Activation of the Superoxide-Generating NADPH Oxidase by Chimeric Proteins Consisting of Segments of the Cytosolic Component p67^{phox} and the Small GTPase Rac1†

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ABSTRACT: Activation of the superoxide (O_2^-)-generating NADPH oxidase of phagocytes is the consequence of the assembly of a membrane-associated flavocytochrome b_{559} with the cytosolic proteins p47^{phox} and p67^{phox} and the small GTPase Rac (1 or 2). We proposed that Rac1 serves as a membrane-targeting molecule for p67^{phox}. This hypothesis was tested by constructing recombinant chimeric proteins, joining various functional domains of p67^{phox} and Rac1, and expressing these in *Escherichia coli*. Chimeras were assayed for the ability to support O_2^- production by phagocyte membranes in an amphiphile-activated cell-free system in the presence or absence of p47^{phox}. A chimera consisting of p67^{phox} truncated at residue 212 and fused to a full-length Rac1 [p67^{phox}(1−212)−Rac1(1−192)] was a potent NADPH oxidase activator. A p67^{phox}(1−212)−Rac1(178−192) chimera, to which Rac1 contributed only the C-terminal polybasic domain, was a weaker but consistent activator. Chimeras comprising the full length of Rac1 bound GTP/GDP, like bona fide GTPases. The activity of p67^{phox}−Rac1 chimeras was dependent on the presence of the tetratricopeptide repeat and activation domains, in the p67^{phox} segment, and on an intact polybasic region, at the C terminus of the Rac1 segment, but not on the insert region of Rac1. Partial activation by chimeras, in the GTP-bound form, was also possible in the absence of p47^{phox}. Evidence is offered in support of the proposal that the GTP- and GDP-bound forms of chimera p67^{phox}(1−212)−Rac1(1−192) have distinct conformations, corresponding to the presence and absence of intrachimeric bonds, respectively.

Phagocytic cells utilize oxygen-derived radicals as major microbicidal agents. Appropriate triggering of membrane receptors is transmitted to an enzyme complex, known as the NADPH oxidase (referred to here as "oxidase"), responsible for the generation of the primordial oxygen radical, superoxide (O_2^-)[1] (reviewed in refs *1* and *2*). The oxidase is composed of a membrane-attached heterodimeric flavocytochrome (cytochrome b_{559}), consisting of two subunits, gp91^{phox} and p22^{phox}, and four cytosolic proteins, p47^{phox}, p67^{phox}, p40^{phox}, and the small GTPase Rac (1 or 2). All of the redox stations of the oxidase are located on gp91^{phox} and, in the resting state, are not engaged in electron transport. O_2^- production is, most likely, the result of a conformational change in gp91^{phox}, consequent to the translocation of cytosolic components to the membrane environment, and the interaction of one or more components with cytochrome b_{559}, a process termed oxidase assembly (reviewed in ref *3*). Oxidase assembly can be induced in a cell-free system by the addition of an anionic amphiphile to a mixture of phagocyte membranes and the cytosolic components p47^{phox}, p67^{phox}, and Rac (*4, 5*).

The identity of the cytosolic component(s) responsible for the induction of a conformational change in gp91^{phox} is unknown. There is firm proof for the interaction of p47^{phox} with p22^{phox} and indirect evidence for its interaction with gp91^{phox}, but these interactions are insufficient for the induction of O_2^- production in vitro. Supplementation of the system with p67^{phox} will still not lead to oxidase activation, which requires the presence of Rac, too.

A new approach to solving this issue is offered by the finding that oxidase activation can be elicited in a cell-free system consisting of membranes or purified cytochrome b_{559}, high concentrations of p67^{phox} and Rac, and amphiphile in the absence of p47^{phox} (*6, 7*). This suggests that p67^{phox} might be the protein directly responsible for the induction of a conformational change in gp91^{phox}, a proposal supported by the identification of an activation domain (residues 199−210) responsible for interaction with cytochrome b_{559} (*8, 9*) and by a recent report demonstrating the binding of p67^{phox} to cytochrome b_{559} (*10*). The emerging model is that of p67^{phox} as the central mediator of oxidase activation, with p47^{phox} and Rac, which both possess binding sites for p67^{phox}, serving as carriers for juxtaposing p67^{phox} with cytochrome b_{559}. Additional proof for this model is provided by our recent finding that prenylated Rac1 recruits p67^{phox} to the membrane and elicits O_2^- production in the absence of p47^{phox} and amphiphile (*11*).

The design of the experiments described in this paper rests principally on two groups of findings. The first relates to

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[1] Abbreviations: O_2^-, superoxide; TPR, tetratricopeptide repeat; PCR, polymerase chain reaction; LiDS, lithium dodecyl sulfate; GTPγS, guanosine 5′-3-*O*-(thio)triphosphate; GDPβS, guanosine 5′-2-*O*-(thio)-diphosphate; mant-GTP, 2′- or 3′-*O*-(*N*-methylanthraniloyl)guanosine 5′-triphosphate; mant-GMPPNP, 2′- or 3′-*O*-(*N*-methylanthraniloyl)-β:γ-imidoguanosine 5′-triphosphate; FPLC, fast protein liquid chromatography; PAGE, polyacrylamide gel electrophoresis; mPAK, mouse p21-activated kinase; PBD, the Rac−CDC42Hs binding domain of mPAK (residues 65−137).

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the fact that cell-free oxidase activation can be achieved with p67^{phox} truncated at residues 246 (*12*) or 210 (*8*). The segment consisting of residues 1−210 includes the tetratricopeptide repeat (TPR) domain, found to be involved in binding of p67^{phox} to Rac (*13*−*15*), and the activation domain (*8, 9*). The second relates to the paramount role of the C-terminal polybasic region of Rac1 in its binding to the plasma membrane (*16*−*19*). The present paper describes the construction of chimeric molecules, joining together various functionally important segments of p67^{phox} and Rac1. Several chimeras were found capable of substituting for the combination of individual p67^{phox} and Rac1 in inducing NADPH-dependent O_2^- production by phagocyte membranes in vitro.

EXPERIMENTAL PROCEDURES

Preparation of Phagocyte Membrane Vesicles. Membranes were prepared from guinea pig peritoneal macrophages by a modification of an earlier procedure (*4*). This modification consisted of resuspending the membranes in 1 M KCl and resedimenting the KCl-washed membranes at 176 000*g* for 2 h at 4 °C (see the Discussion for the rationale behind this step). The membranes were solubilized by 40 mM *n*-octyl-*β*-D-glucopyranoside, and membrane vesicles were generated by the removal of detergent by dialysis, as described before (*20*).

Preparation of Recombinant Proteins. p47^{phox} and p67^{phox} were prepared in baculovirus-infected Sf9 cells, as described (*21*). Nonprenylated Rac1 and p67^{phox}, truncated at residue 212 (p67^{phox}(1−212)), were produced in *Escherichia coli* as described (*11, 21*). The Rac−CDC42Hs binding domain (designated PBD) of mouse p21-activated kinase (mPAK; residues 65−137; *22*) was expressed in *E. coli* as a glutathione *S*-transferase fusion protein and purified by affinity chromatography on glutathione−agarose (Sigma), followed by cleavage by thrombin (*23*). Rac1 Δ[123−133] was produced in *E. coli*, as described (*24*).

