p21*rac* Does Not Participate in the Early Interaction between p47-*phox* and Cytochrome b_{558} That Leads to Phagocyte NADPH Oxidase Activation *in Vitro*†

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ABSTRACT: The phagocyte superoxide-generating NADPH oxidase, a multicomponent, membrane-bound electron transport chain, consists of cytochrome b_{558}, p47-*phox*, p67-*phox*, and p21*rac*1 or p21*rac*2. The mechanisms of oxidase assembly are poorly understood. In previous studies using a cell-free NADPH oxidase system, we showed that preincubation of neutrophil membrane with neutrophil cytosol containing p47-*phox*, but not p67-*phox*, led to formation of a long-lived NADPH oxidase intermediate. This suggested that p47-*phox* interacted with cytochrome b_{558} in the early stages of oxidase assembly while p67-*phox* participated in a later stage. Peptides containing the sequence RGVHFIF (corresponding to amino acids 559–565 of the 91-kDa subunit of cytochrome b_{558}) inhibit NADPH oxidase activity by blocking the early interaction between p47-*phox* and cytochrome b_{558}. In the present study, we examined whether p21*rac* facilitated the interaction between p47-*phox* and cytochrome b_{558}. We preincubated pure recombinant p47-*phox* with neutrophil membrane containing cytochrome b_{558} in the cell-free system. Superoxide-generating activity was subsequently reconstituted by adding pure rp67-*phox* and partially purified p21*rac*. RGVHFIF inhibited superoxide production if added to the cell-free system during preincubation of rp47-*phox* with membrane. RGVHFIF was markedly less inhibitory if added to the cell-free system after membrane was preincubated with pure rp47-*phox*. In contrast to p47-*phox*, preincubation of membrane with either p21*rac* or rp67-*phox* conferred no protection from inhibition of superoxide-generating activity by RGVHFIF added after preincubation. We conclude that p21*rac* does not facilitate interaction of p47-*phox* with cytochrome b_{558} and that p47-*phox* is the first cytosol protein to associate with cytochrome b_{558} during oxidase assembly.

Neutrophils and other phagocytic cells respond to a variety of stimuli with a burst of oxygen consumption and production of superoxide (Rossi, 1986). Superoxide is generated by a membrane-bound NADPH oxidase, a multicomponent complex that catalyzes transfer of electrons from NADPH to molecular oxygen (Segal, 1989; Segal & Abo, 1993). The minimum number of components required for assembly of functional NADPH oxidase has been defined in cell-free models of oxidase formation using recombinant and purified neutrophil membrane and cytosol proteins (Abo et al., 1992; Rotrosen et al., 1992). Superoxide production is initiated in cell-free systems by adding an anionic amphiphile such as sodium dodecyl sulfate or arachidonate. Components required for formation of the oxidase include cytosol proteins of 47 and 67 kDa (p47-*phox* and p67-*phox*, respectively), p21*rac*1 or p21*rac*2, members of the *rho* family of *ras*-related low molecular weight GTP-binding proteins, and an integral membrane cytochrome b_{558} (Abo et al., 1992; Rotrosen et al., 1992). Cytochrome b_{558} is a glycosylated heterodimer with subunits of 91 and 22 kDa (gp91-*phox* and p22-*phox*) (Harper et al., 1985; Kleinberg et al., 1989; Parkos et al., 1987). Cytochrome b_{558} binds both FAD and heme, and gp91-*phox* shares amino acid sequence homology with other NADPH-binding proteins (Rotrosen et al., 1992; Segal et al., 1992).

The NADPH oxidase is assembled from its protein subunits in response to activation of the phagocytic cell by binding of particulate and soluble ligands to cell surface receptors (Clark et al., 1990; Nauseef et al., 1991). Several observations suggest that p47-*phox* interacts with cytochrome b_{558} in the absence of p67-*phox* in one of the first steps of oxidase assembly. Activation of neutrophils by formylmethionylleucylphenylalanine-containing peptides or phorbol esters initiates NADPH oxidase formation following translocation of p47-*phox*, p67-*phox*, and p21*rac* from cytosol to membrane (Clark et al., 1990; Phillips et al., 1993; Quinn et al., 1993). In contrast to normal neutrophils, neither p47-*phox* nor p67-*phox* translocates from cytosol to membranes of neutrophils from patients with the X-linked form of chronic granulomatous disease (CGD)[1] (Heyworth et al., 1991; Park & Babior, 1992). Most patients with this form of CGD fail to express cytochrome b_{558} in membranes of their phagocytic cells. Using neutrophils from patients with another form of CGD containing normal amounts of cytochrome b_{558} and p47-*phox* but deficient in p67-*phox*, Heyworth et al. (1991) showed that p47-*phox* could translocate from cytosol to membrane in the absence of p67-*phox*. In contrast, p67-*phox* could not translocate from cytosol

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[1] Abbreviations: CGD, chronic granulomatous disease; SOD, superoxide dismutase; AS60, supernatant from cytosol made to 60% saturated ammonium sulfate; V_{max}, maximal rate of superoxide generation; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate).

