Ras Effector-Homologue Region on Rac Regulates Protein Associations in the Neutrophil Respiratory Burst Oxidase Complex†

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ABSTRACT: Rac, a small molecular weight GTPase in the Ras superfamily, participates in the activation of the multicomponent superoxide-generating NADPH oxidase of human neutrophils. Rac is 30% identical to Ras overall, but is 75% identical within the sequence corresponding to the effector region of Ras, which regulates mitogenesis through interactions with the protein kinase Raf1. We investigated the role of this region in Rac1 using site-directed mutagenesis. In a cell-free semirecombinant NADPH oxidase system, mutants in the 26, 33, 38, and 45 amino acids showed 20−110-fold reduced binding to the oxidase complex as judged by EC_{50} values and reduced (44−80%) maximal activities in superoxide generation. Only the GTPγS-bound form associated, since the GDP-bound form of Rac neither activated alone nor competed with GTPγS-Rac. EC_{50} values for neither p47-*phox* nor p67-*phox* were affected when mutant Racs were used in place of Rac. Data indicate direct binding of the Rac effector region to one or more components of the respiratory burst oxidase. Results indicate a general role for conserved effector-equivalent regions in small GTPases in the regulation of protein−protein interactions.

Phagocytic cells generate superoxide and secondary oxidants, which participate in microbial killing. The respiratory burst oxidase (NADPH oxidase) is dormant in resting neutrophils, but becomes activated upon exposure to microorganisms or a variety of soluble agonists [reviewed in Lambeth (1988)]. The oxidase can be activated in a cell-free system consisting of cytosol plus plasma membrane by anionic amphiphiles such as arachidonate or sodium dodecyl sulfate (Bromberg & Pick, 1984; Curnutte, 1985; McPhail et al., 1985). In addition, nucleoside triphosphates (Seifert & Schultz, 1987; Gabig et al., 1987; Ligeti et al., 1989) and diacylglycerol (Burnham et al., 1990) augment activity. The nucleoside triphosphate requirement was demonstrated to be specific for guanine nucleotides (Uhlinger et al., 1991; Peveri et al., 1992), implying the involvement of a GTP-binding protein.

The active NADPH oxidase is composed of multiple components that are thought to assemble upon enzyme activation. Components include the heterodimeric cytochrome b_{558}, which is located in the plasma membrane, and three cytosolic proteins, p47-*phox*, p67-*phox*, and a Ras-family small GTPase, Rac. Purified native or recombinant cytochrome b_{558}, reconstituted with lipid and FAD, can replace plasma membrane in the cell-free system (Abo et al., 1992; Rotrosen et al., 1993). The cytochrome contains all the electron-transferring groups including heme(s) and FAD as well as an NADPH binding site (Rotrosen et al., 1992; Segal et al., 1992). p47-*phox* (Lomax et al., 1989) and p67-*phox* (Leto et al., 1990) translocate to the plasma membrane upon oxidase activation (Heyworth et al., 1991; Clark et al., 1990) to form a 1:1:1 complex with the cytochrome b_{558} (Uhlinger et al., 1993). A small molecular weight GTP-binding protein, Rac1 or Rac2 (Didsbury et al., 1989), regulates superoxide generation and is also presumed to assemble with the cytochrome or other oxidase components (Kreck et al., 1994), although fewer data exist on this point. Rac1 (Abo et al., 1991) and Rac2 (Knaus et al., 1991) were identified respectively from macrophage and neutrophil cytosols as the GTP-dependent activating factor. The two are 92% identical and may function interchangeably. In the resting cell, cytosolic Rac is in a complex with rho GDP-dissociation inhibitor (rho GDI) (Abo et al., 1991) and upon oxidase activation translocates to the plasma membrane (Quinn et al., 1993). Recombinant Rac1 expressed in *Escherichia coli*, together with recombinant p47-*phox* and p67-*phox*, substitutes for cytosol in the cell-free assay (Abo et al., 1991, 1992; Kreck et al., 1994) and has permitted the kinetic analysis of its regulatory effects in the present study.