Generation of Chimeric Constructs and Proteins. Seven chimeric constructs were generated, joining residues 1−212 of p67^{phox}, or parts of it, with a full-length Rac1 (residues 1−192), or parts of it. One chimera consisted of residues 1−212 of p67^{phox} and a full-length CDC42Hs (residues 1−191). All of the chimeras are listed in Figure 1. The chimeric constructs were generated by polymerase chain reaction (PCR), combining DNA fragments from p67^{phox} with DNA fragments from Rac1 or CDC42Hs. The following cDNA clones were used as templates: pGEX-2T−p67^{phox}-(1−212) (*11*), pGEX-2T−Rac1 (*24*), pGEX-2T−Rac1 Δ-[123−133] (*24*), and pGEX-2T−CDC42Hs. The cDNAs of chimeras 2−8 were generated by overlapping PCR, using internal sense and antisense primers (encoding identical Rac1 (or CDC42Hs, for chimera 4) sequences) and external sense and antisense primers (binding to the extremes of the two initial p67^{phox} and Rac1 (or CDC42Hs) fragments and containing restriction sites for *Bam*HI and *Eco*RI enzymes, respectively). The chimera 1 cDNA was generated by one-step PCR using an antisense primer which already included the Rac1 fragment to be combined with the p67^{phox} fragment. Both the sense and antisense primers contained restriction sites for *Bam*HI and *Eco*RI, respectively. The resulting chimeric DNAs were digested with *Bam*HI and *Eco*RI enzymes and ligated to the bacterial expression vector pGEX-2T (Amersham Pharmacia Biotech, Piscataway, NJ) that was cut with the same enzymes. The pGEX-2T constructs were then transformed in *an E. coli* DH5α strain (Life Technologies, Grand Island, NY). The chimeric proteins were expressed as glutathione *S*-transferase fusion proteins and purified from IPTG-induced *E. coli* DH5α cells, using gluthatione−agarose, and release by thrombin cleavage (*23*).

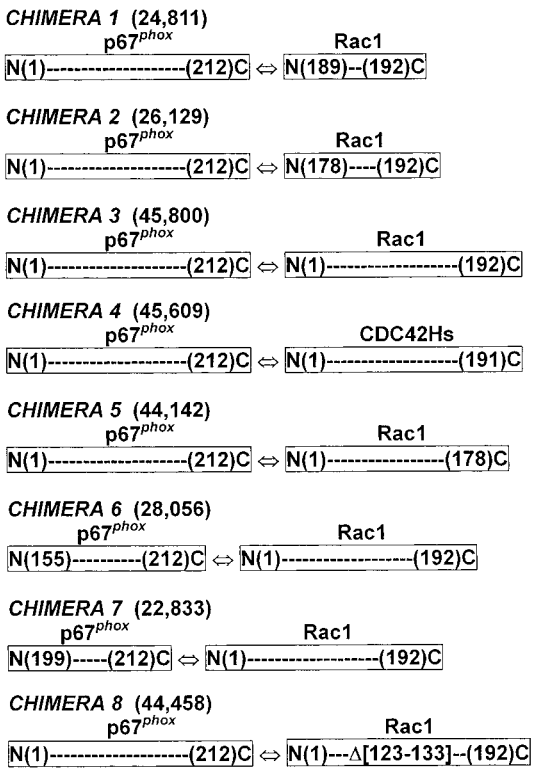

FIGURE 1: Schematic representation of p67^{phox}−Rac1 and p67^{phox}−CDC42Hs chimeras. The numbers in brackets, following the chimera number, are the theoretical M_r values. Within the rectangles are indicated the residue numbers in the native sequences of p67^{phox}, Rac1, or CDC42Hs, representing the N- and C-terminal limits of the segments forming the chimeras.

Cell-Free NADPH Oxidase Assay. Oxidase activation was assayed in a cell-free system consisting of macrophage membrane vesicles and recombinant cytosolic components, with lithium dodecyl sulfate (LiDS) serving as the anionic amphiphilic activator, as described (*24*). Briefly, the basic system contained membranes (equivalent to 5 nM cytochrome b_{559} heme), p47^{phox}, p67^{phox} (or p67^{phox}(1−212)), and Rac1, exchanged to guanosine 5′-3-*O*-(thio)triphosphate (GTPγS), at various concentrations. LiDS was present at a concentration of 130 μM, and incubation was performed in 96-well microplates, in a total volume of 200 μL of assay buffer per well (*24*), for 90 s at 25 °C before the addition of 240 μM NADPH to initiate $O_2^{\bullet -}$ production. This was quantified by the kinetics of cytochrome *c* reduction, as described (*24*). The specificity of cytochrome *c* reduction was ascertained by its prevention by superoxide dismutase. Chimeras were assayed for the ability to support oxidase activation by addition to a mixture of membranes (equivalent to 5 nM cytochrome b_{559} heme) and p47^{phox} (300 nM) and, in another series of experiments, to membranes only (in the absence of p47^{phox}). The cell-free oxidase assay was also utilized for assessing the functional integrity of the component parts of the chimeras, by an "oxidase-activity reconstitution assay" (*11*). Thus, the competence of the p67^{phox}(1−

212) segment was assayed by its ability to support cell-free O_2^- production when chimera (600 nM) was supplemented with a membrane (equivalent to 5 nM cytochrome b_{559} heme), p47^{phox}, and Rac1−GTPγS (the latter two components were at 300 nM); the competence of the Rac1 segment was assayed by its ability to support cell-free O_2^- production when chimera (600 nM) was supplemented with a membrane (equivalent to 5 nM cytochrome b_{559} heme), p47^{phox}, and p67^{phox}(1−212) (the latter two components were at 300 nM). Kinetic parameters (V_{max} and EC_{50}) were determined using the GraphPad Prism, version 3.0, program (GraphPad Software).

Nucleotide Exchange. Rac1 and chimeras were subjected to nucleotide exchange to GTPγS or guanosine 5′-2-*O*-(thio)-diphosphate (GDPβS; both from Roche Molecular Biochemicals, Mannheim, Germany) for use in oxidase-activation assays. Some preparations were subject to mock exchange, performed in the absence of added nucleotide. Chimeras were also exchanged to the fluorescent GTP analogue 2′- or 3′-*O*-(*N*-methylanthraniloyl)guanosine 5′-triphosphate (mant-GTP) or the hydrolysis-resistant GTP analogue, 2′- or 3′-*O*-(*N*-methylanthraniloyl)-β:γ-imidoguanosine 5′-triphosphate (mant-GMPPNP; Molecular Probes, Eugene, OR). All of the exchange reactions were performed at a free Mg^{2+} concentration of 0.5 μM, as described (*11*). GTP/chimera protein stoichiometries were determined on preparations of chimera exchanged to mant-GMPPNP and freed of unbound nucleotide by gel filtration, using an extinction coefficient ($\epsilon_{350\,nm}$) of 5700 M^{-1} cm^{-1} for the mant moiety (*25*). The protein content was determined by the method of Bradford (*26*). The ability of chimera 3 to bind mant-GMPPNP was first tested in pilot experiments, at a free Mg^{2+} concentration of 0.5 μM, by measuring the kinetics of increase in fluorescence (excitation = 361 nm; emission = 440 nm) in a spectrofluorometer (model FP-750; Jasco, Easton, MD), fitted with a magnetically stirred cell (*27*).

Gel Filtration. Chimera p67^{phox}(1−212)−Rac1(1−192), exchanged to GTPγS or mant-GMPPNP, was subjected to gel filtration on a Superdex 75 HR 10/30 fast protein liquid chromatography (FPLC) column (Amersham Pharmacia Biotech). The column was eluted with a buffer consisting of 65 mM sodium potassium phosphate (pH 7.0), 2 mM NaN_3, 1 mM ethylene glycol bis(2-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid, 1 mM $MgCl_2$, and 1 μM flavin adenine dinucleotide. Chromatography was performed on an HPLC system (Waters Corp., Milford, MA), at a flow rate of 0.2 mL/min at 4 °C, and absorbance at 280 nm was measured continuously by a diode array detector (MD-1510; Jasco). When the chimera was labeled with mant-GMPPNP, the fluorescent signal (excitation = 361 nm; emission = 440 nm) was also recorded continuously, by passing the column eluate through a spectrofluorometer (FP-750; Jasco) fitted with an HPLC flow cell (MFC-132; Jasco). Forty 0.6 mL fractions were collected, and the chimera-containing fractions were identified by their ability to support cell-free oxidase activation, as described above. The column was standardized with molecular mass markers (range 6 500−66 000; Sigma, St. Louis, MO).