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to membrane in the absence of p47-*phox* in similar experiments using CGD patient neutrophils containing cytochrome b_{558} and p67-*phox* but deficient in p47-*phox*. Using a cell-free system, we showed that formation of the NADPH oxidase was accelerated by preincubation of neutrophil membrane containing cytochrome b_{558} with neutrophil cytosol from a patient with a form of CGD containing normal amounts of p47-*phox* but deficient in p67-*phox* (Kleinberg et al., 1990). No change in the rate of oxidase formation was seen when membrane was similarly preincubated with CGD cytosol containing p67-*phox* but not p47-*phox*.

Other evidence supporting an interaction between p47-*phox* and cytochrome b_{558} in an early stage of oxidase assembly was seen in experiments examining the effect of synthetic peptides containing the sequence RGVHFIF (corresponding to amino acids 559–565 of gp91-*phox*) which specifically inhibited superoxide generation *invitro* as well as by intact permeabilized neutrophils (Rotrosen et al., 1990; Kleinberg et al., 1992). RGVHFIF-containing peptides did not inhibit superoxide production directly, but rather, blocked assembly of the NADPH oxidase. Superoxide production in the cell-free system was insensitive to inhibition by RGVHFIF if neutrophil membrane containing cytochrome b_{558} was preincubated in the absence of RGVHFIF with CGD cytosol containing p47-*phox* but deficient in p67-*phox*. In contrast, preincubation of membrane with CGD cytosol containing p67-*phox*, but not p47-*phox*, did not protect against inhibition of NADPH oxidase activity by RGVHFIF (Kleinberg et al., 1990). RGVHFIF-containing peptides blocked translocation of p47-*phox* from cytosol to membrane *in vitro* (Park et al., 1992), suggesting that these inhibitory peptides may inhibit physical association between p47-*phox* and cytochrome b_{558}.

The above observations suggested that formation of the NADPH oxidase could be divided into an early interaction between p47-*phox* and cytochrome b_{558} followed by subsequent participation of p67-*phox*, leading to completion of oxidase assembly. Moreover, experimental data examining kinetics of oxidase formation (Kleinberg et al., 1990), inhibition of oxidase activity by RGVHFIF-containing peptides (Rotrosen et al., 1990; Kleinberg et al., 1990), and translocation of p47-*phox* from cytosol to membrane (Heyworth et al., 1991) suggested that interaction of p47-*phox* with cytochrome b_{558} was essentially irreversible. We hypothesize that p47-*phox* interacts with cytochrome b_{558} and forms a long-lived oxidase intermediate which is insensitive to inhibition by RGVHFIF-containing peptides. Several laboratories have reported recently that p21*rac*1 or p21*rac*2, members of the *rho* family of *ras*-related proteins, are required for NADPH oxidase activity in cell-free systems that support superoxide production (Abo et al., 1991; Knaus et al., 1991). The purpose of the present study was to determine the role of p21*rac* in oxidase assembly, with particular emphasis on whether p21*rac* interacts with p47-*phox* and cytochrome b_{558} during the early stage of NADPH oxidase assembly.

