The superoxide (O$_2^-$)-generating NADPH oxidase complex of phagocytes consists of a membrane-associated flavocytochrome (cytochrome $b_{558}$) and four cytosolic proteins, p47$^\text{phox}$, p67$^\text{phox}$, p40$^\text{phox}$, and the small GTPase Rac (Rac1 or -2). NADPH oxidase activation (O$_2^-$ production) is elicited as the consequence of assembly of some or all cytosolic components with cytochrome $b_{558}$. This process can be reproduced in an in vitro system consisting of phagocyte membranes, p47$^\text{phox}$, p67$^\text{phox}$, and Rac, activated by an anionic amphiphile. We now show that posttranslationally processed (prenylated) Rac1 initiates NADPH oxidase assembly, expressed in O$_2^-$ production, in a cell-free system containing phagocyte membrane vesicles and p67$^\text{phox}$, in the absence of an activating amphiphile and of p47$^\text{phox}$. Prenylated Cdc42Hs, a GTPase closely related to Rac, is inactive under the same conditions. Results obtained with phagocyte membrane vesicles can be reproduced fully by replacing these with partially purified cytochrome $b_{558}$, incorporated in phosphatidylcholine vesicles. Prenylated, but not nonprenylated, Rac1 binds spontaneously to phagocyte membrane vesicles and also to artificial, protein-free, phosphatidylethanolamine vesicles, a process counteracted by GDP dissociation inhibitor for Rho. Binding of prenylated Rac1 to membrane vesicles is accompanied by the recruitment of p67$^\text{phox}$ to the same location and the formation of an assembled NADPH oxidase complex, producing O$_2^-$ upon the addition of NADPH. Amphiphile and p47$^\text{phox}$-independent NADPH oxidase activation by prenylated Rac1 is inhibited by Rho GDP dissociation inhibitor and by phosphatidylcholine vesicles, both competing with membrane for prenylated Rac1. We conclude that, in vitro, targeting of Rac to the phagocyte membrane is sufficient for the induction of NADPH oxidase assembly, suggesting that the principal or, possibly, the only role of Rac is to recruit cytosolic p67$^\text{phox}$ to the membrane environment, to be followed by the interaction of p67$^\text{phox}$ with cytochrome $b_{558}$.

The production of reactive oxygen species represents the major microbicidal mechanism of professional phagocytes. The primordial oxygen radical is superoxide (O$_2^-$), and it is produced by the NADPH-derived one-electron reduction of molecular oxygen, by an enzyme complex known as the NADPH oxidase (referred to here as “oxidase”; reviewed in Refs. 1 and 2). This consists of a membranal heterodimeric flavocytochrome (cytochrome $b_{558}$), comprising two subunits, gp91$^\text{phox}$ and p22$^\text{phox}$, and four cytosolic proteins, p47$^\text{phox}$, p67$^\text{phox}$, p40$^\text{phox}$, and the small GTPase Rac (Rac1 or -2). Elicitation of O$_2^-$ production in vitro involves the stimulus-dependent translocation of some or all cytosolic components to the plasma membrane and their assembly with cytochrome $b_{558}$. Oxidase assembly can be induced in vitro by exposing a mixture of phagocyte membranes and the cytosolic components p47$^\text{phox}$, p67$^\text{phox}$, and Rac to a critical concentration of an anionic amphiphile (3, 4).

Rac1 or -2 is absolutely required for oxidase assembly in the amphiphile-activated cell-free system (5, 6). It is also clearly involved in O$_2^-$ production in intact phagocytes, as shown by the inhibitory effect of Rac antisense oligonucleotides (7) and by selective defects in O$_2^-$ production by neutrophils of Rac2-deficient mice (8) and of a patient with an inhibitory mutation in Rac2 (9). It is the consensus opinion that, in the course of oxidase assembly, Rac is translocated to the membrane (10), although lack of translocation (11) or the lack of relevance of translocation to assembly (12) was also claimed. In the cytosol, Rac is found as a C-terminally prenylated (geranylgeranylated) protein (13), forming a heterodimer with the regulatory protein GDP dissociation inhibitor for Rho (Rho GDI) (5). Dissociation of prenylated Rac from Rho GDI was proposed to be an obligatory step preceding translocation of Rac from the cytosol to the membrane (14–16). The role of Rac in oxidase assembly was studied extensively in the semirecombinant amphiphile-activated cell-free system (4). Both nonprenylated (5, 17) and prenylated (17–19) Rac are capable of supporting oxidase activation in vitro. It was recently suggested (20) that, whereas membrane association of prenylated Rac (Rac1 or -2) is mediated principally by hydrophobic interaction between the geranylgeranyl tail and membrane lipids, nonprenylated Rac1 binds by electrostatic interaction between a C-terminal polybasic region (21) and negative charges on the membrane.