Polyacrylamide Gel Electrophoresis (PAGE) and Immunoblotting. The chimeric proteins were analyzed by SDS−PAGE and immunoblotting (*28*). The gels were stained with GELCODE blue stain reagent (Pierce, Rockford, IL). For the detection of the p67^{phox} portion in the chimera, we used a goat polyclonal anti-p67^{phox} antiserum. For the detection of the Rac1 portion in the chimeras, we used an affinity-purified rabbit polyclonal anti-Rac1 antibody, raised against a peptide corresponding to residues 178−191 of Rac1 (Santa Cruz Biotechnology, Santa Cruz, CA).

Binding of Chimeras to Membrane. This was measured by gel filtration, as recently described (*11*). Briefly, chimeras were subjected to nucleotide exchange to mant-GTP, as described previously, and aliquots of 1500 pmol were mixed with membrane vesicles (250 pmol equivalent of cytochrome b_{559} heme) in a volume of 0.6 mL. The mixture was injected in a Superose 12 HR 10/30 FPLC gel filtration column (Amersham Pharmacia Biotech), and chromatography was performed as described previously for the Superdex 75 column except for the fact that the membrane−chimera mixture was chromatographed once in the absence and once in the presence of the amphiphilic activator, LiDS (130 μM), added to the column buffer. Fluorescence was recorded continuously, as described previously, and the amounts of free and membrane-bound chimera were calculated by the integration of the fluorescent signal of the free chimera and that associated with the membrane vesicles eluting in the excluded volume of the column.

Synthetic Rac1 Peptide. A peptide corresponding to the C-terminal polybasic segment of Rac1 (residues 178−192) was synthesized by Mimotopes Ltd. (Melbourne, Australia) at a purity of 92%. The peptide was dissolved in 75% dimethyl sulfoxide/25% water (v/v) at a concentration of 1.5 mM and further diluted in oxidase-assay buffer (*24*) to the desired final concentration.

RESULTS

Properties of Chimeras. Seven chimeric proteins consisting of parts of p67^{phox} and Rac1 were constructed; one chimera consisted of part of p67^{phox} and the small GTPase CDC42Hs (Figure 1). The rationale behind the design of the chimeras was to include in or exclude from the fused-proteins domains, belonging to p67^{phox} or Rac1, found in the past to participate in one of the following functions: activation of the oxidase, interaction with cytochrome b_{559}, interaction between p67^{phox} and Rac1, or binding to the plasma membrane. All of the proteins were expressed in *E. coli*, an organism in which post-translational modification of small GTPases does not take place. This means that the chimeras, whether containing the C terminus of Rac1 or CDC42Hs, were not prenylated.

All of the chimeras, with the exception of nos. 6 and 7, contained p67^{phox} residues 1−212 as their N-terminal half. This region comprises the entire TPR domain (residues 1−154) as well as the activation domain (residues 199−210) but is lacking the N-terminal proline-rich domain and both of the src homology 3 (SH3) domains. To the C end of p67^{phox}(1−212) were fused the C-terminal residues 189−192 of Rac1, representing the signal motif for geranylgeranylation (*29*), (inactive in *E. coli*; chimera 1); the C-terminal residues 178−192 of Rac1, comprising the six-residue polybasic motif (chimera 2); a full-length Rac1 (chimera 3); a full-length CDC42Hs (chimera 4); a Rac1, truncated at residue 178, which removes both the polybasic and prenylation motifs (chimera 5); and a full-length Rac1 containing a deletion of the insert region (residues 123−133), which was proposed to be involved in oxidase activation possibly

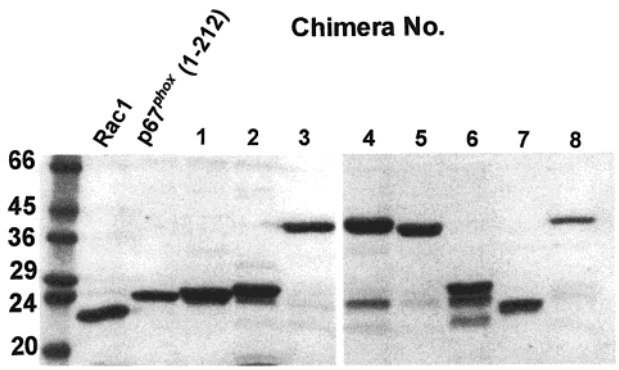

FIGURE 2: SDS−PAGE analysis of chimeras. Amounts (2−5 μg) of chimeric or parent proteins were loaded on the gels (12.7% acrylamide). The first lane contained the molecular weight markers, with $M_r \times 10^{-3}$ indicated to the left of the lane (SDS−PAGE standards, 14 000−70 000; Dalton Mark VII-L; Sigma).

by direct interaction with cytochrome b_{559} (*30*−*32*; chimera 8). Two chimeras contained smaller segments of p67^{phox}(1−212) at their N terminus. Thus, chimera 6 consisted of p67^{phox}(155−212), lacking the TPR domain (residues 1−154), but with an intact activation domain and an additional segment, C terminal to the missing TPR domain, fused to a full-length Rac1. Chimera 7 consisted of the activation domain of p67^{phox} only (residues 199−212), the C terminus of which was fused to a full-length Rac1.

All of the chimeras were analyzed by SDS−PAGE, as a check of purity and proper M_r. As seen in Figure 2, the chimeras were of sizes corresponding closely to theoretical predictions (listed in Figure 1). Chimeras 1, 2, 4, and 6 were contaminated with what appears to be a degradation product of close to 24 kDa in size; preparations of chimera 6 contained an additional contaminant of less than 24 kDa. Varying the conditions of thrombin cleavage had no effect on the level of contaminants. All of the chimeras, with the exception of no. 7, were recognized on an immunoblot by a polyclonal anti-p67^{phox} antibody and, with the exception of nos. 4 and 5, by an anti-Rac1 (residues 178−191) antibody (results not shown).

Chimeras were also tested for the ability of the p67^{phox} and Rac1 segments to function independently of their fusion partners by an oxidase-activity reconstitution assay, performed as described under the Experimental Procedures section. Thus, all of the chimeras, with the exception of nos. 6 and 7, were found to contain a functionally competent p67^{phox} segment, as shown by the ability to support O_2^- production when combined with a membrane, p47^{phox}, and Rac1−GTPγS (Table 1; column 3). Chimeras 3 and 6−8 contained a functional Rac1 segment, as shown by the ability to support O_2^- production when combined with a membrane, p47^{phox}, and p67^{phox}; chimeras 1, 2, 4, and 5 (containing CDC42Hs) exhibited no Rac1-associated activity (Table 1; column 4).

Activation of NADPH Oxidase by Chimeras 1−*3.* First, we tested the first three chimeras for the ability to support amphiphile-dependent oxidase activation in a cell-free system consisting of a membrane and p47^{phox} and lacking p67^{phox} and Rac1. Chimeras 1 and 2 were tested in native form only because the Rac1 segment of these does not include the GTP/GDP binding sites. As seen in Figure 3A and Table 1, chimera 2 was capable of moderate, dose-dependent, oxidase activation, with a V_{max} of 12.00 ± 0.54 mol of O_2^- s^{-1} (mol of cytochrome b_{559} heme)$^{-1}$ and an EC_{50} of 79.95 ± 16.49 nM chimera (mean values ± SEM of three experiments).

Chimera 3, which comprised a full-length segment of Rac1, was potentially capable of GTP/GDP exchange, and its ability to support cell-free oxidase activation was investigated following its exchange to GTPγS or GDPβS. As is apparent in Figure 3B and Table 1, chimera 3, in the GTPγS-bound form, was a potent oxidase activator, with a V_{max} of 71.27 ± 2.16 mol of O_2^- s^{-1} (mol of cytochrome b_{559} heme)$^{-1}$ and an EC_{50} of 216.1 ± 20.2 nM chimera (mean values ± SEM of three experiments). This approached the activity of a combination of equimolar amounts of p67^{phox}-(1−212) and Rac1−GTPγS, which exhibited a similar V_{max} of 69.17 ± 1.62 mol of O_2^- s^{-1} (mol of cytochrome b_{559} heme)$^{-1}$ but a fourfold lower EC_{50} (50.27 ± 6.48 nM p67^{phox}-(1−212) + Rac1; mean values ± SEM of three experiments). These results represent mere approximations, because the dose/response curves of the chimeras did not reach a proper plateau, even at a concentration of 1000 nM.