MATERIALS AND METHODS

Cell-Free System. Neutrophil membrane and cytosol fractions were prepared from normal donor granulocyte pheresis packs as described (Kleinberg et al., 1990). Cytochrome b_{558} concentrations were 0.14–1.0 nmol/mg of total membrane protein in different preparations using reduced minus oxidized $\Delta\epsilon_{427-413} = 160\,mM^{-1}\,cm^{-1}$ (Capeillere-Blandin et al., 1991) and assuming 2 hemes/cytochrome b_{558} (Segal & Abo, 1993). Membrane was diluted with relaxation buffer to a final concentration of 1.0 mg of protein/mL (approximately 4.0×10^8 cell equivalents/mL) and solubilized with deoxycholate buffer as described except that the deoxycholate/membrane protein weight ratio was 5:1. Partially purified p21*rac* (AS60) was made as follows. Cytosol (6 mg of protein/mL, equivalent to 2×10^8 cell equivalents/mL) was made 60% final concentration in ammonium sulfate and incubated overnight at 4 °C. Cytosol was centrifuged at 13000*g* for 2 min, and the supernatant was dialyzed using 1000 molecular weight cutoff membrane against two changes of water and a final 0.9% NaCl and 83 mM sodium phosphate, pH 7.0, solution. Final dialyzed solution was stored at –80 °C with no loss of activity over at least 3 months. Recombinant p47-*phox* and p67-*phox* were purified from lysates of Sf9 cells infected with baculovirus containing transcripts encoding p47-*phox* or p67-*phox* as described (Leto et al., 1991). Protein amounts were quantitated using a commercial bicinchoninic acid assay (Pierce Chemical, Rockville, IL).

The cell-free assay for superoxide production was performed in 96-well microtiter plates as described (Kleinberg et al., 1990) with the following modifications. Superoxide was generated only in the presence of a complete cell-free system. A complete cell-free system (100 μL final volume) consisted of deoxycholate-solubilized neutrophil membrane (1.0 μg of protein/well), rp47-*phox* (0.2–0.4 μg/well), rp67-*phox* (0.4–0.6 μg/well), and AS60 (1.2–3.6 μg/well), in reaction mixture [final concentrations 75 mM potassium phosphate, pH 7.0, 200 μM acetylated ferricytochrome *c*, 1.0 μM FAD, 1 mM EGTA, 4 mM $MgCl_2$, 1.0 μM GTPγS, 30 μM sodium arachidonate, and 200 μM NADPH (Nunoi et al., 1988)]. Superoxide dismutase (SOD) was added to control wells at a final concentration of 50 μg/mL. Superoxide generation was quantitated as SOD-inhibitable reduction of acetylated ferricytochrome *c* in duplicate wells at 550 nm on a Molecular Devices (Menlo Park, CA) Thermomax microtiter plate reader. The maximal rate of superoxide production (V_{max}) was determined from the maximal rate of ΔA_{550} as described (Kleinberg et al., 1990, 1992). For these experiments, particular concentrations of p47-*phox*, p67-*phox*, and AS60 for each lot were optimized in three-way dose-response experiments where varying concentrations of the three cytosol components were added to membrane in the reaction mixture and the amount of superoxide produced was measured. p47-*phox*, p67-*phox*, and AS60 concentrations were optimized such that superoxide production was not limited by the amount of any one cytosol protein. In addition, incomplete cell-free systems containing membrane and only two cytosol components but lacking either p47-*phox*, p67-*phox*, or AS60 generated little to no superoxide unless supplemented with the missing cytosol proteins. No p47-*phox* was detected in our membrane preparations when analyzed by immunoblotting with anti-p47-*phox* sera (data not shown). Neutrophil membrane contained as much as 6% p67-*phox*/mg of total protein compared to p67-*phox* levels in cytosol (data not shown). However, in our system the amount of membrane protein used is 30-fold less than the equivalent amount of cytosol protein in cell-free systems where normal neutrophil cytosol is used as the source for p47-*phox*, p67-*phox*, and *rac* (Kleinberg et al., 1990). For the experiments described in this study, these low membrane levels of p67-*phox* were insufficient to facilitate oxidase formation in the cell-free system in the absence of added rp67-*phox* (Figure 3, ▲).

Inhibition of Superoxide Production by RGVHFIF. RGVHFIF was synthesized by the Biopolymer Laboratory at the University of Maryland School of Medicine and purified by reverse-phase high-performance liquid chromatography

(purity >97%). Amino acid composition analysis (Pico-Tag method, Waters, Bedford, MA) yielded ratios of amino acids consistent with correct synthesis of RGVHFIF. RGVHFIF was dissolved in water, and stock solutions were stored at −80 °C. Inhibition of superoxide production by RGVHFIF (final concentration 96 μM) was compared under two conditions. In the first condition, RGVHFIF was preincubated for 5 min with membrane and either p47-*phox*, p67-*phox*, or AS60. NADPH oxidase activity was then reconstituted by adding to the cell-free system the other two essential cytosol components absent from the preincubation mixture (p67-*phox* + AS60, p47-*phox* + AS60, or p47-*phox* + p67-*phox*). In the second condition, membrane and either p47-*phox*, p67-*phox*, or AS60 were preincubated for 5 min without RGVHFIF. RGVHFIF was then added to each well immediately before adding the other two cytosol components absent from the preincubation mixture. V_{max} observed with inhibition of superoxide production by RGVHFIF for each condition was normalized with respect to control wells lacking RGVHFIF.