Ras, the prototypical small GTP-binding protein, functions in the pathway for growth factor regulation of mitogenesis (Marshall, 1993). Activating mutations are oncogenic, resulting in uncontrolled growth, but certain other mutations can block these mitogenic effects. Such inhibitory mutations define the Ras effector region, comprised of residues within the region from amino acids 26−48. Specific residues within this region mediate protein interactions with Raf-1, a protein kinase in the MAP kinase cascade (Warne et al., 1993; Koide et al., 1993). Overall, Rac1/2 and Ras are approximately 30% identical. However, the effector residues of Ras are approximately 75% identical with corresponding residues in the Racs (below), suggesting functional conservation in this region:

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          21          31          41
Ras   IQLIQNHFVD EYDPTIEDSY RKQVVIDGET
      |   | |   || ||: | |   | :||
Rac1  ISYTTNAFPG EYIPTVFDNY SANVMVDGKP
          ↓       ↓   ↓       ↓
          H       N   N       T
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Table 1: Characterization of Rac1 Mutations with Respect to Superoxide Generation, Kinetic Parameters, and GTPγS Binding[a]

	superoxide generation		EC_{50}			
mutation	relative rate at 1 μM Rac (%)	relative V_{max} (Rac→∞) (%)	Rac (nM)	p47-*phox* (μM)	p67-*phox* (μM)	GTPγS binding (mol/mol of Rac)
none	100	100	42	0.66	0.33	0.91
N26H	22	66	2970	0.57	0.29	0.56
I33N	35	58	830	0.65	0.36	0.69
D38N	8	44	4700	0.57	0.13[b]	0.67
M45T	37	80	1510	0.62	0.29	0.49

[a] The indicated mutations were constructed in Rac1 and expressed as in the Experimental Procedures. Superoxide generation and GTPγS binding were determined as in the Experimental Procedures. Data shown are representative of a minimum of two experiments. [b] Because of very low activity, this EC_{50} should not be considered accurate but representative of a rough approximation.

The effector region residues in Ras are denoted by a double underline, and identical residues shared by Ras and Racs are denoted by a vertical line or by dots for conservative changes. Herein, we have made the indicated site-specific mutations of effector region equivalent residues in Rac1 (arrows). These residues were chosen based on known homologous mutations in Ras and also to span representative residues within the putative Rac effector region. We report herein that the effector-equivalent region in Rac is critical for protein−protein interactions within the NADPH oxidase complex.

EXPERIMENTAL PROCEDURES

Preparation of Cell Fractions and Recombinant Proteins. Human neutrophils were isolated from healthy adult donors as described (Pember et al., 1983) after informed consent was obtained. Plasma membranes were prepared and stored as described (Kreck et al., 1994). These preparations typically contained approximately 0.2 nmol of cytochrome b_{558}/mg of protein, based on heme analysis and the assumption of two hemes per cytochrome (Uhlinger et al., 1993). Recombinant p47-*phox* and p67-*phox* were expressed and purified from baculovirus-infected sf9 insect cells according to Uhlinger et al. (1992, 1993). Rac1 and its mutants were expressed in *E. coli* as the glutathione *S*-transferase fusion proteins, purified using glutathione *S*-agarose beads, and released from the glutathione *S*-transferase domain by thrombin cleavage (Kreck et al., 1994). All recombinant proteins were purified to greater than 95% homogeneity.

Mutagenesis. Rac1 was previously engineered to include a 5′*Bam*HI and 3′*Eco*RI restriction enzyme site (Kreck et al., 1994) and to eliminate the prenylation site by converting Cys189 to Ser. The change had no effect on cell-free activity but increased stability during storage; this form of Rac1 was therefore used as the parent for the further mutations. For Rac1(M45T) and Rac1(I33N), mutagenesis was achieved by sequential PCR site-directed mutagenesis (Cormack, 1991). The resulting DNA was digested with *Bam*HI and *Eco*RI and ligated into the pGEX-2T expression vector, and the complete gene was sequenced by the dideoxy method confirming the mutation. For Rac1(N26H) and Rac1(D38N), the *Bam*HI/*Eco*RI-digested Rac1 above was cloned into the multiple-cloning site in M13mp19 (Greenstein & Brent, 1991). Oligonucleotide-directed mutagenesis was carried out according to Kunkel et al. (1987) followed by production of double-stranded DNA in DH5α *E. coli*. Sequencing was carried out as above to verify the mutation prior to subcloning into the pGEX-2T plasmid.