It is assumed that the reason for the superior activity of Rac1−GTPγS in the cell-free system is that high affinity binding to p67^{phox} requires Rac1 to be in the GTPγS-bound form (*13*). Because, in chimera 3, the two interacting proteins are fused, this requirement might no longer apply. We found, indeed, that chimera 3 was also active in the GDPβS-bound and mock-exchanged forms (Figure 3B) and even in native form (results not shown). At high concentrations of chimera, there was little difference in the activity among these preparations and the GTPγS-bound form, but the latter form was clearly more active at low concentrations. The reason for this difference was investigated further.

In the absence of amphiphile, neither chimera 2 nor 3 was active.

Activation of Oxidase by Chimera 3 in the Absence of p47^{phox}. p67^{phox} and Rac1, when present in micromolar concentrations, can support cell-free oxidase activation in the absence of p47^{phox} (*6*, *7*). p47^{phox} (and amphiphile)-independent oxidase activation is also induced by prenylated Rac1 (*11*). We, consequently, examined the ability of chimera 3 to activate the oxidase in the absence of p47^{phox}. Chimera 3, in the GTPγS-bound form, was indeed capable of supporting amphiphile-dependent O_2^- production by membranes in the absence of p47^{phox} (Figure 3C). In fact, the activity of chimera 3, in the absence of p47^{phox}, exceeded that of a combination of equimolar amounts of p67^{phox}(1−212) and Rac1−GTPγS in the absence of p47^{phox}. A feature characteristic of the p47^{phox}-independent activation by chimera 3 was that the GTPγS-bound form was the only one active; chimera 3 exchanged or mock exchanged to GDPβS had much lower activities. The dose/response curves of chimera 3-GTPγS, in the absence of p47^{phox}, were nonhyperbolic and made accurate kinetic analysis impossible.

Molecular Characteristics of Chimera 3. We first confirmed the ability of chimera 3 to bind GTP by employing the fluorescent GTP analogue, mant-GMPPNP. Figure 4A shows that chimera 3 binds mant-GMPPNP in a manner indistinguishable from Rac1. Identical results were obtained when uptake of mant-GDP by chimera 3 was assessed (results not shown). The stoichiometries of mant-GMPPNP/chimera 3 and mant-GDP/chimera 3, determined on preparations freed of unbound mant nucleotides by gel filtration on

Table 1: Activation of NADPH Oxidase by p67^{phox}−Rac1 and p67^{phox}−CDC42Hs Chimeras[a]

		NADPH oxidase activity [mol of O_2^- s^{-1} (mol of cytochrome b_{559} heme)$^{-1}$]			
		activity of complete chimera		activity	
chimera no.	composition	+ p47^{phox}	w/o p47^{phox}	p67^{phox} segment	Rac1 segment
1	p67^{phox}(1−212)−Rac1(189−192)	4.99 ± 0.47	3.09 ± 0.13	83.03 ± 4.04	8.44 ± 0.75
2	p67^{phox}(1−212)−Rac1(178−192)	10.99 ± 0.39	6.04 ± 0.30	85.90 ± 3.57	21.72 ± 3.62
3	p67^{phox}(1−212)−Rac1(1−192)	54.68 ± 1.47	35.76 ± 0.96	84.06 ± 3.68	79.89 ± 7.14
4	p67^{phox}(1−212)−CDC42Hs(1−191)	4.38 ± 0.07	2.10 ± 0.05	70.67 ± 4.34	5.51 ± 0.92
5	p67^{phox}(1−212)−Rac1(1−178)	4.27 ± 0.39	2.06 ± 0.10	69.98 ± 3.47	4.79 ± 0.60
6	p67^{phox}(155−212)−Rac1(1−192)	3.50 ± 0.34	2.27 ± 0.06	5.72 ± 0.45	70.88 ± 6.88
7	p67^{phox}(199−212)−Rac1(1−192)	3.83 ± 0.12	2.18 ± 0.08	6.02 ± 0.46	62.90 ± 6.06
8	p67^{phox}(1−212)−Rac1(1−Δ[123−133]−192)	70.03 ± 0.17	44.79 ± 1.58	88.98 ± 3.08	85.11 ± 9.85
	mixture of p67^{phox}(1−212) + Rac1(1−192)	64.08 ± 3.24	16.49 ± 0.81		
	mixture of p67^{phox}(1−212) + Rac1(1−Δ[123−133]−192)	64.50 ± 0.82	5.08 ± 0.46		

[a] Cell-free, amphiphile-dependent, NADPH oxidase activation was performed as described in the Experimental Procedures section. The concentrations of components were as follows: membrane, equivalent of 5 nM cytochrome b_{559} heme; all of the chimeras, 600 nM; p47^{phox} (when present), 300 nM. In the mixtures of p67^{phox}(1−212) + Rac1 [or Rac1(1−Δ[123−133]−192)], both of the components were 600 nM. The results were derived by assaying chimeras and Rac1 in the GTPγS-bound form. The concentration of LiDS was 130 μM. The abilities of the p67^{phox} and Rac1 segments of the chimeras to function independently of their fusion partners (columns 3 and 4) were measured as described in the Experimental Procedures section. The results represent the mean values ± SEM of three experiments.

Superdex 75 (Figure 4B), were above 0.9 mol/mol, indicating a behavior identical to that of canonical small GTPases.

We next performed a more detailed analysis of chimera 3 by FPLC gel filtration on Superdex 75. Chimera 3 was exchanged to either GTPγS or GDPβS, and the two preparations were injected in the column; fractions of the eluates were analyzed for the ability to support cell-free oxidase activation, as described in the Experimental Procedures section, and by SDS−PAGE. As seen in Figure 5A, chimera 3, exchanged to GTPγS, exhibited a chromatographic pattern different from that of the chimera exchanged to GDPβS. Both absorbance curves at 280 nm revealed two peaks, eluting at 10.20 ± 0.02 and 10.94 ± 0.03 mL, corresponding to M_r values of 44 021 ± 588 and 32 080 ± 401, respectively (mean values ± SEM, derived from the fractionations of chimera 3 exchanged to GTPγS or GDPβS; four and five experiments, respectively); however, the 44 kDa peak was dominant in chimera 3-GTPγS preparations, whereas the 32 kDa peak was dominant in chimera 3-GDPβS preparations. A third peak, eluting at 11.50−11.62 mL, was also detected. The 32 kDa peak was also found dominant when preparations of native chimera 3 (not subjected to nucleotide exchange) were chromatographed on Superdex 75. Native chimera 3 is expected to be predominantly in the GDP-bound form. Rechromatography of pooled fractions centered on the 44 kDa peak on a fresh Superdex 75 column resulted in the recovery of the same molecular species (44 kDa). Attempts to reseparate the 32 kDa peak met with difficulties because of poor recovery and the appearance of lower M_r species, probably representing degradation products.

The fractions corresponding to the area under the first two peaks contained material which supported amphiphile-dependent O_2^- production by the membranes in the presence of p47^{phox}; the fractions derived from the separation of chimera 3 in the GTPγS-bound form were considerably more active (Figure 5B). SDS−PAGE analyses of the oxidase-activating fractions originating from the separation of both GTPγS-bound and GDPβS-bound forms of chimera 3 revealed the presence of only one major band, slightly less than 45 kDa, in all of the fractions (parts C and D of Figure 5). This corresponds closely to the size of the protein eluting as the first peak, as determined by gel filtration standards; however, no protein, 32 kDa in size, was detected by SDS−PAGE analysis in the fractions comprising the second peak or in any other fraction. The oxidase-activating ability of a particular fraction was in close correlation with the presence and intensity of the <45 kDa band. The nature of the material making up the third peak is unknown; it is likely to consist of proteolytic degradation products of the chimera.