SDS–PAGE and Immunoblotting. Proteins were separated by SDS–PAGE, transferred to nitrocellulose, and visualized with Fast Green as described (Kleinberg et al., 1989). Some immunoblots were incubated overnight with 1:1000 dilutions of anti-p47-*phox* or anti-p67-*phox* goat sera (Leto et al., 1991). These immunoblots were then incubated with 1 μg/mL peroxidase-conjugated rabbit anti-goat IgG (Kirkegaard and Perry, Gaithersburg, MD) and bands developed with 4-chloro-1-naphthol and H_2O_2 (Kleinberg et al., 1989). Other immunoblots were incubated overnight with 1 μg/mL rabbit polyclonal IgG raised against a synthetic peptide corresponding to the carboxyl-terminal 11 amino acids of p21*rac*2 (Santa Cruz Biotechnologies, Santa Cruz, CA). These blots were then incubated with 1 μg/mL alkaline phosphatase-conjugated goat anti-rabbit IgG (Kirkegaard and Perry, Gaithersburg, MD) and developed with a commercial 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium solution (Kirkegaard and Perry, Gaithersburg, MD). Relative mobilities of protein bands were compared to those of known protein markers (Novex, Encinitas, CA).

RESULTS

Use of pure recombinant p47-*phox* and p67-*phox* and partially purified p21*rac* enabled us to examine the role of p21*rac* in assembly of the NADPH oxidase in a manner not possible in earlier experiments using neutrophil cytosol derived from patients with CGD deficient in either p47-*phox* or p67-*phox*. Specifically, we asked whether p21*rac* was required for formation of the RGVHFIF-insensitive oxidase intermediate resulting from preincubation of p47-*phox* with cytochrome b_{558} in the cell-free system. As outlined earlier, a long-lived, partially assembled oxidase intermediate forms in an incomplete cell-free system containing p47-*phox* and cytochrome b_{558} but lacking p67-*phox* (Kleinberg et al., 1990). RGVHFIF-containing peptides appear to block the interaction between p47-*phox* and cytochrome b_{558} at an early step in NADPH oxidase formation. In the present study, we detected the presence of this oxidase intermediate by determining sensitivity of *in vitro* NADPH oxidase activity to inhibition by RGVHFIF-containing peptides.

We examined participation of p21*rac* in assembly of the NADPH oxidase in a cell-free system consisting of neutrophil membrane containing cytochrome b_{558}, rp47-*phox*, rp67-*phox*, and AS60 (p21*rac*). Neutrophil membrane was used as the source for cytochrome b_{558} since several laboratories have shown that cytochrome b_{558} is the only membrane protein

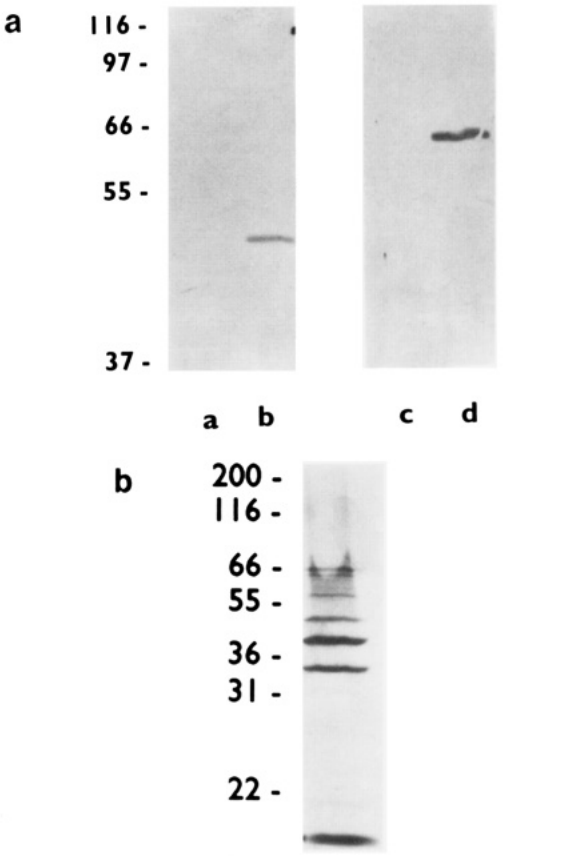

FIGURE 1: AS60 is devoid of detectable amounts of p47-*phox* and p67-*phox*. (a) AS60 (30 μg) (lanes a and c) and normal neutrophil cytosol (30 μg) (lanes b and d) were separated by SDS–PAGE on 10% polyacrylamide gels, transferred to nitrocellulose, probed with anti-p47-*phox* serum (lanes a and b) or anti-p67-*phox* serum (lanes c and d) and developed as described in Materials and Methods. Positions of relative mobilities of protein markers are shown. (b) AS60 (15 μg) was separated by SDS–PAGE on a 10% polyacrylamide gel and stained with Coomassie blue.