Assays. To quantify binding, 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, pH 7.0, at 25 °C for 15 min. Four volumes of buffer without EDTA, but containing 10 mM $MgCl_2$, were then added, and the mixture was chromatographed on a 25 × 1 cm Sephadex G-50/80 column. Fractions (0.5 mL) were collected, protein was assayed (Bradford, 1976), and protein-containing fractions were pooled. [^{35}S]GTPγS in the pool was quantified by scintillation counting. Data are expressed as a molar ratio of GTPγS to Rac. Superoxide generation was monitored continuously by superoxide dismutase inhibitable reduction of cytochrome *c* (Burnham et al., 1990; Kreck et al., 1994), using a Thermomax kinetic microplate reader (Molecular Devices, Menlo Park, CA).

Kinetic Analysis. All rates were determined from a linear portion of the kinetic trace, which followed a 5-min preincubation with activators and protein components prior to the addition of NADPH and cytochrome *c*. This preincubation eliminated the lag that is seen prior to achieving linear cytochrome *c* reduction. Theoretical lines through the data which are shown in the figures were calculated using a nonlinear least squares fit of the data using the Michaelis−Menten equation and were calculated and plotted using Sigma Plot. Kinetic constants are reported as V_{max} and EC_{50} (Effective Concentration at 50% of V_{max}). *Apparent* binding constants are reported as EC_{50} rather than K_m values because it is assumed that the Rac and other cytosolic factors are functioning as regulatory factors, the binding of which modulates activity, and do not themselves participate in the catalytic mechanism.

RESULTS

The mutant proteins were first compared with Rac1 in the semirecombinant cell-free activation system, using a single fixed concentration (1 μM) of Rac or mutant Rac. These data are summarized in column 1 of Table 1. Effector region mutations produced significantly lower activity under these conditions, ranging from 8% to 37% of the activity using Rac1. To rule out the possibility that these effects were due to the interaction of Rac with some other membrane component, we also utilized highly purified, lipid-reconstituted cytochrome b_{558} in place of plasma membranes. Results (not shown) were essentially the same as those using plasma membrane.

To investigate the kinetic mechanism for the lowered activity, superoxide generation was determined as a function of the concentration of Rac1 or of mutant Racs, and kinetic parameters were determined. As shown in Figure 1 and Table 1, Rac effector mutations affected both the V_{max} and the EC_{50} (apparent binding constant). While effects on the