The most likely explanation for the association of two apparent molecular masses of chimera 3 by gel filtration, with a single mass on electrophoresis following denaturation, is that chimera 3 is homogeneous in size but exists in two conformations, depending on the type of bound nucleotide. The GTP-bound form of the chimera exists predominantly in the conformation eluting in peak 1, true to its molecular mass of 44 kDa; the GDP-bound form elutes predominantly in peak 2, with an apparent mass of 32 kDa, probably resulting from retention on the column due to electrostatic or hydrophobic interactions (see the Discussion section for an elaboration of this model).

Oxidase-Activating Abilities of Chimeras 4−8. We constructed a number of additional p67^{phox}−Rac1 chimeras, with the purpose of establishing the structural elements required for endowing these with oxidase-activating capacity. The chimeras were tested in native and GTPγS-bound forms in both the presence and absence of p47^{phox}. In preliminary experiments, we ascertained that chimeras 4−8 all bound mant-GMPPNP, at ratios similar to those found with chimera 3. Chimeras in which the Rac1 segment was replaced by a full-length CDC42Hs (chimera 4), the Rac1 segment was C-terminally truncated at residue 178 (chimera 5), the TPR domain of the p67^{phox} segment was missing (chimera 6), or only the activation domain of p67^{phox} was present (chimera 7) were all inactive (Figure 6A and Table 1). The lack of activity of chimeras 6 and 7, both containing the p67^{phox} activation domain and missing the TPR domain, came as a surprise (see the Discussion section for further treatment of this issue).

Chimera 8 (lacking the insert region in the Rac1 segment), in the GTPγS-bound form, was a potent activator of the oxidase (Figure 6B and Table 1). Its activity (V_{max} of 89.88 ± 2.52 mol of O_2^- s^{-1} (mol of cytochrome b_{559} heme)$^{-1}$ and EC_{50} of 138.4 ± 14.3 nM chimera; mean values ± SEM

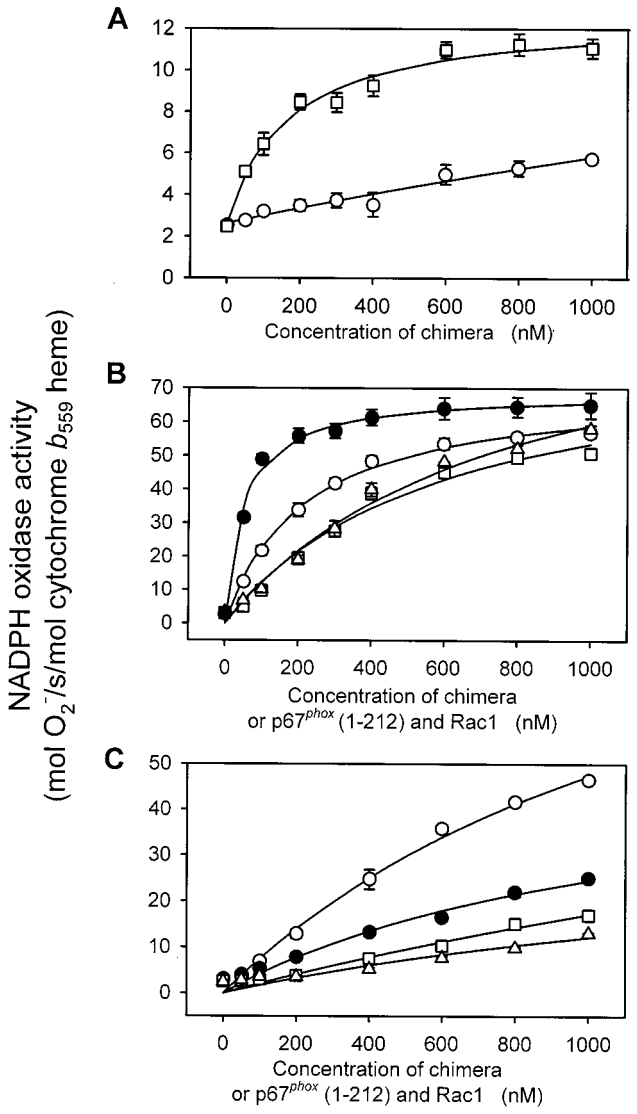

FIGURE 3: NADPH oxidase activation by p67^{phox}−Rac1 chimeras 1−3. (A) Oxidase activation by chimeras 1 and 2 in the native form (without nucleotide exchange). The cell-free assay consisted of a membrane (equivalent of 5 nM cytochrome b_{559} heme), p47^{phox} (300 nM), LiDS (130 μM), and chimeras 1 (open circles) or 2 (open squares) at concentrations from 0 to 1000 nM. (B) Oxidase activation by chimera 3, in the presence of p47^{phox}. The chimera was exchanged to GTPγS (open circles) or GDPβS (open squares) or mock exchanged (open triangles). As a control, we tested mixtures of p67^{phox}(1−212) and Rac1−GTPγS at equimolar concentrations varied from 0 to 1000 nM (filled circles). Assay conditions were as those in panel A. (C) Oxidase activation by chimera 3 in the absence of p47^{phox}. The chimera was exchanged to GTPγS (open circles) or GDPβS (open squares) or mock exchanged (open triangles). As a control, we tested mixtures of p67^{phox}(1−212) and Rac1−GTPγS at equimolar concentrations varied from 0 to 1000 nM in the absence of p47^{phox} (filled circles). Conditions were as those described in panel A, but only a membrane, chimera, and LiDS were present. The results in all of the panels are mean values ± SEM of three experiments.

of three experiments) was slightly superior to that of a mixture of p67^{phox}(1−212) and Rac1 Δ[123−133]−GTPγS. Similarly to chimera 3, in the presence of p47^{phox}, it was also active in the native form, albeit with a lesser potency (results not shown).

Chimera 8-GTPγS was also active in the absence of p47^{phox}, as found before with chimera 3. Surprisingly, its potency markedly exceeded that of a mixture of equimolar

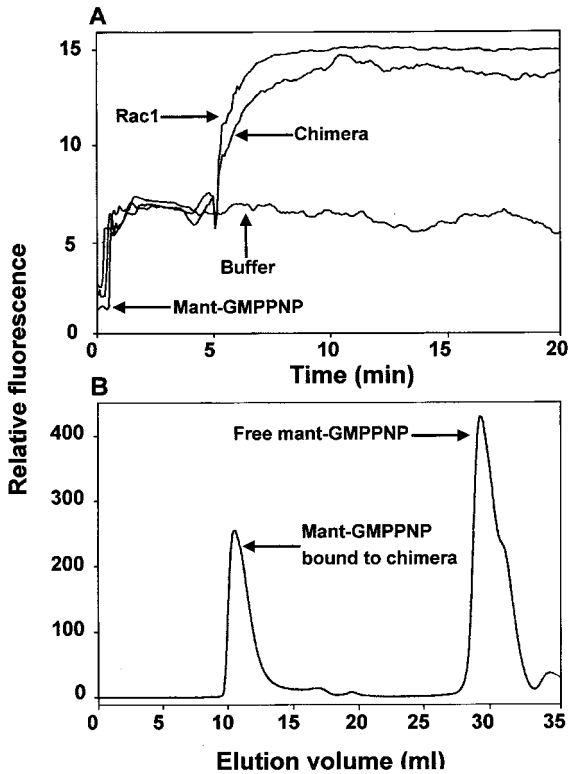

FIGURE 4: Binding of fluorescent GTP analogue to chimera 3. (A) Chimera 3 or Rac1 (both 1 μM) were exposed to 0.2 μM mant-GMPPNP in a buffer containing 0.5 μM free Mg^{2+}, and the binding of mant-GMPPNP was followed kinetically in a spectrofluorometer, as described in the Experimental Procedures section. The tracings are representative results out of three experiments. (B) In separate experiments, chimera 3 was incubated with a fivefold molar excess of mant-GMPPNP, and 5000 pmol of chimera was injected in a Superdex 75 FPLC column. The tracing represents the fluorescent signal recorded in the eluate from the column. In this particular experiment (representative of two), chimera 3 with bound mant-GMPPNP elutes at a volume corresponding to an M_r of 45 336; the free (unbound) mant-GMPPNP is retained on the column.

amounts of p67^{phox}(1−212) and Rac1 Δ[123−133]−GTPγS, which was incapable of oxidase activation, up to a concentration of 1000 nM (Figure 6B and Table 1). Chimera 8 was inactive in the absence of amphiphile.