required for NADPH oxidase activity (Knoller et al., 1991; Rotrosen et al., 1992; Abo et al., 1992). p21*rac* was separated from p47-*phox* and p67-*phox* by precipitating p47-*phox* and p67-*phox* from neutrophil cytosol made 60% w/v in ammonium sulfate (Figure 1). Even though AS60 still contained a large number of proteins compared to cytosol, AS60 was devoid completely of p47-*phox* or p67-*phox* in most preparations. A faint band suggesting trace amounts of p67-*phox* was seen in a few preparations of AS60. In contrast, p21*rac* was present in AS60 and cytosol as well as in membrane (Figure 2).

AS60 could not substitute for neutrophil cytosol in the cell-free system even in those membrane preparations that contained trace amounts of p67-*phox*. Complete cytosol activity in the cell-free system was restored only when AS60 was supplemented with recombinant p47-*phox* and p67-*phox* (Figure 3). Concentrations of AS60 as low as 1.2 μg/well combined with rp47-*phox* and rp67-*phox* generated equivalent amounts of superoxide as 30 μg/well normal neutrophil cytosol (1×10^6 cell equivalents) in the cell-free system. Low levels of superoxide production were seen consistently in wells containing neutrophil membrane, rp47-*phox*, and rp67-*phox* but not AS60 (Figure 3). This suggested that p21*rac* in neutrophil membrane (Figure 3) was partially active in the cell-free system since no superoxide generation was seen when rp47-*phox*, rp67-*phox*, and purified cytochrome b_{558} were present in the cell-free system in the absence of p21*rac* (Rotrosen et al., 1992).

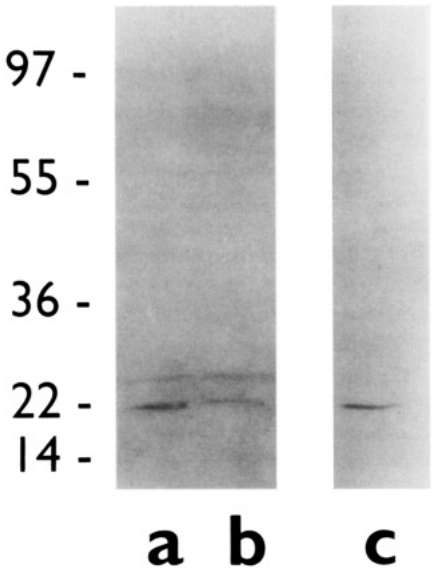

FIGURE 2: AS60 contains p21*rac*2. 45 μg/lane of each of neutrophil cytosol (lane a), AS60 (lane b), and neutrophil membrane (lane c) were separated on an 8–16% SDS–PAGE gel (Novex, Encinitas, CA), transferred to nitrocellulose, and incubated with rabbit anti-p21*rac*2 carboxyl-terminal peptide IgG. Immunoblot was developed as described in Materials and Methods. Positions of relative mobilities of protein markers are shown. The nature of the faint staining upper band seen in lanes a and b is unknown, but its presence is seen in other cells besides neutrophils (Leto, unpublished data).

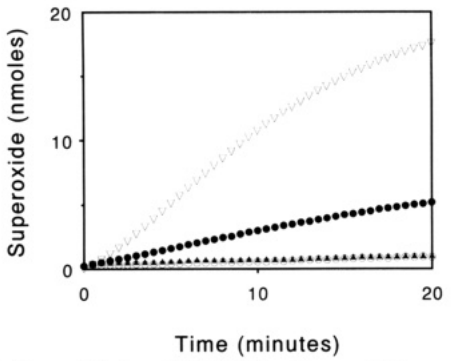

FIGURE 3: Reconstitution of NADPH oxidase activity requires p47-*phox*, p67-*phox*, and p21*rac*. Neutrophil membrane and reaction mixture were added to wells containing rp47-*phox* and AS60 (▲), rp67-*phox* and AS60 (○), rp47-*phox* and rp67-*phox* (●), and rp47-*phox*, rp67-*phox*, and AS60 (▽). Concentrations of individual components were rp47-*phox* 0.2 μg/well, rp67-*phox* 0.6 μg/well, and AS60 3.6 μg/well. Amount of superoxide generated was quantitated as described in Materials and Methods. This experiment is representative of 4 similar experiments. Membrane and reaction mixture added to wells with AS60 alone (not shown) generated trace quantities of superoxide similar to that seen with p47-*phox* and AS60 (▲) and p67-*phox* and AS60 (○).

