding of downstream effector enzymes such as Raf (24). Contained within this span is the Switch I region, which undergoes a conformational change depending on whether GTP or GDP is bound. The amino acid sequence of Rac in this region is highly homologous to that in Ras, suggesting conservation of the effector binding function of this region. Site-specific mutations (25–27) and chimeric proteins of Rac/Cdc42 (28) assayed for the ability to support superoxide generation demonstrate that this region is important for the interactions within the NADPH oxidase complex. Rac can bind directly to p67phox, and mutations in amino acids within this region eliminate this interaction (27).

One of the major differences between Rac and Rho family proteins is the presence of an additional 12-amino acid "insert region" in the Rho family proteins (Fig. 1). This region is predicted by molecular modeling to form a surface loop near the GTP binding site,2 but the function of this region has not been investigated. In the present studies, we have used site-directed mutagenesis and deletion analysis to examine the effector function of the insert region, using both the NADPH oxidase and PAK as target enzymes. We find that for the NADPH oxidase but not for PAK, the insert region functions as a novel effector region.

* This work was supported by National Institutes of Health Grant AI22809. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Supported by National Institutes of Health Predoctoral Training Grant GM08367.
‡ To whom correspondence should be addressed: Tel.: 404-727-5875; Fax: 404-727-2738; E-mail: dlambe@bimcore.emory.edu.

1 The abbreviations used are: GTP·γS, guanosine 5′-O-(3-thiotriophosphate); PAK, p21-activated kinase; Mant, N-methylanthraniloyl; Gpp(NH)p, 5′-guanylylimidodiphosphate; PCR, polymerase chain reaction; PIPES, 1,4-piperazinediethanesulfonic acid.

2 J. D. Lambeth and J. L. Freeman, unpublished results.
Fig. 1. Comparison of amino acid sequence of Rho family proteins with Ras. The sequences of these small GTPases were aligned using the Pretty Sequence program of the Wisconsin Package Interface.

EXPERIMENTAL PROCEDURES

Materials—Hespan (6.2% hetastarch in 0.9% NaCl) was obtained from American Hospital Supply Corp. Lymphocyte separation medium (6.2% Ficol, 9.4% sodium diatrizoate) was purchased from Organon Tekniker. N-Methylisatoc anhydride (Mant) was obtained from Molecular Probes. NADPH, FAD, cytochrome b_565 was purified by on a Sephadex LH-20 column. The concentration of Mant-GppNHp was determined according to Bradford (33). hPAK65 was expressed and purified as described by Uhlinger et al. (12, 32). Protein concentration was determined according to Bradford (33). hPAK65 was expressed and purified as described by Uhlinger et al. (12, 32).

Rac Insert Region Regulates NADPH Oxidase, Not PAK65

3 J. D. Lambeth, unpublished results.
Rac Insert Region Regulates NADPH Oxidase, Not PAK65

Characteristics of Mant-GppNHp binding and GTP hydrolysis of Rac1 mutations

The indicated mutations were constructed in Rac1, protein was expressed as a GST-fusion protein, and GTP binding and the first order rate constant for GTP hydrolysis were determined as described under "Experimental Procedures." Data shown are representative of two or three experiments.

| Mutation | Apparent K_d | Apparent stoichiometry | GTP hydrolysis |
|----------|--------------|------------------------|---------------|
| None     | 5            | 1.0                    | 0.35          |
| E127Q    | ND           | ND                     | 0.29          |
| K130N    | ND           | ND                     | 0.37          |
| K132E    | ND           | ND                     | 0.29          |
| L134R    | 11           | 1.1                    | ND            |
| T135N    | 6            | 1.0                    | 0.35          |
| Delta    | 34           | 1.3                    | 0.35          |

* The K_d and stoichiometry are reported as apparent because the GDP that is associated with Rac during purification may compete with Mant-GppNHp, causing an overestimation of the K_d and the stoichiometry. Because the apparent stoichiometry is close to the theoretical value of 1:1, influence of GDP does not appear to be substantial.