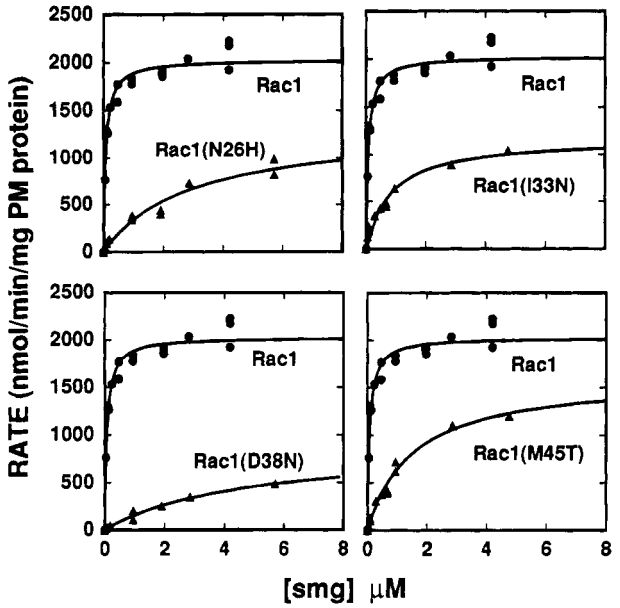

FIGURE 1: Concentration dependence for Rac1 and Rac1 effector mutants in superoxide generation in a semirecombinant cell-free NADPH-oxidase system. Plasma membrane (10 μg) was preincubated at 25 °C with recombinant p47-*phox* (1.3 μM) and p67-*phox* (1.3 μM), 10 μM GTPγS, 260 μM arachidonate, and the indicated concentration of Rac1 (*circles*) or Rac1 mutant protein (*triangles*) in a volume of 50 μL. The Rac proteins were preloaded by incubating 0.5 μM Rac with 100 μM GTPγS for 15 min at 25 °C. 10 μL of the incubation mixture was diluted to a total volume of 250 μL in buffer containing NADPH (200 μM) and 80 μM cytochrome *c*, and superoxide generation was determined spectrophotometrically as in the Experimental Procedures. SMG refers to the small molecular weight GTPase. Data shown are representative of a minimum of two experiments.

V_{max} were relatively small (on the order of 2-fold, see Table 1, column 2), effects on apparent binding ranged from 20-fold for the I33N mutation to 110-fold for the D38N mutation (Table 1, column 3). Mutations at positions 26 and 38 produced the largest effects on binding.

Figure 2 shows the concentration dependence for p47-*phox* in the presence of 1 μM Rac1 or its effector mutants. Figure 3 shows the corresponding concentration dependence for p67-*phox* using Rac1 or effector region mutants. EC_{50} values are summarized in Table 1. As is shown, mutations in the Rac effector region caused no significant effect on the EC_{50} values for either p47-*phox* or p67-*phox*.

As summarized in Table 1, all of the Rac mutants bound GTPγS, although the extent of binding differed. Rac1 bound nucleotide approximately stoichiometrically, while the mutants had equal to or greater than half the normal binding. This may indicate that a portion of the expressed protein is not active or that GTPγS is released during chromatography. Although either explanation might account for up to a 2-fold effect on the EC_{50} for Rac, differences in GTPγS binding would not account for the up to 110-fold effects that were observed. In addition, neither EC_{50} values nor V_{max} values correlated with guanine nucleotide binding.

We also investigated whether the guanine nucleotide itself regulates the binding of Rac to the oxidase complex. GDP-preloaded Rac1 (1 μM) did not itself activate and, at an equimolar concentration, did not inhibit oxidase activation by 1 μM GTPγS-Rac1 (data not shown). Both complexes were formed by preincubating Rac with the appropriate nucleotide at low Mg^{2+}. Thus, only the nucleoside triphosphate form of Rac binds to the oxidase. These data also eliminated the possibility that some residual GDP bound to the mutant proteins might introduce a competing species, which could have decreased apparent binding.

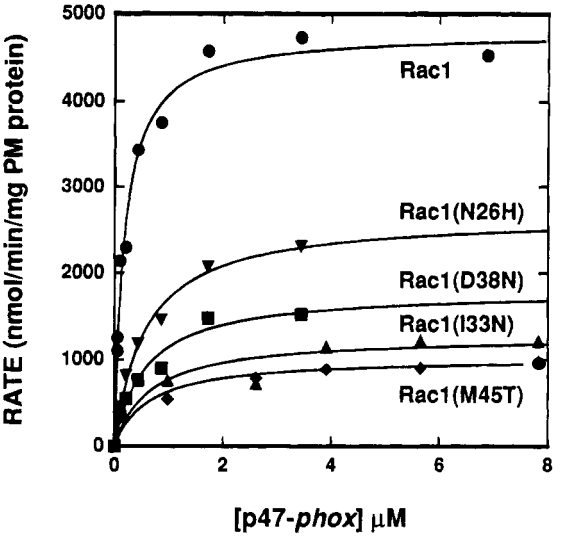

FIGURE 2: Concentration dependence for p47-*phox* in superoxide generation in a semirecombinant cell-free NADPH oxidase system using native and mutant Rac1. Conditions were as in Figure 1, except that the final concentration of Rac1 or Rac1 mutant proteins was 1 μM, and the concentration of recombinant p47-*phox* was varied as indicated. Experiments were carried out using GTPγS-preloaded Rac1 (*circles*) or Rac1 mutant proteins N26H (*inverted triangles*), I33N (*triangles*), D38N (*squares*), and M45T (*diamonds*). The rate of superoxide generation was measured as described in Figure 1. Data shown are representative of a minimum of two experiments.