We intended to test the hypothesis that binding of Rac to the membrane is a crucial event in the initiation of oxidase assembly. We found indeed that prenylated, but not nonprenylated, Rac1 initiates oxidase assembly and NADPH-dependent O$_2^-$ production in a cell-free system, containing phagocyte membranes and p67$^\text{phox}$, in the absence of an amphiphilic activator.
and of p47phox. This demonstrates that, under certain conditions, targeting of Rac1 to the phagocyte membrane is sufficient for the initiation of oxidase assembly and suggests that the principal (or only) role of Rac is to act as a carrier for p67phox from the cytosol to the membrane.

EXPERIMENTAL PROCEDURES

Preparation of Phagocyte Membranes and Membrane Vesicles—Phagocyte membranes were prepared from guinea pig peritoneal macrophages elicited by the injection of mineral oil, as described (3). The membranes were solubilized by 40 mM n-octyl-β-D-glucopyranoside (octyl glucoside; Sigma) in buffer A (250 mM sodium chloride, 5 mM MgCl2, and 1 mM sodium, potassium phosphate, pH 7.0, 1 mM EGTA, 1 µM calmodulin). The membrane vesicles were prepared by dialysis against buffer lacking detergent, as described (3). These vesicles were subjected to a final centrifugation at 43,000 × g for 1 h at 4 °C. The partially purified cytochrome b559 preparations had a specific content of 4–6 nmol of heme/mg of protein.

Preparation of PC Vesicles—Soybean PC (the same type as the one used for relipidation of cytochrome b559) was dissolved, at a concentration of 10 mg/ml, as described before (4). Soybean phosphatidylcholine (PC) (10–20% phosphatidylcholine, type II-S, product 5638; Sigma), at a concentration of 200 µg/ml, as described before (23), and vesicles were prepared by dialysis of relipidated cytochrome b559 against detergent-free buffer for 18 h at 4 °C. The partially purified cytochrome b559 preparations had a specific content of 4–6 nmol of heme/mg of protein.

Preparation of Recombinant Oxidase Components—p67phox and p47phox were prepared in Sf9 cells, infected with baculovirus carrying E. coli (DH5α competent cells; Life Technologies) and purified by affinity chromatography on a HiTrap Hepar-arin column (5 ml; Amersham Pharmacia Biotech), essentially as described before (23) with the following modifications: (a) batch absorption by DEAE-Sepharose was replaced by passage through a HiTrap DEAE-Sepharose column (5 ml; Amersham Pharmacia Biotech) placed in line ahead of the heparin column, and (b) the hydroxyapatite chromatography step was omitted. The purified cytochrome b559 was reconstituted with cytochrome c, phosphatidylcholine (PC) (10–20% phosphatidylcholine, type II-S, product 5638; Sigma), at a concentration of 200 µg/ml, as described before (23), and vesicles were prepared by dialysis of relipidated cytochrome b559 against detergent-free buffer for 18 h at 4 °C. The partially purified cytochrome b559 preparations had a specific content of 4–6 nmol of heme/mg of protein.

Preparation of PC Vesicles—Soybean PC (the same type as the one used for relipidation of cytochrome b559) was dissolved, at a concentration of 10 mg/ml, as described before (4). Soybean phosphatidylcholine (PC) (10–20% phosphatidylcholine, type II-S, product 5638; Sigma), at a concentration of 200 µg/ml, as described before (23), and vesicles were prepared by dialysis of relipidated cytochrome b559 against detergent-free buffer for 18 h at 4 °C. The partially purified cytochrome b559 preparations had a specific content of 4–6 nmol of heme/mg of protein.