Characterization of Rac1 mutations with respect to superoxide generation and kinetic parameters

The indicated mutations were constructed in Rac1 and expressed as GST fusion proteins. Superoxide generation and kinetic parameters were determined as described under "Experimental Procedures." Data are representative of three experiments.

| Mutation | Superoxide generation | EC_{50} |
|----------|----------------------|---------|
|          | Rate at 1 μM | V_{max} | Rac | 47 | 67 |
| None     | ND | ND | ND | ND |
| E127Q    | ND | ND | ND | ND |
| K130N    | 83 | 690 | 1380 | 0.6 | 0.8 |
| K132E    | 58 | 89 | 1530 | 0.6 | 1.3 |
| L134R    | 52 | 85 | 1350 | 0.4 | 0.8 |
| T135N    | 60 | 88 | 820 | 0.6 | 1.2 |
| Delta    | 52 | 68 | 950 | 0.7 | 0.6 |

Characterization of Mutant Proteins: Binding and Hydrolysis of Guanine Nucleotides—To determine whether the mutations in Rac caused a major perturbation in the structure, Rac and Rac mutants were tested for their ability to bind and hydrolyze guanine nucleotides. Using a fluorescent analog of GppNHp, a nonhydrolyzable analogue of GTP, the binding of the nucleotide was determined as in Fig. 2. The reaction was terminated by adding 10 μl of 5 × SDS-polyacrylamide gel electrophoresis sample buffer and heating in boiling water for 5 min. Samples were applied to a 14% SDS-polyacrylamide gel, 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 ^32P was counted.

RESULTS

Characterization of Rac1 and Rac1 insert mutants in superoxide generation in a semicombinant cell-free NADPH oxidase system. Plasma membrane (10 μg) was preincubated at 25°C with recombinant p47^{phox} (1.3 μM) and p67^{phox} (1.3 μM) in 10 μM GTP-γS, 160 μM arachidonate, and the indicated concentration of preloaded Rac1 or Rac1 mutant protein in a volume of 50 μl. The Rac proteins were preincubated by incubating 0.5 μl of Rac with 100 μM GTP-γS at 15 min at 25°C. 10 μl of the incubation mixture was diluted to a total volume of 250 μl in buffer containing 200 μM NADPH and 80 μM cytochrome c. Superoxide generation was determined spectrophotometrically as described under "Experimental Procedures." Data shown are representative of a minimum of two experiments.

Fig. 3. Concentration dependence for Rac1 and Rac1 insert mutants in superoxide generation in a semicombinant cell-free NADPH oxidase system. Plasma membrane (10 μg) was preincubated at 25°C with recombinant p47^{phox} (1.3 μM) and p67^{phox} (1.3 μM) in 10 μM GTP-γS, 160 μM arachidonate, and the indicated concentration of preloaded Rac1 or Rac1 mutant protein in a volume of 50 μl. The Rac proteins were preincubated by incubating 0.5 μl of Rac with 100 μM GTP-γS at 15 min at 25°C. 10 μl of the incubation mixture was diluted to a total volume of 250 μl in buffer containing 200 μM NADPH and 80 μM cytochrome c. Superoxide generation was determined spectrophotometrically as described under "Experimental Procedures." Data shown are representative of a minimum of two experiments.
less, the finding that the stoichiometry approach the theoretical value of 1:1 suggests that the affinity of Rac for the Mant GppNHp is significantly higher than for GDP. This method gives higher stoichiometry than the more commonly used filter binding assay and demonstrates that all of the recombinant proteins are capable of binding GTP tightly. Apparent K_d values were calculated based on the average of several points (open circles, Fig. 2) near the end point, as described under “Experimental Procedures.” Apparent K_d values and apparent stoichiometries for GppNHp binding to Rac and mutant Rac proteins are summarized in Table I (columns 1 and 2). The apparent K_d values for RacL134R and RacT135N are the same within experimental error as wild-type Rac. Deletion of the entire insert region resulted in a 7-fold increase in the apparent K_d. However, binding was still tight, and the concentration of guanine nucleotide used in the assays was sufficient to fully saturate this form of Rac.