Importance of the Rac1 C-Terminal Polybasic Domain. Several of the results obtained so far point to the paramount importance of the polybasic C terminus of Rac1 in conferring activity to the chimeras. Thus, chimera 5 was inactive despite the presence of most of the Rac1 sequence, with the exception of residues 179−192, whereas chimera 2, containing the Rac1 C-terminal sequence missing in chimera 5, was partially active. The lack of activity of chimera 4, in which the Rac1 sequence was replaced by CDC42Hs, could also be the result of the lesser-positive charge of the C terminus of CDC42Hs (four as opposed to six basic residues). We further investigated the role of the polybasic domain by making use of the observation that synthetic polybasic peptides, corresponding to the C-terminal sequence of Rac1, inhibit cell-free oxidase activation (*16*−*18*). As seen in Figure 7, the Rac1 peptide (178−192) inhibited oxidase activation by all three of the active chimeras (2, 3, and 8) in a dose-dependent manner. The peptide was also inhibitory when activation by chimeras 3 and 8 took place in the absence of p47^{phox} (results not shown). The peptide did not inhibit activation when added after the 90 s incubation

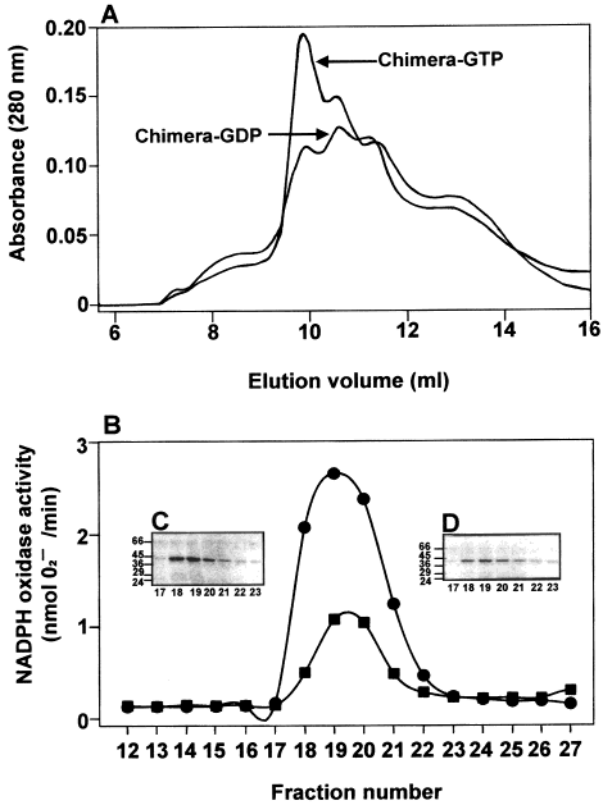

FIGURE 5: Conformational heterogeneity of chimera 3 analyzed by gel filtration. (A) A total of 5000 pmol of chimera 3, exchanged to GTPγS or GDPβS, was injected in a Superdex 75 column. Tracings represent the absorbances at 280 nm of two such separations. (B) Several 0.6 mL fractions were collected (filled circles, chimera 3-GTPγS; filled squares, chimera 3-GDPβS), and 50 μL aliquots were assayed for the ability to support oxidase activation in a cell-free system, also containing a membrane, p47^{phox}, and LiDS. (C and D) Aliquots (20 μL) were analyzed by SDS−PAGE (panel C, chimera 3-GTPγS; panel D, chimera 3-GDPβS). The lines to the left of the gels indicate the migration of molecular weight markers ($M_r \times 10^{-3}$). Results are representative of four experiments performed with chimera 3-GTPγS and five experiments with chimera 3-GDPβS.

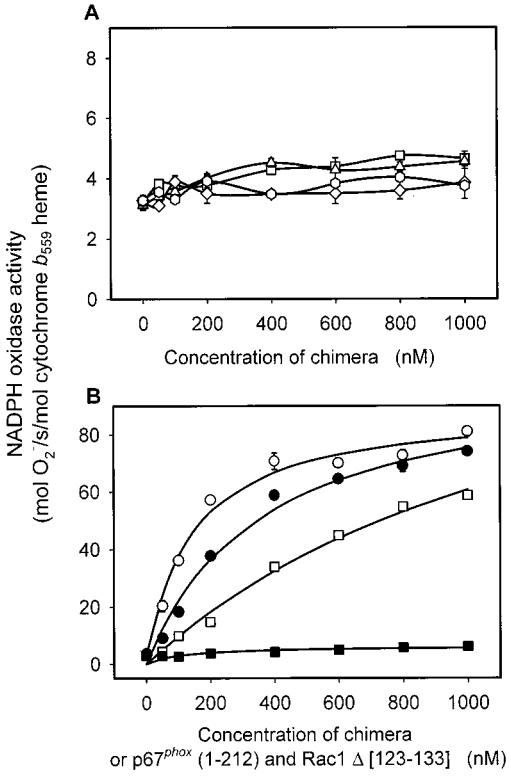

FIGURE 6: NADPH oxidase activation by p67^{phox}−Rac1 chimeras 5−8 and p67^{phox}−CDC42Hs chimera 4. (A) Oxidase activation by chimeras 4 (open squares), 5 (open triangles), 6 (open diamonds), and 7 (open hexagons). All of the chimeras were exchanged to GTPγS. The cell-free assay consisted of a membrane (equivalent of 5 nM cytochrome b_{559} heme), p47^{phox} (300 nM), chimeras (at concentrations from 0 to 1000 nM), and LiDS (130 μM). (B) Oxidase activation by chimera 8 exchanged to GTPγS in the presence (open circles) or absence (open squares) of p47^{phox}. As controls, we tested mixtures of p67^{phox}(1−212) and Rac1 Δ[123−133]−GTPγS, at equimolar concentrations varied from 0 to 1000 nM in the presence (300 nM; filled circles) or absence (filled squares) of p47^{phox}. Conditions were as those described in panel A. The results in both panels are the mean values ± SEM of three experiments.

interval of membranes with chimeras and amphiphile. All of this suggests that the peptide acts by competing with the C terminus of the chimera for attachment to the membrane.

Binding of Chimera to the Membrane. The results obtained so far are amenable to the interpretation that the Rac1 segment serves as a carrier for bringing the chimeras to the membrane and allowing a secondary interaction of the p67^{phox} segment with cytochrome b_{559}. With the purpose of finding direct evidence for such a role, we performed experiments in which chimera 3 and control Rac1 preparations were subjected to exchange to mant-GTP, mixed with membrane vesicles, and subjected to FPLC gel filtration on a Superose 12 column, in the presence and absence of the activating amphiphile LiDS in the column buffer. In-line recording of fluorescence permits the determination of the amounts of free and membrane-bound chimera. This procedure was used by us before for quantifying the binding of prenylated Rac1 to membrane vesicles (*11*). Figure 8A illustrates the translocation of a significant proportion of mant-GTP-labeled chimera 3 (18.10%) to membrane vesicles, eluting in the excluded volume of the column (peak 1) when LiDS was present; in the absence of LiDS, only 9.32% of the chimera associated with the membrane. The binding to the membrane occurred in the absence of p47^{phox}. Control Rac1, not fused with p67^{phox}(1−212), did not exhibit amphiphile-dependent membrane binding (Figure 8B); the amounts of mant-GTP-labeled Rac1 bound to membrane vesicles were practically identical in the presence (14.45%) and absence (13.65%) of LiDS (see peak 1). In similar experiments, we also found that, unlike chimera 3, chimera 4 (in which CDC42Hs replaces the Rac1 segment) did not exhibit amphiphile-dependent membrane association.