Preparation of Recombinant Oxidase Components—p67phox and p47phox were prepared in Sf9 cells, infected with baculovirus carrying cDNAs of the oxidase components (human), as described (24). Recombinant prenylated Rac1 (human) and Rho GDI (bovine) were isolated from Escherichia coli as fusion proteins with glutathione S-transferase (GST), as described (24). In some experiments, we used bacterial prenylated p67phox (human) that was also isolated from E. coli as a fusion protein with GST. cDNA of truncated p67phox (residues 1–212) was obtained by polymerase chain reaction, and the fragment was cloned into the pGEX-2T vector (4). The GST-p67phox (1–212) fusion protein was expressed in E. coli (DE5α competent cells; Life Technologies) and purified by affinity chromatography on glutathione-agarose (Sigma).

Construction and Isolation of His-tagged Prenylated and Nonprenylated Rac1—The cDNA for Rac1 was isolated from the pGEX-2T clone, used for the expression of the protein in E. coli, by digestion with the restriction enzymes BamHI and EcoRI. In order to express His6-Rac1 in insect cells, the cDNA was cloned into the BamHI and EcoRI sites of the baculovirus transfer vector pBacPAK-His1 (CLONTECH). Rac1 cDNA was introduced into the baculovirus genome using the BaculoGold system (PharMingen). Positive colonies from plaque assays were used for the production of virus stocks that served for the infection of Sf9 cell monolayers. The amounts of membrane-bound Rac1 and p67phox were monitored by Western blotting using an NADPH oxidase antibody raised in rabbits against a truncated oxidase complex (see below), and the relative amounts of membrane-bound and free p67phox were measured by an enzyme-linked immunosorbent assay (ELISA).

NADPH Oxidase Activity Reconstitution Assay—In this assay, the membrane association of a certain cytosolic oxidase component was monitored by the expression of the oxidase complex in the absence of amphiphiles. The oxidase complex was reconstituted from purified oxidase components (human) and membranes and cytosol were separated by sonication in buffer A (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 5 mM MgCl2, and 1 mM sodium, potassium phosphate, pH 7.0, 2 mM NaN3, 1 mM EGTA, 1 mM MgCl2, and 1 µM FAD). Its functional integrity was ascertained by its ability to form a heterodimer with Rho GDI, this being identified by gel filtration on Superdex 75 and anion exchange chromatography on Mono Q. His6-tagged prenylated Rac1 was purified from the cytosol by the same technique, except for the use of 50 mM imidazole in buffer A, lacking sodium cholate.

Cdc42Hs—His6-tagged prenylated Cdc42Hs was a kind gift from Dr. R. A. Cerione (Cornell University). It was produced by baculoviral expression in Sf21 cells and purified from the membrane fraction. The protein was found to react on immunoblot with a polyclonal anti-Cdc42Hs antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA; product sc-87). Its functional integrity was ascertained by its ability to form a complex with Rho Hs. The assembly of the oxidase complex was assessed by measuring NADPH-dependent O2 production in a cell-free system, consisting of 250 pmol of cytochrome b559 (human) or recombinant p67phox (human) and 0.5 nM of Rac1 (0–500 nM) subjected to nucleotide exchange with either GTPyS or GDP[S], and recombinant p67phox (250–1600 nM), in 96-well microplates, in a volume of 200 µl of assay buffer (25) per well. No free GTPyS was added to the reaction. In some experiments, p47phox was included, at a concentration of 300 nM. The mixtures were incubated for 90 s at 25 °C, in the absence of an amphiphilic activator, and O2 generation was identified by the addition of NADPH (240 µM). O2 production was measured by the kinetics of cytochrome c reduction, as described before (25). Specificity of cytochrome c reduction was controlled by its inhibition by superoxide dismutase (Sigma). Amphiphilic-dependent cell-free oxidase activation was performed using the same concentrations of components as indicated above but with the addition of p47phox (300 nM) and lithium dodecyl sulfate (LIDS) (130 µM).