To further characterize the mutant proteins, the GTPase rates were determined as described under “Experimental Procedures.” As summarized in Table I (column 3), the rate constants for the mutated and deletion forms of Rac were identical within experimental error to those of the wild-type Rac.

The Insert Region of Rac Participates in NADPH Oxidase Activity—When 1 μM Rac containing either a single amino acid mutation in the insert region or a complete deletion in the insert region was used in place of wild-type Rac in the cell-free assay for superoxide generation, the amount of superoxide dismutase inhibitable superoxide production was reduced by approximately 50% (Table II, column 1). This concentration of wild-type Rac should be saturating, indicating that the mutant Rac proteins either bind or activate more poorly than the wild-type Rac.

The lower activity of these mutants as compared with wild-type Rac was further investigated by conducting detailed kinetic analyses of the mutant proteins to determine the EC50 and V_max values. Fig. 3 shows rate plots measuring superoxide generation in a semirecombinant NADPH oxidase system using native and mutant Rac1. Conditions were as described in the legend to Fig. 1, except that the final concentration of Rac1 was 1 μM, the Rac mutants was 3 μM, and the concentration of p47phox was varied as indicated. The rate of superoxide generation was measured as described in the legend to Fig. 1, and the percentage of V_max is plotted versus the p47phox concentration. Data shown are representative of a minimum of two experiments.

---

4 J. L. Freeman, unpublished results.
generation in which the concentration of Rac or Rac mutant proteins were varied, while the other oxidase components were held constant at saturating levels. Fig. 4 shows the same experiment using the insert region deletion mutant. The data were fit to the Michaelis-Menten equation using a nonlinear least squares method to obtain the \( V_{\text{max}} \) and \( \text{EC}_{50} \) values. \( \text{EC}_{50} \) values are formally equivalent to the \( K_d \) values for binding to the oxidase complex if it is assumed that the Rac is functioning as an activator and does not itself participate in the catalytic reaction. As shown in Table II (column 3), \( \text{EC}_{50} \) values for single site mutants of the insert region were at least 13–25-fold higher than wild-type Rac, whereas the deletion of the entire insert region resulted in a 17-fold increase in \( \text{EC}_{50} \) compared with wild type. The \( V_{\text{max}} \) values for the single site mutant proteins approached that of wild-type Rac (74–88%), but the deletion mutant showed a slightly lower \( V_{\text{max}} \), 68% (Table II, column 2), perhaps due to a more general effect on the structure of the deletion mutant.

The above assays utilized isolated plasma membranes as a source of the cytochrome. To eliminate the possibility that the decrease in NADPH oxidase activity seen with the mutant Rac proteins was due to an interaction with some membrane component other than cytochrome b\(_{558}\), the latter was purified, reconstituted with phospholipid and FAD, and used in combination with the other purified recombinant NADPH oxidase components. Fig. 5A shows that at a concentration near the \( \text{EC}_{50} \) for native Rac (47 nM), the activity of the mutant proteins is considerably diminished compared with the wild-type proteins. In Fig. 5B, 470 nM of wild-type Rac produces maximal superoxide generation, but this concentration of mutant Racs is still subsaturating. Fig. 5C shows that at very high concentrations (1400 nM), the wild-type and mutant Racs both produce similar rates of superoxide production. These data confirm that the major effect of the insert region mutations is on the binding of Rac to the NADPH oxidase complex.