Inhibition of Chimera Activity by PBD of PAK. PAK was found to interact with Rac1 in GTP-bound form; the two sites in Rac1 involved in this interaction were also found to be required for oxidase activation: the canonical N-terminal effector region (residues 22−45) and a downstream region (residues 143−175; *33*). The PBD of PAK interacts principally with the effector region of Rac, which undergoes a conformational change upon GTP binding, explaining the marked preference of PBD for the GTP-bound form of Rac (*22*).

We recently showed that PBD inhibits oxidase activation in vitro, by competing with p67^{phox} for binding to Rac1 (*34*). Because, in chimera 3, the p67^{phox} and Rac1 segments are joined by a "covalent" bond, it was of interest to see whether

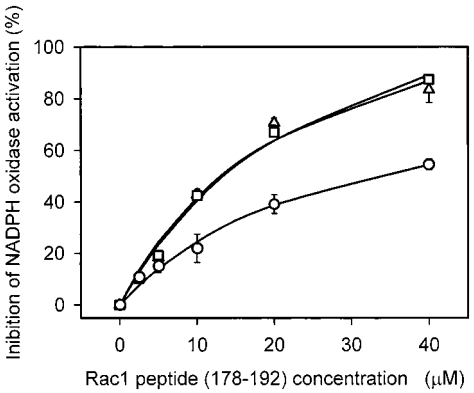

FIGURE 7: Synthetic C-terminal Rac1 peptide inhibiting oxidase activation by p67^{phox}−Rac1 chimeras. Synthetic Rac1 peptide (178−192) was added as the first component at varying concentrations to the cell-free NADPH oxidase assay, consisting of a membrane (equivalent of 5 nM cytochrome b_{559} heme), p47^{phox} (300 nM), LiDS (130 μM), and chimeras 2 (open circles), 3 (open squares), and 8 (open triangles) all at a concentration of 600 nM. The inhibition of NADPH oxidase activation was calculated in relation to a control preparation in which the Rac1 peptide was replaced by a buffer. Results are the mean values ± SEM of three experiments.

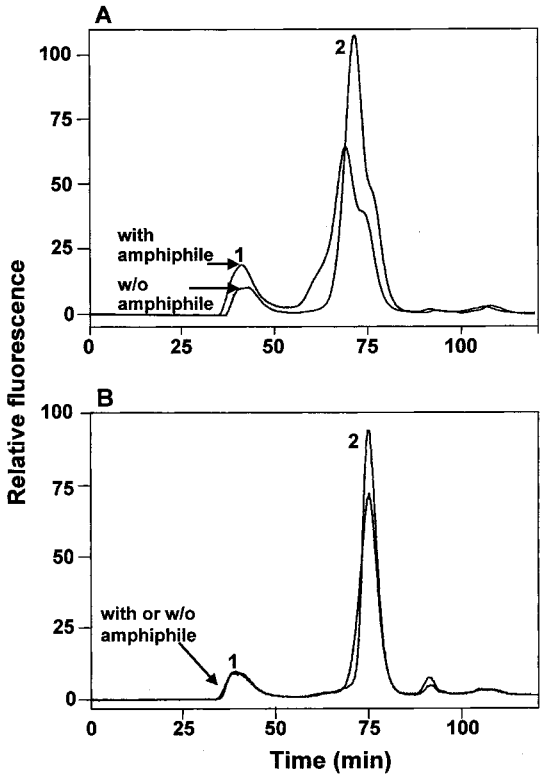

FIGURE 8: Amphiphile-dependent translocation of chimera 3 but not Rac1 to membrane vesicles. Chimera 3 (panel A) or Rac1 (panel B; 1500 pmol each) were exchanged to mant-GTP, mixed with phagocyte membrane vesicles (equivalent of 250 pmol of cytochrome b_{559} heme), and subjected to gel filtration on a Superose 12 FPLC column in the presence and absence of LiDS (130 μM) in the column buffer. The eluates were analyzed for fluorescence by passage through a spectrofluorometer fitted with an HPLC flow cell. Peak 1 represents the fraction bound to membrane vesicles; peak 2 represents the free (unbound) component. The experiments illustrated are representative of two performed with chimera 3 and two others with Rac1.

PBD also inhibits oxidase activation by the chimera. In investigating the effect of PBD, we made use of the fact that chimera 3 is capable of oxidase activation in both the

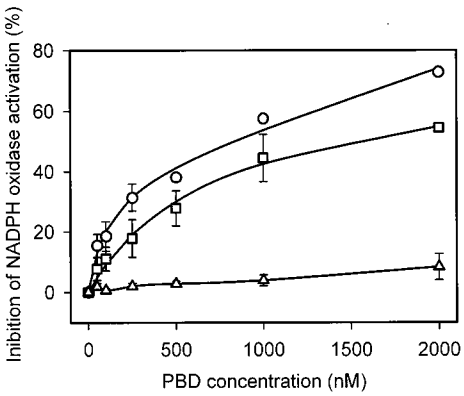

FIGURE 9: PBD of PAK inhibiting oxidase activation by chimera 3. The PBD of PAK was added as the first component at varying concentrations to the cell-free NADPH oxidase assay, consisting of membrane (equivalent of 5 nM cytochrome b_{559} heme), LiDS (130 μM), and chimeras (300 nM) in the presence or absence of p47^{phox} (300 nM). The curves represent the effect of PBD on chimera 3-GTPγS in the absence of p47^{phox} (open circles), chimera 3-GTPγS in the presence of p47^{phox} (open squares), and native chimera 3 in the presence of p47^{phox} (open triangles). The inhibition of NADPH oxidase activation was calculated in relation to a control preparation in which PBD was replaced by a buffer. The results are the mean values ± SEM of three experiments.

GTP-bound and native or mock-exchanged forms (Figure 3B). As seen in Figure 9, PBD interfered, in a dose-dependent manner, with oxidase activation by chimera 3, exchanged to GTPγS, both in the presence and in the absence of p47^{phox}. PBD exerted no inhibitory effect on oxidase activation by chimera 3 in the native form. The seemingly obvious interpretation of this result is that PBD interacts with the GTP-bound, but not with the native, form of the chimera, in accordance with its known preference for Rac−GTP. However, the very occurrence of the inhibition is unexpected and provides useful information on the structure of chimera 3 (see the Discussion section).

DISCUSSION

We demonstrate that several chimeric proteins, consisting of segments of p67^{phox} and Rac1, are capable of replacing the individual cytosolic components in eliciting O_2^- generation in a cell-free system consisting of membranes, p47^{phox}, and an anionic amphiphile.

To ensure that no Rac1 was carried into the cell-free assay by the membranes, these were subjected to washing with 1 M KCl before solubilization. This procedure was shown to result in the effective removal of all traces of potentially membrane-bound Rac (*35*). The absence of Rac from KCl-washed membranes was also demonstrated by the lack of O_2^- production by such membranes combined with p47^{phox} and p67^{phox}, when no Rac was added (see ref *11*).

The two most active chimeras were nos. 3 and 8. These contained p67^{phox}(1−212) and either the full length of Rac1 (chimera 3) or Rac1 lacking the insert region (residues 123−133; chimera 8). Both were active in the GTP-bound, GDP-bound, and native forms, with the GTP-bound form being the most active. When in the GTP-bound form, considerable oxidase-activating capacity was evident in the absence of p47^{phox}, demonstrating that, in vitro, p67^{phox}−Rac1 chimeras contain all of the elements required for the induction of a conformational change in cytochrome b_{559}, leading to electron flow.