In Line Fluorescence Assay—Prenylated or nonprenylated Rac1 were subjected to nucleotide exchange with the fluorescent analogues 2’-(or 3’)-O-(N-methylnaphthylamino) guanosine 5’-triphosphate (mant-GTP) or mant-GDP (26), as described for nonfluorescent nucleotides, except for not removing unbound nucleotides. Rac1 labeled with fluorescent analogues was also mixed or not with either membrane vesicles (250 pmol of cytochrome b559 heme) or PC vesicles (0.6 mg of lipid), injected in a Superose 12 HR 10/30 FPLC gel filtration column (Amersham Pharmacia Biotech), and eluted with a buffer consisting of 65 mM sodium, potassium phosphate, pH 7.0, 2 mM NaN3, 1 mM EGTA, 1 mM MgCl2, and 1 µM FAD. Its composition is, in essence, identical to reaction buffer used in the cell-free oxidase assay (25). Chromatography was performed on an HPLC system (Waters), at a flow rate of 0.2 ml/min, at 4 °C, and the fluorescent signal (excitation = 556 nm; emission = 445 nm) was measured continuously, by passing the column eluate through a spectrophotofluorometer (model FP-770; Jasco), fitted with an HPLC flow cell.
Cell-free oxidase activation was performed as described under “Experimental Procedures.” The concentrations of components were as follows: membrane or partially purified repurified cytochrome b_{559}, equivalent of 5 nm cytochrome b_{559} heme; Rac1-GTPyS, 300 nm; p47^{phox}, 300 nm; and p67^{phox}, 300 nm. Results represent means ± S.E. of five experiments.

**TABLE I**

| Combination of NADPH oxidase components                                         | NADPH oxidase activity (mol O_2 s^{-1} mol cytochrome b_{559} heme) |
|-------------------------------------------------------------------------------|---------------------------------------------------------------------|
| Membrane                                                                      | 1.01 ± 0.18                                                          |
| Membrane + p47^{phox}                                                          | 1.83 ± 0.11                                                          |
| Membrane + p67^{phox}                                                          | 1.03 ± 0.15                                                          |
| Membrane + prenylated Rac1                                                      | 1.78 ± 0.30                                                          |
| Membrane + p47^{phox} + p67^{phox}                                             | 2.03 ± 0.05                                                          |
| Membrane + p47^{phox} + prenylated Rac1                                        | 2.35 ± 0.21                                                          |
| Membrane + p67^{phox} + prenylated Rac1                                        | 55.12 ± 3.95                                                         |
| Membrane + p67^{phox} + prenylated Rac1 + SOD                                 | 1.84 ± 0.42                                                          |
| Denatured membrane + p67^{phox} + prenylated Rac1                              | 0.67 ± 0.03                                                          |
| Membrane + denatured p67^{phox} + prenylated Rac1                             | 1.80 ± 0.39                                                          |
| Membrane + p67^{phox} + denatured prenylated Rac1                              | 1.77 ± 0.05                                                          |
| Membrane + p47^{phox} + p67^{phox} + prenylated Rac1                           | 62.34 ± 2.62                                                         |
| Membrane + p47^{phox} + p67^{phox} + prenylated Rac1 + SOD                    | 2.18 ± 0.43                                                          |
| Membrane + p67^{phox} + nonprenylated Rac1                                    | 1.00 ± 0.11                                                          |
| Membrane + p47^{phox} + p67^{phox} + nonprenylated Rac1                       | 2.00 ± 0.15                                                          |
| Cytochrome b_{559} + p67^{phox} + prenylated Rac1                             | 96.05 ± 1.83                                                         |
| Cytochrome b_{559} + p47^{phox} + p67^{phox} + prenylated Rac1                | 105.96 ± 1.27                                                        |
| Membrane + p47^{phox} + p67^{phox} + prenylated Rac1 activated by LiDS^{c}    | 69.78 ± 2.84                                                         |

| a | Derived from Sf9 cell membranes. |
| b | SOD, superoxide dismutase, 500 units/ml. |
| c | Derived from E. coli. |
| d | These experiments are representative of the canonical amphiphile-activated cell-free system. The concentration of LiDS was 100 μM. |

Amphiphile-independent activation of NADPH oxidase by prenylated Rac1

**RESULTS**

Amphiphile-independent Activation of NADPH Oxidase by Prenylated Rac1

The initial aim of this investigation was to reexamine the influence of the state of prenylation of Rac1 on its ability to support cell-free assembly of the oxidase complex. Earlier work seemed to indicate that prenylation of Rac is essential for oxidase activation (18), a result that disagreed with reports describing activation by nonprenylated Rac, exchanged to GTPyS (5, 17). A more recent report clearly indicates that prenylated Rac is a superior activator because of its higher affinity for the phagocyte membrane, mediated by hydrophobic interaction between the geranylgeranyl group and membrane lipids (20). Common to all of these findings is the requirement for an anionic amphiphile and for the presence of both p47^{phox} and p67^{phox}, for activation to take place.