**Effect of Rac Insert Region Mutations on the Binding of p47\(_{\text{phox}}\) and p67\(_{\text{phox}}\)**

To further investigate the role of the amino acids in the insert region, the concentrations of either p47\(_{\text{phox}}\) or p67\(_{\text{phox}}\) were varied in the presence of either 1 \( \mu M \) wild-type Rac or 3 \( \mu M \) Rac insert region mutations (Figs. 6 and 7, respectively, for single site mutants and Fig. 8 for the deletion mutant). The \( \text{EC}_{50} \) value for p47\(_{\text{phox}}\) did not change significantly with a saturating concentration of the mutant Racs (Table II, column 4). The \( \text{EC}_{50} \) value for p67\(_{\text{phox}}\), however, increased by 2–4-fold when the mutant Racs were used in place of wild-type Rac (Table II, column 5). This increase, although relatively small, was highly reproducible. Variation in the concentration of wild-type Rac did not result in similar changes (data not shown), indicating that the effect was not due to the degree of saturation of Rac with the NADPH oxidase complex.

Mutations in Rac Alter PAK and NADPH Oxidase Activi-

---

5 The \( \text{EC}_{50} \) for wild-type Rac is likely to be less than 60 nM (see Table II), which represents an upper limit. Data fits to values less than this are not accurate because the Michaelis-Menten assumption that \( [S]_\text{free} = [S]_\text{total} \) is violated. Thus, the \( \text{EC}_{50} \) value for the wild-type Rac is reported as less than or equal to 60 nM.
Using Rac or Rac mutants, the rate of superoxide production by the NADPH oxidase was compared with the activity of PAK (Fig. 9). The concentrations of Rac used in each assay were below the respective EC50 values for both PAK and the NADPH oxidase. Wild-type Rac and the R102E mutants activated both superoxide generation and PAK, whereas mutations disrupting guanine nucleotide binding (T17N and A59T) were inactive in both systems. Mutations I33N, D38N, and M45T in the Ras homologous effector region (amino acids 26–45) and a previously uncharacterized mutation, T75N, also drastically reduce the ability to activate both the NADPH oxidase and PAK. The N26H mutation reduced NADPH oxidase activity but did not significantly affect activation of PAK. In contrast, mutants in the insert region (amino acids 124–135) showed a marked decrease in the ability to support NADPH oxidase activity but had no significant effect on PAK activity.

DISCUSSION

Like other members of the Rho family, Rac contains 12 amino acids (residues 124–135) C-terminal to the GTP binding domain that are not found in other small GTPases. This insert region represents one of several areas of divergence among Rho family members (41). Because Rac is the only Rho family member able to support significant NADPH oxidase activity and because other regions of sequence divergence between Rac and the other Rho family members are important for its specificity, we examined these 12 amino acids (124–135) by mutational and deletional analysis. Single site mutants of amino acids in this region, as well as deletion of the entire insert region, result in a significantly diminished ability of Rac to support superoxide generation by the NADPH oxidase. This effect is largely due to a decreased affinity of Rac for the NADPH oxidase complex (as reflected in an increased EC50 for the small GTPase). In contrast, there was little or no effect of these mutations on the ability of Rac to activate PAK. The latter can also be activated by Cdc42 (22, 23). Additional evidence for a role for this region in NADPH oxidase function comes from recently published "peptide scanning" studies (42), in which peptides spanning this region of Rac inhibited cell-free NADPH oxidase activity. In contrast, studies using chimeric expressed proteins composed of Rac and Rho failed to identify this region as being functionally relevant but did identify an additional region near the C terminus (contained within residues 143–175), which, like the Ras-like effector region (residues 26 to 45), was found to mediate the interaction of Rac with both p67phox and PAK (43).