Neutrophil membrane was preincubated for 5 min with either rp47-*phox*, rp67-*phox*, or AS60 followed by reconstitution of NADPH oxidase activity by addition to the cell-free system of the two cytosol components not included during preincubation. Superoxide production was markedly inhibited under all preincubation conditions whenever RGVHFIF was included in the preincubation mixture of neutrophil membrane with rp47-*phox*, rp67-*phox*, or AS60. When pure rp47-*phox* alone was preincubated with membrane for 5 min in the absence of peptide, RGVHFIF failed to inhibit superoxide production when RGVHFIF was added to the cell-free system immediately before addition of AS60 and rp67-*phox* (Table 1). In contrast, preincubation of AS60 alone with membrane for 5 min in the absence of peptide did not protect oxidase activity from inhibition by RGVHFIF added to the cell-free

Table 1: p21*rac* Does Not Participate in the Early Interaction between p47-*phox* and Cytochrome b_{558}[a]

preincubation	reconstitution	NADPH oxidase activity (normalized to controls)
p47-*phox* + RGVHFIF	p67-*phox* + AS60	0.32 ± 0.12 (13)
p47-*phox*	p67-*phox* + AS60 + RGVHFIF	0.83 ± 0.13 (13)
p67-*phox* + RGVHFIF	p47-*phox* + AS60	0.35 ± 0.11 (14)
p67-*phox*	p47-*phox* + AS60 + RGVHFIF	0.23 ± 0.07 (14)
AS60 + RGVHFIF	p47-*phox* + p67-*phox*	0.12 ± 0.05 (7)
AS60	p47-*phox* + p67-*phox* + RGVHFIF	0.19 ± 0.05 (7)

[a] Neutrophil membrane, arachidonate, and reaction mixture were preincubated with either rp47-*phox*, rp67-*phox*, or AS60 for 5 min. Following preincubation, NADPH oxidase activity was reconstituted by adding to each well those cytosol components not included in preincubation (see Materials and Methods). Concentrations of rp47-*phox*, rp67-*phox*, and AS60 as described in Materials and Methods. RGVHFIF (96 μM) either was included in the preincubation mixture or was added during reconstitution immediately before addition of the two cytosol components not included in preincubation. Superoxide production was quantitated as the V_{max} of SOD-inhibitable reduction of acetylated ferricytochrome *c* superoxide production as described in Materials and Methods. NADPH oxidase activity remaining after inhibition by RGVHFIF was expressed as (V_{max} of wells containing RGVHFIF)/(V_{max} of simultaneous control wells lacking peptide) ± standard errors of the means. The number of replicate experiments is indicated in parentheses.

system just before addition of rp47-*phox* and rp67-*phox*. Similarly, preincubation of rp67-*phox* with membrane in the absence of peptide did not prevent inhibition of superoxide generation by RGVHFIF added to the cell-free system immediately before AS60 and rp47-*phox* during reconstitution. The above data show that NADPH oxidase activity *in vitro* became insensitive to inhibition by RGVHFIF only after preincubation of rp47-*phox* with neutrophil membrane in the absence of peptide. This indicated that p21*rac* is not required to facilitate interaction of p47-*phox* with cytochrome b_{558} in the early stage of oxidase formation.

Preincubation of rp47-*phox* with neutrophil membrane in the absence of peptide also rendered *in vitro* superoxide production insensitive to inhibition by RGVHFIF at higher concentrations of RGVHFIF (Figure 4). Membrane and rp47-*phox* were preincubated for 5 min with or without various concentrations of RGVHFIF. NADPH oxidase activity was reconstituted *in vitro* by addition of rp67-*phox* and AS60 to the cell-free system. In wells preincubated without peptide, RGVHFIF was added to the cell-free system immediately before addition of rp67-*phox* and AS60. Even at peptide concentrations of 192 μM, preincubation of membrane with p47-*phox* in the absence of peptide substantially protected NADPH oxidase activity from inhibition by RGVHFIF compared to samples where RGVHFIF was included in the preincubation mixture.