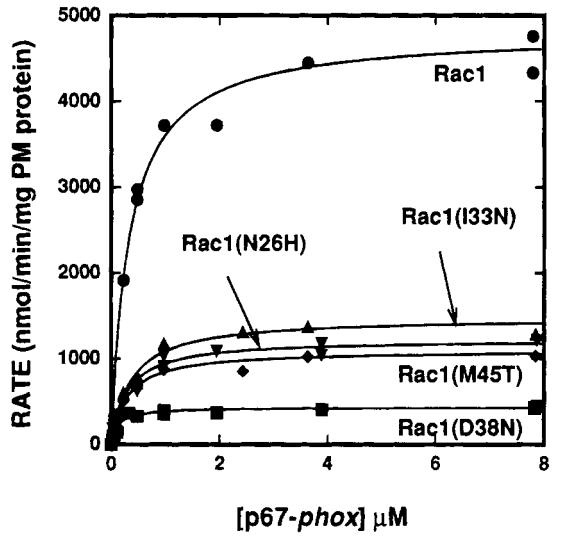

FIGURE 3: Concentration dependence for p67-*phox* in superoxide generation in a semirecombinant cell-free NADPH oxidase system using native and mutant Rac1. Conditions were as in Figure 1, except that the final concentration of Rac1 or Rac1 mutant proteins was 1 μM, and the concentration of recombinant p67-*phox* was varied as indicated. Experiments were carried out using GTPγS-preloaded Rac1 (*circles*) or Rac1 mutant proteins N26H (*inverted triangles*), I33N (*triangles*), D38N (*squares*), and M45T (*diamonds*). The rate of superoxide generation was measured as described in Figure 1. Data shown are representative of a minimum of two experiments.

DISCUSSION

Rac, a member of the Ras superfamily of small molecular weight GTPases, shows a relatively high degree of sequence

identity with Ras in the Ras effector region. This region in Ras is required for mitogenic effects and seems to function via GTP-regulated binding to Raf-1 (Warne et al., 1993; Marshall, 1993). In the present studies, we have shown that mutations in homologous residues in Rac inhibit its function as a participant in the multicomponent NADPH oxidase, indicating that this region of Rac is also functionally important.

To test the hypothesis that the mutations diminished the binding to the NADPH oxidase complex, we determined the concentration dependence for the normal and mutant Rac in the semirecombinant cell-free system. For all of the mutations, there was a weakened interaction of Rac with the oxidase complex, as judged by a higher EC_{50} value. In particular, mutations at the 26 and 38 positions produced large effects (70- and 110-fold, respectively) corresponding to 2.6 and 2.8 kcal binding energy. Assuming that about 12 residues comprise the effector site (as in Ras), then each residue contributes an average 0.8 kcal to the total binding energy, implying that residues 26 and 38 are particularly important. Effects of the mutations at positions 33 and 45 were less striking but were reproducible. Residues 33 and 38 are predicted to be within the equivalent of the Ras "Switch I" region, which undergoes a large conformational change upon GTP binding (Milburn et al., 1990), whereas residues 26 and 45 are outside this region. Thus, the strength of the mutation did not correlate with its position within the Switch I region.