The fact that chimera 2, containing only a brief Rac1 C-terminal sequence, is moderately active is in accordance with the proposal that at least one of the roles of Rac is to carry p67^{phox} to the membrane or to serve as a membrane anchor for p67^{phox}. Support for this is also offered by the lack of activity of chimera 5, which is identical to the active chimera 3 except that it lacks the Rac1 C-terminal polybasic sequence. Additional evidence for this contention is provided by the finding that chimera 3 translocates to membrane vesicles in an in vitro assay in the presence of oxidase-activating concentrations of amphiphile, as opposed to the lack of translocation of Rac1 not fused to p67^{phox}. It is likely that the p67^{phox} segment stabilizes an initial electrostatic association of the chimera with the membrane by binding to cytochrome b_{559}. The inferior activity of chimera 2, in comparison to chimeras 3 and 8, suggests either that Rac possesses an additional function in oxidase assembly, carried by residues upstream to the C terminus, or that the conformation of chimera 2 is less than optimal. The lack of activity of chimera 4 is also open to two interpretations; it could be due to the fact that the C terminus of CDC42Hs contains only four, noncontiguous, basic residues and would, therefore, possess a lesser affinity for the membrane or that there is involvement in the oxidase activation of other domains present in Rac1 but absent in CDC42Hs.

On the basis of evidence that the activation domain in p67^{phox} is the key element mediating the interaction of p67^{phox} with cytochrome b_{559} (*8*, *9*), we expected chimeras 6 and 7 to be active. Chimera 7 contains the activation domain proper, whereas chimera 6 also includes an additional region, C terminal to the TPR domain, thought to participate in intramolecular interactions with a 20-residue sequence in the TPR domain (*15*). Both of the chimeras are lacking the TPR domain, which, albeit essential for the interaction of p67^{phox} with Rac1, was expected to be dispensable when the two components were fused artificially.

We also expected the GTP- and GDP-bound forms of chimera 3 to be equally potent activators, on the basis of the concept that the bound nucleotide determines the affinity of Rac1 for p67^{phox}, an issue which becomes irrelevant when the two components are fused.

A likely explanation for these two paradoxes is that, in active chimeras such as nos. 3 and 8, the TPR domain of the p67^{phox} segment participates in intrachimeric interactions with residues in the Rac1 segment, the residues involved being those recently defined by mutational studies (*14*) and the analysis of p67^{phox}−Rac1 crystals (*15*). As found in the canonical intermolecular interaction between p67^{phox} and Rac (*13*), intrachimeric interaction between the corresponding segments is expected to be of the highest affinity when the chimera is in the GTP-bound form. We propose the existence of two conformations of chimera 3: a "closed" conformation due to the formation of intrachimeric bonds when the chimera is in the GTP-bound form and an "open" conformation lacking intrachimeric bonds when the chimera is in the GDP-bound form (Figure 10). It is likely that these two forms represent the two extremities of the spectrum and that, in both the GTP-bound and GDP-bound forms, both structural species are present, albeit at different frequencies. Strong support for this proposal comes from the gel filtration analysis of the GTP- and GDP-bound forms of chimera 3 (Figure 5), directly demonstrating the existence of two

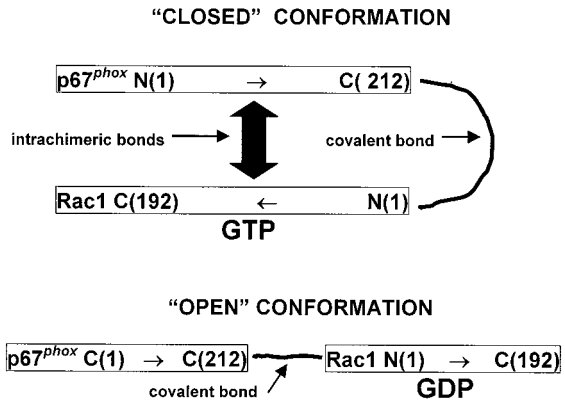

FIGURE 10: Hypothetical structures of the GTP- and GDP-bound forms of chimera 3.

structural forms of chimera 3: the GTP-bound form, consisting predominantly of a 44 kDa species, and the GDP-bound form, consisting predominantly of molecules retained on the column, with an apparent mass of 32 kDa. We cannot establish whether the chimeras in both conformations are capable of oxidase activation, with different potencies, or only the closed conformation is active. The total lack of activity of chimeras 6 and 7, incapable of forming intrachimeric bonds, supports the second option. Further, support for such a model is provided by the finding that PBD inhibits oxidase activation by the GTP-bound form of chimera 3. Such inhibition would only be expected if one assumes that intrachimeric bonds between the p67^{phox} and Rac1 segments are important for activity and are disrupted by PBD. The molecular basis of the functional superiority of chimeras in which the engineered fusion of p67^{phox} and Rac1 is supplemented by natural bonds between the two segments of the chimera remains to be elucidated. Recently, the generation of chimeric proteins between either Ras or Rap1 and the Ras-binding domain of Raf1 was reported (*36*). When the GTPase segments were in the GTP-bound form, these interacted with the Raf1 segment, generating the equivalent of our closed conformation, an event detected by fluorescent markers.

The ability of chimeras 3 and 8 to activate the oxidase, in the absence of p47^{phox}, agrees well with earlier reports of p47^{phox}-independent oxidase activation in vitro at high concentrations of p67^{phox} and Rac1 (*6*, *7*) or in the presence of prenylated Rac1 *(11)*. Obviously in the physiological context, oxidase activation in the absence of p47^{phox} represents an artifactual situation and the main value of its exploration is to provide information on the action mechanisms of p67^{phox} and Rac1. It is, therefore, of interest that the GTP-bound forms of chimeras 3 and 8, in the absence of p47^{phox}, were more potent activators than the combination of individual components, under the same conditions. This is, probably, the result of the fact that, in the absence of p47^{phox}, the translocation of p67^{phox} to the membrane is dependent entirely on Rac; therefore, when the two proteins are fused, the chances of p67^{phox} to be carried to the membrane and to stay attached are better. The fact that, in the absence of p47^{phox}, only the GTP-bound forms of the chimeras were active probably reflects, again, the importance of intrachimeric bonds in ensuring maximal activity.

The ability of chimera 8 to activate the oxidase to a degree equal to chimera 3 came as a surprise. Chimera 8 is lacking

the insert region of Rac1, which was suggested to be essential for oxidase activation in the amphiphile-activated cell-free system. This claim was based on our own "peptide walking" results (*18*) on mutational studies on Rac1 (*30*, *31*) and on recent results demonstrating a direct interaction of Rac2 with cytochrome b_{559}, involving the Rac insert region (*32*). Our finding that chimera 8 is a potent activator of the oxidase, in both the presence and absence of $p47^{phox}$, is in contradiction with the above findings but is in good agreement with our earlier result that the deletion of the insert region of Rac1 has no significant effect on oxidase activation in the cell-free system (*24*). The lack of involvement of the Rac insert region in oxidase activation is also in agreement with earlier studies describing normal oxidase activation by Rac−Rho (*33*) and Rac−CDC42Hs (*37*) chimeras in which the Rac1 insert region was replaced by the corresponding regions in Rho and CDC42Hs. However, caution should be exercised in interpreting the ability of chimera 8 to activate the oxidase; the experiments here described were performed with octyl glucoside-solubilized membranes, whereas in the work described in refs *30* and *31* supporting the importance of the insert region, purified nonsolubilized plasma membranes were used.

The chimeras described in this report were nonprenylated. Prenylated Rac has a superior ability to bind to membranes (*11*, *19*) and represents the physiological molecule actually involved in the activation of oxidase in intact cells. For a true comparison of the relative potencies of the various $p67^{phox}$−Rac1 chimeras to activate the oxidase, the use of prenylated constructs offers several advantages. Such experiments are in progress.

The design of chimeras incorporating functional domains derived from distinct oxidase components represents a new approach to the study of this particular enzyme. As shown by the results of the present study and by a recent report describing the construction of $p47^{phox}$−$p67^{phox}$ chimeras (*38*), it holds considerable promise and should be applicable to the generation of more complex constructs, containing segments from more than two oxidase components. The chimeras described in the present study also illustrate the potential of two more general principles of genetic engineering: the introduction of a GTP/GDP switch into a protein which is not a canonical GTPase and the addition of a membrane-targeting sequence to a cytosolic component lacking such an element.

ACKNOWLEDGMENT

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