We found that, surprisingly, a cell-free system consisting of phagocyte membrane vesicles and recombinant prenylated Rac1, p67^{phox}, and p47^{phox} produces O_2 upon the addition of NADPH, in the absence of any activator. Next, we found that the presence of p47^{phox} is not required, its absence leading to only a minor reduction in the amount of O_2 produced; however, no O_2 is produced if p67^{phox} is replaced by p47^{phox}, either in the absence or presence of amphiphile. The level of oxidase activation by prenylated Rac1 in the absence of amphiphile and p47^{phox}, as expressed by O_2 production, is comparable with that customary in the complete amphiphile-activated cell-free system. Amphiphile and p47^{phox}-independent oxidase activation, of the same or superior intensity, is also occurring when membrane vesicles are replaced by partially purified cytochrome b_{559} incorporated in PC vesicles. The characteristics of this novel oxidase activation system are summarized in Table I. Thus, the absence or heat inactivation (95 °C for 30 min) of any of the three obligatory components of the reaction results in a total lack of O_2 production. Prenylation of Rac1 is a sine qua non condition for activity; nonprenylated Rac1 is incapable of inducing O_2 production, whether derived from E. coli (Table I; Fig. 1, A and B) or from the cytosol of baculovirus-infected Sf9 cells and containing a His tag (results not shown). Most of the experiments here described were executed with vesicles derived from solubilized macrophage membrane, but identical results were obtained with nonsolubilized membrane suspension. Maximal activation is achieved by 90 s; prolonging incubation of components for up to 10 min does not alter the level of activation. In the presence of a constant amount of membrane or purified cytochrome b_{559}, oxidase activity is proportional...
Membrane Targeting of Rac1 Induces NADPH Oxidase Assembly

with the concentration of prenylated Rac1 (Fig. 1, A and B) and p67phox (Fig. 1C). It is of interest that, in the presence of either total membrane or purified cytochrome b559, both the GTP-S- and the GDP/S-bound forms of prenylated Rac1 are active, with Rac1-GTP/S being only marginally more effective (Fig. 1, A and B). The possible reasons for this finding are addressed under “Discussion.” Recombinant p67phox proteins, isolated from baculovirus-infected SF9 cells or from E. coli are both capable of cooperation with prenylated Rac, and p67phox truncated at residue 212 is as potent as full-length protein (Fig. 1C), in accordance with the finding that the Rac-binding domain is located within the first 199 residues of p67phox (28).

No GTPase other than Rac1 or -2 was found capable of supporting NADPH oxidase activation in the canonical amphiphile-dependent system (17). To find out whether this also applied to amphiphile-independent oxidase activation, we tested prenylated (geranylgeranylated) Cdc42Hs, a member of the Rho subfamily of GTPases closely related to Rac. The cell-free system consisted of vesicles derived from solubilized membrane (5 nM cytochrome b559 heme), prenylated Cdc42Hs, exchanged to GTPγS (0–100 nM), and p67phox (300 nM). After incubation for 90 s and the addition of NADPH, no significant amounts of O2 were generated; at the highest concentration of Cdc42Hs tested (100 nM), O2 production was 3.57 ± 0.14 mol of O2/s/mol of cytochrome b559 heme (mean ± S.E. of three experiments).