It has become increasingly evident that each of the small GTPases can bind to and regulate multiple effector targets. Ras, for example, interacts not only with Raf but also with PI 3-kinase (44), Ral GDS (45–47), and Rin (48). This raises important and as-yet unanswered questions regarding the maintenance of specificity and regulatory integrity in a multiply branching pathway. Our studies indicate that insert region amino acids comprise a novel effector region for Rac and imply...
that this region may also be important in some of the functions of the other Rho family proteins. The use of the classical Ras-like effector region in combination with the insert effector region may provide the Rho family proteins with flexibility in terms of the spectrum of proteins with which they interact. For example, Rac and Cdc42, which are 80% homologous (70% identical) in the Ras-like effector region, both activate PAK. The Ras-like effector region participates in this interaction, but the insert region does not influence binding (see below). In contrast, Rac but not Cdc42 effectively binds to and activates the NADPH oxidase. Rac and Cdc42 are 50% homologous (50% identical) within the insert region. The combined use of both the Ras-like effector region and the insert effector region may convey the required specificity and affinity to permit Rac but not Cdc42 to function as an NADPH oxidase activator. Thus, the use of two or more effector regions on small GTPases permits Rac and Cdc42 to utilize a combinatorial approach to specificity.

Titrations with cytosolic components p47phox and p67phox in the semirecombinant cell-free system demonstrate that the Rac insert region mutations have a modest but reproducible effect to increase the EC50 of p47phox for the NADPH oxidase complex. No effect was seen on the EC50 for p47phox. Insert region mutations had very little effect on the Vmax (saturating Rac concentrations). In contrast, mutations in the Ras-like effector region have no observable effect on the EC50 for p47phox or p47phox (26). Thus, the kinetic consequences of mutations in these two effector regions are significantly different. Although there are several molecular interpretations for these kinetic differences, they are likely to reflect different functions for these two regions. By molecular modelling based on the Ras structure, the insert region is predicted to form a surface loop that is nearly on the opposite side of Rac from the Ras-like effector region. An attractive model is that Rac binds through these two regions to different surfaces within the NADPH oxidase and possibly to different components of this multicomponent enzyme. The use of two regions may permit Rac to participate in the assembly or orientation of oxidase components to form the activated NADPH oxidase complex.

In this regard, there is evidence for the binding of Rac to at least two components of the oxidase. A direct interaction between p67phox and Rac has been reported by several laboratories using p67phox affinity chromatography (27), binding to nitrocellulose-blotted protein (43, 49), and the yeast two-hybrid method (50). In these studies, changes in residues 26–45 and 143–175 inhibited binding to p67phox, implying that the interaction occurs directly with the Ras homologous effector region and an effector region C-terminal to the GTP binding domain. In studies of Rac translocation to the plasma membrane using normal and chronic granulomatous disease neutrophils, Rac translocation was somewhat diminished in cells lacking cytochrome b558 (5). This result was not seen in cells lacking either p47phox or p67phox, and data were interpreted as indicating a direct interaction between cytochrome b558 and Rac. Although these binding models are often viewed as competing theories, the finding of an additional binding surface that is important for oxidase activity makes it plausible that both binding models are correct and that Rac serves to bridge between the cytochrome b558 and p67phox.

In light of the above model, it is important to interpret the effect of insert region mutations on the EC50 for p67phox. This could result from one of at least three mechanisms: 1) this region of Rac could be directly interacting with p67phox, 2) the insert region might bind to and alter the conformation of another component of the NADPH oxidase to which p67phox binds directly, or 3) Rac may bind in close proximity but not directly to p67phox, and the altered binding of p67phox might be due to electrostatic effects when some of the positive charges in the insert region are changed to negative charges. The latter possibility can be ruled out, because the deletion mutation that does not introduce any charges also results in the increased EC50 for p67phox. As yet, there is no evidence that the insert region binds directly to p67phox. Thus, the present data do not distinguish between a direct binding to p67phox and an indirect effect due to conformational or other changes in the cytochrome. They are therefore consistent with a model in which Rac binds to both the cytochrome and to p67phox. Studies are underway to investigate the direct interactions of Rac with other components of the respiratory burst oxidase.