We have previously proposed that the C-terminal region of Rac is involved in its interactions with the oxidase complex (Kreck et al., 1994). A C-terminal peptide of Rac1 inhibits cell-free oxidase activity. Inhibition is competitive with respect to Rac1, but noncompetitive with respect to p47-*phox* and p67-*phox*. Order of addition experiments suggest that the peptide inhibits oxidase assembly rather than the activity *per se*. The participation of the C-terminus in protein−protein interactions has recently been confirmed (M. L. Kreck, unpublished experiments) using site-directed mutagenesis. By molecular modeling (J. D. Lambeth, unpublished model), the C-terminus is predicted to be on the face of the Rac molecule opposite the effector region. Thus, we propose that at least two faces of the Rac molecule are involved in simultaneous interactions with other components of the NADPH oxidase.

According to current models, p47-*phox* and p67-*phox* form a complex, and p47-*phox* may bind directly to cytochrome b_{558}. Such a model is based on the finding that both in intact cells and in a cell-free system, p67-*phox* translocation requires p47-*phox* (Heyworth et al., 1991). In addition, the two are found in association in a large cytosolic complex (Park et al., 1992). Nevertheless, a function for Rac in such a scheme has not been proposed. One possibility is that Rac may promote cytochrome−cytosolic factor interactions by binding simultaneously to both the cytochrome and to either p47-*phox* or p67-*phox*. Alternatively, Rac might bind to one of these proteins, causing a conformational change which enhances that protein's affinity for the flavocytochrome. In either case, a mutation in a binding region of Rac should alter the binding of either p47-*phox* or p67-*phox* in the active oxidase complex. However, the mutant Racs produced no effects on the EC_{50} for either protein, ruling out these two models. The large effects of some of the mutations on the EC_{50} for Rac itself is consistent with a direct binding of Rac to cytochrome b_{558}. An alternative explanation is that Rac binds to p47-*phox* or p67-*phox* in a region which is not involved in cytochrome interactions. However, it is difficult to envision how such a thermodynamically silent binding to a distant site would affect the electron transfer rate within the flavocytochrome.

In summary, the present studies demonstrate that as in Ras, the effector-equivalent region in Rac is critical for regulated protein−protein interactions. The Rac is proposed to bind to cytochrome b_{558} to promote electron transfer among pyridine nucleotide, flavin, and/or heme groups. Conserved effector regions in small molecular weight GTPases may provide a general mechanism for guanine nucleotide-regulated protein−protein interactions in a variety of small GTPase-regulated systems.

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REFERENCES

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.

Bradford, M. (1976) *Anal. Biochem. 72*, 248−254.

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.

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

Cormack, B. (1991) in *Current Protocols in Molecular Biology* (Ausubel, F. M., et al., Eds.) pp 8.5.1−8.5.9, John Wiley & Sons, New York.

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.

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

Greenstein, D., & Brent, R. (1991) in *Current Protocols in Molecular Biology* (Ausubel, F. M., et al., Eds.) pp 1.14.1−1.14.5, John Wiley & Sons, New York.

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.

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

Koide, H., Satoh, T., Nakafuku, M., & Kaziro, Y. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 8683−8686.

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.

Lambeth, J. D. (1988) *J. Bioenerg. Biomembr. 20*, 709−733.

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.

Marshall, M. S. (1993) *Trends Biochem. Sci. 18*, 250−254.

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

Milburn, M. V., Tong, L., DeVos, A. M., Brunger, A., Yamaizumi, Z., Nishimura, S., & Kim, S. H. (1990) *Science 247*, 939−945.

Park, J., Ma, M., Ruedi, J. M., Smith, R. M., & Babior, B. M. (1992) *J. Biol. Chem. 267*, 17327−17332.

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

Peveri, P., Heyworth, P. G., & Curnutte, J. T. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 2494−2498.

Quinn, M. T., Evans, T., Loetterle, L. R., Jesaitis, A. J., & Bokoch, G. M. (1993) *J. Biol. Chem. 268*, 20983−20987.

Rotrosen, D., Yeung, C. L., Leto, T. L., Malech, H. L., & Kwong, C. H. (1992) *Science 256*, 1459−1462.

Rotrosen, D., Yeung, C. L., & Katkin, J. P. (1993) *J. Biol. Chem. 268*, 14256−14260.

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.

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.

Warne, P. H., Viciana, P. R., & Downward, J. (1993) *Nature 364*, 352−355.