Prenylated but Not Nonprenylated Rac1 Binds to Phagocyte Membrane Vesicles

These findings are compatible with a model, first proposed by Lambeth (20), in which the extent of membrane association of Rac is a key factor in determining the level of oxidase activation. To test this model, we designed a system permitting the direct measurement of the binding of prenylated and nonprenylated Rac1 to membrane vesicles. In this technique, prenylated and nonprenylated Rac1 were labeled with the fluorescent GTP analogue, mant-GTP, or with mant-GDP; mixed with membrane vesicles; and subjected to gel filtration on a Superose 12 column. Free Rac1 and Rac1 associated with membrane vesicles were identified by their elution volumes, using an in line fluorescence detector. Free prenylated Rac1 elutes at 14 ml, corresponding to a Mr of 21,000 (Fig. 2A); the fluorescence peak starting at 25 ml represents mant-GTP not bound to Rac1. When prenylated Rac1 is mixed with membrane vesicles and injected in the column, the bulk of Rac1 elutes with the membrane in the exclusion volume (7.6 ml, corresponding to molecules with a Mr > 2 × 106) (Fig. 2B). Recruitment of prenylated Rac1 to the membrane was independent of the nature of bound nucleotide; Rac1-mant-GTP and Rac1-mant-GDP attach to membrane in equal amounts (results not shown). Nonprenylated Rac1 does not bind to membrane vesicles, most of it eluting as free Rac1 (Fig. 2C). Fig. 2D illustrates an experiment in which artificial protein-free PC vesicles (replacing phagocyte membrane vesicles) were mixed with prenylated Rac1 and subjected to gel filtration. This approach is based on the work of Araki et al. (29), showing that prenylated RhoA binds to phospholipid-coated beads by hydrophobic interaction. It is apparent that the majority of prenylated Rac1 elutes in association with PC vesicles, indicating that binding of prenylated Rac1 to membrane vesicles is mediated by Rac1-lipid interaction. Preincubation of prenylated Rac1 with Rho GDI before mixing with either phagocyte membrane (Fig. 2E) or PC (Fig. 2F) vesicles, followed by gel filtration, results in a reduction in the relative amounts of prenylated Rac1 associated with membrane or PC vesicles and the appearance of a new fluorescence peak, eluting at 12.4 ml, corresponding to the Mr of the Rac1-Rho GDI heterodimer (52,000). A comparison of E and F in Fig. 2 indicates that Rho GDI is less able to compete for Rac1 with PC vesicles than with membrane vesicles, suggesting that prenylated Rac1 has a higher affinity for artificial soybean PC vesicles than for native phagocyte membrane vesicles (the total amounts of lipid present in the two types of vesicles injected in the column were comparable). Variations in relative fluorescence readings between individual experiments are probably due to variable losses of prenylated Rac1 during

3 The size of the Rac1-Rho GDI heterodimer is larger than expected because of the presence of a remaining fusion linker in recombinant Rho GDI, prepared by thrombin digestion of the Rho GDI-GST fusion protein.
Membrane Targeting of Rac1 Induces NADPH Oxidase Assembly

FIG. 2. Prenylated, but not nonprenylated, Rac1 binds to membrane and PC vesicles, in the absence of amphiphile; Rho GDI competes with membrane and PC vesicles for binding of prenylated Rac1. Prenylated or nonprenylated Rac1 were subjected to nucleotide exchange to mant-GTP, mixed with membrane or PC vesicles, and separated by FPLC gel filtration on a Superose 12 column. In some experiments, prenylated Rac1 was preincubated with Rho GDI for 10 min at 30 °C at a 3-fold molar excess of Rho GDI over Rac1, before mixing with membrane or PC vesicles. The eluates were monitored in line for the fluorescent signal of mant-GTP. Bound Rac1 represents the fraction bound to membrane or PC vesicles, eluting in the exclusion volume. Free Rac1 represents the fraction not bound to vesicles, detected at the expected elution volume (M, of 21,000). Rac1-Rho GDI represents the heterodimer of Rac1 and Rho GDI, detected at an elution volume corresponding to a M, of 52,000. The excess of mant-GTP left from nucleotide exchange is retained on the column, due to hydrophobic interaction. A, prenylated Rac1 injected in the absence of membrane vesicles. B, prenylated Rac1 mixed with membrane vesicles. C, nonprenylated Rac1 mixed with membrane vesicles. D, prenylated Rac1 mixed with PC vesicles. E, prenylated Rac1 preincubated with Rho GDI before the addition to membrane vesicles. F, prenylated Rac1 preincubated with Rho GDI before the addition to PC vesicles. The panels illustrate representative individual experiments out of three experiments performed for each combination of components.