The truncated peptide GVHFIF does not inhibit superoxide production in the cell-free system possibly because the amino-terminal arginine in RGVHFIF is critical for inhibition (Kleinberg et al., 1992). No inhibition of superoxide production was seen when GVHFIF was substituted for RGVHFIF even when preincubated with neutrophil membrane and rp47-*phox* (data not shown). Therefore, protection against RGVHFIF-mediated inhibition of superoxide production conferred by preincubation of rp47-*phox* with neutrophil membrane was specific for the RGVHFIF amino acid sequence.

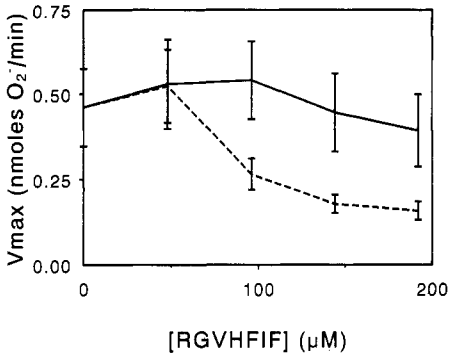

FIGURE 4: Preincubation of rp47-*phox* with neutrophil membrane in the absence of peptide leads to formation of an RGVHFIF-insensitive oxidase intermediate even at high RGVHFIF concentrations. Neutrophil membrane was preincubated for 5 min with rp47-*phox* (solid line) or rp47-*phox* + RGVHFIF (dashed line) in the reaction mixture. Superoxide production was initiated by subsequent addition of rp67-*phox* + AS60 + RGVHFIF (solid line) or rp67-*phox* + AS60 (dashed line) (see Materials and Methods). Concentrations of individual components were rp47-*phox* 0.2 μg/well, rp67-*phox* 1.0 μg/well, and AS60 2.0 μg/well. Concentrations of RGVHFIF were varied from 0 to 192 μM. Results shown represent means ± standard errors for 3 experiments.

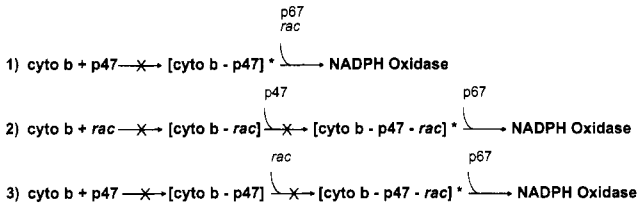

FIGURE 5: Three possible mechanisms to describe the role of p21*rac* in the assembly of the NADPH oxidase *in vitro*. See text for discussion of reaction schemes. Possible oxidase intermediates are indicated by square brackets. Possible stable RGVHFIF-insensitive intermediates are marked with an *. Interactions that are potentially inhibited by RGVHFIF are denoted with an X. Only reaction scheme 1 is consistent with our observation that an RGVHFIF-insensitive intermediate is formed by preincubation of neutrophil membrane with rp47-*phox* alone.

DISCUSSION

Recent studies from several laboratories have identified p21*rac* as a required component for assembly and function of the NADPH oxidase (Abo et al., 1991; Knaus et al., 1991; Rotrosen et al., 1992). p21*rac* may regulate oxidase function through a GTP-dependent process since reconstitution of NADPH oxidase activity *in vitro* requires p21*rac* in its GTP-bound form (Mizuno et al., 1992; Abo et al., 1992). We considered three possible mechanisms for participation of p21*rac* in oxidase assembly that could account for observations from previous experiments using CGD patient neutrophil cytosol which indicated that interaction of p47-*phox* with cytochrome b_{558} led to formation of a long-lived RGVHFIF-insensitive intermediate in the early stage of NADPH oxidase formation (Figure 5). First, p21*rac* may have no role in the initial interaction between p47-*phox* and cytochrome b_{558}. In this model, both p21*rac* and p67-*phox* participate in oxidase assembly subsequent to initial interaction between p47-*phox* and cytochrome b_{558} (Figure 5, reaction scheme 1). Second, p21*rac* may be the first cytosol protein to interact with cytochrome b_{558} preceding participation of p47-*phox* in oxidase assembly. In this model, the long-lived oxidase intermediate is formed after subsequent p47-*phox* interaction with a p21*rac*/cytochrome b_{558} intermediate (Figure 5, reaction scheme 2). Third, p47-*phox* may be the first cytosol protein to interact with cytochrome b_{558} but leads to formation of an unstable intermediate. Subsequent participation of p21*rac* leads to formation of a long-lived RGVHFIF-insensitive oxidase intermediate that can go on to form NADPH oxidase after addition of p67-*phox* (Figure 5, reaction scheme 3).