In summary, the present studies have described a novel effector region present on Rac that is important for activation of the respiratory burst oxidase but not PAK. This region, along with the Ras-equivalent effector region, may permit small GTPases to utilize a combinatorial approach to target specificity. The use of opposing molecular surfaces on Rac to activate the multicomponent respiratory burst oxidase suggests a model in which Rac functions to link two or more other components of the oxidase in a GTP-dependent manner.

Acknowledgments—We thank Mary L. Kreck for sharing unpublished data and for helpful discussion, Mitch Balish for technical advice, and Yukio Nisimoto for advice with cytochrome b558 purification. We also thank Michelle Demain for excellent secretarial assistance.

REFERENCES
1. Quinn, M. (1995) J. Leukocyte Biol. 58, 263–276
2. Heyworth, P. G., Curnutte, J. T., Nuse, W. M., Volpp, B. D., Pearson, D. W., Rosen, H., and Clark, R. A. (1993) J. Clin. Invest. 87, 352–356
3. Clark, R. A., Volpp, B. D., Leidal, K. G., and Nuse, W. M. (1990) J. Clin. Invest. 85, 714–723
4. Bokoch, G., Bohl, B., and Chuang, T. (1994) J. Biol. Chem. 269, 31674–31679
5. Heyworth, P., Bohl, B., Bokoch, G., and Curnutte, J. (1994) J. Biol. Chem. 269, 30749–30752
6. Leto, T., Adams, A., and Mendez, I. (1994) Proc. Natl. Acad. Sci. U.S.A. 91, 10560–10564
7. Finan, P., Shimizu, Y., Gout, I., Hsuan, J., Truong, O., Butcher, C., Bennett, P., Waterfield, M. D., and Kelle, S. (1994) J. Biol. Chem. 269, 13752–13755
8. Uhlinger, D., Taylor, K., and Lambeth, J. D. (1994) J. Biol. Chem. 269, 22095–22098
9. Abe, A., Pick, E., Hall, A., Totty, N., Teahan, C. G., and Segal, A. W. (1991) Science 253, 668–670
10. Abe, A., Boyhan, A., West, I., Thrasarher, A. J., and Segal, A. W. (1992) J. Biol. Chem. 267, 16767–16770
11. Kreck, M. L., Uhlinger, D. J., Tyagi, S. R., Ine, K. L., and Lambeth, J. D. (1994) J. Biol. Chem. 269, 4161–4168
12. Uhlinger, D. J., Tyagi, S. R., Ine, K. L., and Lambeth, J. D. (1993) J. Biol. Chem. 268, 8624–8631
13. Quinn, M. T., Evans, T., Lotetter, L. R., Isetalis, A. J., and Bokoch, G. M. (1993) J. Biol. Chem. 268, 20983–20987
14. Ridley, A. (1995) Curr. Opin. Genet. Dev. 5, 24–30
15. Hall, A. (1994) Annu. Rev. Cell Biol. 10, 31–54
16. Takai, Y., Sasaki, T., Tanaka, K., and Nakanishi, H. (1995) Trends Biochem. Sci. 20, 227–230
17. Qiu, R., Chen, J., Kirk, D., McCormick, F., and Simons, M. (1995) Nature 374, 457–459
18. Vojtek, A., and Cooper, J. (1995) Cell 82, 527–529
19. Bowman, E. P., Uhlinger, D. J., and Lambeth, J. D. (1993) J. Biol. Chem. 268, 21509–21512
20. Malcolm, K., Ross, A., Qiu, R., Simons, M., and Exton, J. (1994) J. Biol. Chem. 269, 25951–25954
21. Kwak, J.-Y., Lopez, I., Uhlinger, D. J., Ryu, S. H., and Lambeth, J. D. (1995) J. Biol. Chem. 270, 27090–27098
22. Martin, G., Bonlack, G., McCormick, F., and Abe, A. (1995) EMBO J. 14, 1770–1778
23. Manser, E., Leung, T., Salihuddin, H., Zhao, Z., and Lim, L. (1994) Nature 370, 40–46
24. Marshall, M. S. (1993) Trends Biochem. Sci. 18, 250–254
25. Xu, X., Barry, D., Settlemier, J., Schwartz, M., and Bokoch, G. (1994) J. Biol. Chem. 269, 23569–23576
26. Freeman, J. L., Uhlinger, D. J., and Lambeth, J. D. (1994) Biochemistry 33, 13431–13435
27. Diederich, D., Abo, A., Jhonsen, C., Segal, A. W., and Hall, A. (1994) Science 265, 531–532
28. Kwong, C., Adams, A., and Leto, T. (1995) J. Biol. Chem. 270, 19868–19872
29. Bowman, E., Uhlinger, D., and Lambeth, D. (1995) in Small GRPases and Their Regulators, Part B: Rho Family Proteins, Curr. Top. Biochem. Pharmacol. 7, 137–170
30. Segal, A. W., West, I., Wientjes, F., Nugent, J. H., A. Chavan, A. J., Haley, B., Garcia, R. C., Rosen, H., and Scrase, G. (1992) Biochem. J. 284, 781–788
31. Nisimoto, Y., Otsubo-Murakami, H., and Lambeth, D. (1995) J. Biol. Chem. 270, 33–54
Rac Insert Region Regulates NADPH Oxidase, Not PAK65