Membrane Association of Prenylated Rac1 Leads to Amphiphile-independent Translocation of p67phox to the Membrane

The simplest explanation for the induction of oxidase assembly by prenylated Rac1, in the absence of amphiphile and p47phox, is that prenylated Rac1 is responsible for the translocation of p67phox to the membrane by virtue of the double affinity of Rac1 for p67phox and for membrane lipid. To demonstrate this in quantitative terms, membrane vesicles were mixed with prenylated or nonprenylated Rac1 and p67phox and injected in a Superose 12 column. The collected fractions were analyzed for the presence of membrane-associated p67phox by the NADPH oxidase activity reconstitution assay and by ELISA (Fig. 3). These assays demonstrate that (a) prenylated, but not nonprenylated, Rac1 binds to membrane vesicles containing cytochrome b559 (Fig. 3, A, C, and D), confirming the results illustrated in Fig. 2; (b) in the absence of Rac1 or in the presence of nonprenylated Rac1, only a minor fraction of p67phox translocates to membrane vesicles (Fig. 3, B, D, and E), and (c) in the presence of prenylated Rac1, a significantly larger amount of p67phox becomes associated with membrane vesicles (Fig. 3, C and E). The quantitative difference between p67phox recruited to the membrane, in the absence and presence of prenylated Rac1, appears to be more pronounced when assessed by the NADPH oxidase activity reconstitution assay (Fig. 3, comparison of B with C) than by ELISA (Fig. 3E). A possible explanation for this is that, whereas the activity assay offers a direct measure of components in solution, the ELISA results are influenced by secondary parameters, such as the affinity of components for the surface of the plate wells, the geometry of their attachment, and the accessibility of antigenic epitopes to the detecting antibody.

The mere addition of NADPH to samples taken from fractions containing membrane vesicles with bound Rac1 and p67phox (such as shown in Fig. 3C) results in spontaneous O2− production, demonstrating that these fractions contain a fully assembled and activated oxidase complex (Fig. 4A). Adding NADPH to fractions containing membrane vesicles preincubated with p67phox only (such as shown in Fig. 3B) or with nonprenylated Rac1 and p67phox (such as shown in Fig. 3D) does not elicit O2− production (Fig. 4A).

Incomplete oxidase assembly, however, can be brought to completion; thus, the addition of p67phox and NADPH to fractions containing membrane vesicles with bound Rac1 (such as shown in Fig. 3A) results in O2− production; the addition of p47phox has no effect (Fig. 4B). Interestingly, the ability to bring
incomplete oxidase assembly to completion is dependent on the order of association of the cytosolic components with the membrane. Thus, in the presence of sodium cholate, at concentrations above the critical micellar concentration (8 mM), p67phox is incorporated in membrane vesicles and can be detected in such vesicles, upon gel filtration on Superose 12, in a fully functional state by the NADPH oxidase activity reconstitution assay (Fig. 4C; filled squares). However, the addition of prenylated Rac1 and NADPH to fractions containing membrane-associated p67phox does not result in O2 production (Fig. 4C, filled triangles), in contrast to the situation, illustrated in Fig. 4B, in which the addition of p67phox and NADPH to membrane vesicles with bound Rac1 led to amphiphile-independent O2 generation. That this difference is not due to eventual damage to the membrane or p67phox by sodium cholate is shown by the ability of the same membrane vesicles with bound p67phox to be active in the NADPH oxidase activity reconstitution assay. Furthermore, in experiments identical to those illustrated in Fig. 4B, but in which prenylated Rac1-GTP-S is mixed with membrane vesicles in the presence of 9 mM sodium cholate before gel filtration on Superose 12, the outcome is the same; the addition of p67phox and NADPH results in O2 production (results not shown).

Amphiphile-independent NADPH Oxidase Activation

Involving Prenylated Rac1 Is Subject to Inhibition by Agents Competing with the Membrane for Binding of Rac1

Inhibition by Rho GDI—Rac1 was originally identified as a component of the oxidase complex by its purification from macrophage cytosol as a heterodimer with Rho GDI (5, 14). Both native and recombinant Rac1-Rho GDI dimers are potent activators of O2 production in the amphiphile-dependent cell-free system (14, 30). We found that, in amphiphile-independent oxidase activation by prenylated Rac1, Rho GDI acts in a diametrically opposite fashion, as an inhibitor (Fig. 5A). Inhibition by Rho GDI is evident whether Rac1 is in the GTP- or GDP-bound form (results not shown). Upon supplementation of the system with p47phox and LiDS, Rho GDI is no longer inhibitory. Inhibition by Rho GDI is maximal when it is added simultaneously with the components of the cell-free system; adding Rho GDI 5 min after the initiation of oxidase assembly leads to a marked reduction in its inhibitory effect (Fig. 5C). Interference by Rho GDI with oxidase activation was found with both total membrane and purified cytochrome b559 preparations (results not shown). The ability of Rho GDI to prevent amphiphile-independent oxidase activation by prenylated Rac1 is in good agreement with the experiments, illustrated in Fig. 2, E and F, showing that Rho GDI is capable of counteracting the recruitment of Rac1 to both membrane and PC vesicles.