RGVHFIF-containing peptides inhibit superoxide generation in the cell-free system. In this paper, we showed that preincubation of neutrophil membrane with rp47-*phox* alone in the absence of RGVHFIF was sufficient to render *in vitro* NADPH oxidase activity insensitive to inhibition by RGVHFIF (Table 1). Preincubation of membrane with AS60 alone in the absence of peptide did not protect NADPH oxidase activity from inhibition by RGVHFIF added subsequent to the preincubation step. Inclusion of AS60 with rp47-*phox* and membrane during preincubation in the absence of peptide did not increase the magnitude of NADPH oxidase activity recovered after subsequent addition of RGVHFIF and rp67-*phox* compared to preincubation of membrane with rp47-*phox* alone (data not shown). This suggests that p21*rac* does not facilitate interaction of p47-*phox* with cytochrome b_{558} *in vitro*.

Our results are consistent with predictions of the first of the above models for p21*rac* participation in oxidase assembly (Figure 5, reaction scheme 1). We propose that the first stage of oxidase assembly involves an interaction between p47-*phox* and cytochrome b_{558} which is not dependent on activities of p67-*phox* or p21*rac*. RGVHFIF-containing peptides inhibit this interaction possibly by blocking binding of the native gp91-*phox* RGVHFIF-containing carboxyl-terminus with a site either on p47-*phox* (Rotrosen et al., 1990; Nakanishi et al., 1992) or on an internal binding site of cytochrome b_{558}. In subsequent steps, p21*rac* and p67-*phox* interact with the p47-*phox*/cytochrome b_{558} intermediate leading to completion of NADPH oxidase assembly and acquiring the capacity to generate superoxide. This model is consistent with the observation by Uhlinger et al. (1993) that p47-*phox* translocation from cytosol to membrane in the cell-free system was not GTPγS or p67-*phox* dependent. In contrast, p67-*phox* translocation to membrane was dependent on GTPγS, suggesting that p21-*rac* participated with p67-*phox* in the later stages of oxidase assembly.

Several studies suggest that p47-*phox* and p67-*phox* may exist as complexes in neutrophil cytosol (Leto et al., 1991; Park et al., 1992). Our *in vitro* studies do not preclude the possibility that p47-*phox*, p67-*phox*, and p21*rac* translocate as a high molecular weight complex from cytosol to membrane rather than as individual cytosolic proteins. However, our findings support the concept that NADPH oxidase assembly proceeds *functionally* through a sequence of reactions beginning with an initial interaction between cytochrome b_{558} and p47-*phox*. Whether this interaction is between cytochrome b_{558} and free p47-*phox* or p47-*phox* bound in a complex with p21*rac* and p67-*phox* remains to be determined.

The phagocyte NADPH oxidase is uniquely regulated compared to other electron transport chains. By virtue of its ability to bind FAD, NADPH, and heme, purified cytochrome b_{558} in the absence of p47-*phox*, p67-*phox*, and *rac* can be made to function as a complete electron-transfer protein under certain *in vitro* conditions (Koshkin & Pick, 1993). However, *in vivo* and under general *in vitro* conditions such as those used in our studies, cytochrome b_{558} cannot transfer electrons from NADPH to O_2 in the absence of p47-*phox*, p67-*phox*, and p21*rac*. We speculate that p47-*phox* and p21*rac* may modulate superoxide production in phagocytes by regulating different stages of NADPH oxidase assembly. Activation of neutrophils in response to soluble and particulate stimuli leads to rapid phosphorylation of p47-*phox* and translocation of

p47-*phox* from cytosol to membrane (Heyworth & Segal, 1986; Okamura et al., 1988; Heyworth et al., 1989; Rotrosen & Leto, 1990). Phosphorylation of p47-*phox* may regulate the first step of oxidase formation by initiating association of p47-*phox* with cytochrome b_{558}. Further control of NADPH oxidase activity may be exerted through p21*rac* GTP/GDP exchange which may regulate the final steps of oxidase assembly. GTP/GDP binding to p21*rac* is regulated by a family of accessory proteins (Mizuno et al., 1992). By these machanisms, phagocytes may tightly control NADPH oxidase activity to ensure that the respiratory burst is initiated only when confronted by microbial infection.

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