32. Uhlinger, D. J., Inge, K. L., Kreck, M. L., Tyagi, S. R., Neckelmann, N., and Lambeth, J. D. (1992) Biochem. Biophys. Res. Commun. 186, 509–516
33. Bradford, M. (1976) Anal. Biochem. 72, 248–254
34. Hiratsuka, T. (1983) Biochim. Biophys. Acta 742, 496–508
35. Leonard, D., Evans, T., Hart, M., Cerione, R., and Manor, D. (1994) Biochemistry 33, 12323–12328
36. Randazzo, P., and Kahn, R. (1994) J. Biol. Chem. 269, 10758–10763
37. Burnham, D. N., Uhlinger, D. J., and Lambeth, J. D. (1990) J. Biol. Chem. 265, 17550–17559
38. Lambeth, J. D., Burnham, D. N., and Tyagi, S. R. (1988) J. Biol. Chem. 263, 3818–3822
39. Rotrosen, D., Yeung, C. L., and Katkin, J. P. (1993) J. Biol. Chem. 268, 14256–14260
40. Feig, L., and Cooper, G. (1988) Mol. Cell. Biol. 8, 3235–3243
41. Kwong, C. H., Malech, H. L., Rotrosen, D., and Leto, T. L. (1993) Biochemistry 32, 5711–5717
42. Joseph, G., Gorzalczany, Y., Koshkin, V., and Pick, E. (1994) J. Biol. Chem. 269, 29024–29031
43. Diekmann, D., Nobes, C., Burbezo, P., Abo, A., and Hall, A. (1995) EMBO J. 14, 5297–5305
44. Rodriguez-Viciana, P., Warne, P., Dhand, R., Vanhaesebroeck, B., Gout, I., Fry, M., Waterfield, M., and Downward, J. (1994) Nature 370, 527–532
45. Spaargaren, M., and Bischoff, J. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 12609–12613
46. Hofler, F., Fields, S., Schneider, C., and Martin, G. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 11089–11093
47. Kikuchi, A., Demo, S., Ye, Z., Chen, Y., and Williams, L. (1994) Mol. Cell. Biol. 14, 7483–7491
48. Han, L., and Colicelli, J. (1995) Mol. Cell. Biol. 15, 1318–1323
49. Prigmore, E., Ahmed, S., Best, A., Kozma, R., Manser, E., Segal, A., and Lim, L. (1995) J. Biol. Chem. 270, 10717–10722
50. Dorscell, O., Rebell, L., Bokoch, G., Cannonis, J., and Gacon, G. (1996) J. Biol. Chem. 271, 83–88