Inhibition by PC Vesicles—The fact that prenylated Rac1 binds to PC vesicles (Fig. 2D) suggested that such vesicles might compete with bona fide membrane vesicles for Rac1. Indeed, the addition of PC vesicles to a mixture of membrane vesicles, prenylated Rac1, and p67phox, in the absence of amphiphile, causes a marked, dose-dependent inhibition of oxidase activation (Fig. 5B). PCvesicles have no effect on amphiphile-independent oxidase activation involving prenylated or nonprenylated Rac1, p67phox, and p47phox. A calculation of the relative amounts of added PC to the amount of membrane phospholipid present in the reactions (based on data appearing in Ref. 22) shows that, at 40 μg/ml PC (which results in almost complete inhibition of activation), the ratio is close to 1:1. PC vesicles, like Rho GDI, are inhibitory only when added together with the components of the cell-free system; PC added 5 min after the initiation of assembly exerts a much lesser inhibitory effect (Fig. 5D). Inhibition of oxidase activation by PC vesicles also takes place when membrane is replaced by purified cytochrome b559 (results not shown).

Effect of Polybasic Rac1 Peptide—Membrane association of Rac1 also involves electrostatic interaction between a polybasic region at the C terminus of Rac1 and negative charges on the membrane (20, 21), as shown by the ability of synthetic peptides, incorporating the polybasic region, to interfere with amphiphile-dependent oxidase activation (21, 31). We investigated the effect of a 15-mer peptide, corresponding to residues 177–191 of Rac1 (LCPPPVKKRKRKCLL) and containing six basic residues, on amphiphile-independent oxidase activation supported by prenylated Rac1. This (21) and related (32) polybasic Rac1 peptides were found to be potent inhibitors of the amphiphile-activated cell-free system, containing nonprenylated Rac1 (IC50 in the 1–2 μM range). Rac1 peptide 177–191 exerts no inhibitory effect on amphiphile-independent oxidase...
assembly involving prenylated Rac1 and p67phox, up to a concentration of 40 μM peptide. Thus, percentage inhibition of oxidase activity by 40 μM Rac1 peptide 177–191 is −5.5 ± 3.9 (mean ± S.E. of three experiments; negative arithmetic value is the result of minor enhancement of activation).

**DISCUSSION**

We describe the induction of oxidase assembly and consequent NADPH-driven O$_2^-$ production in a cell-free system, consisting of phagocyte membranes, recombinant p67phox, and recombinant prenylated Rac1, in the absence of an amphiphilic activator and of p47phox. Identical results are obtained when total membrane is replaced by partially purified and relipidated cytochrome b$_{559}$, suggesting that the only functional protein contributed by the membrane is cytochrome b$_{559}$ and that “nonphysiological” lipids can successfully replace native phagocyte lipids.

Prenylated Cdc42Hs, a GTPase exhibiting about 70% overall identity with Rac1 and -2, is incapable of supporting amphiphile-independent oxidase activation. Prenylated, but not nonprenylated, Rac1 was found to attach avidly to phagocyte membrane vesicles and to artificial PC vesicles. Binding of prenylated Rac1 to membrane vesicles could be demonstrated by the use of Rac labeled with a fluorescent marker and by an

NADPH oxidase activity reconstitution assay. Attachment occurs in the absence of amphiphile, is independent of the type of nucleotide (GTP or GDP) bound to Rac, and appears to be mediated exclusively by the geranylgeranyl tail.

In mixtures of membrane vesicles, prenylated Rac1 and p67phox, binding of prenylated Rac1 to membrane is accompanied by the parallel binding of a fraction of p67phox. Only minor binding of p67phox to membrane is seen in the absence of prenylated Rac1 or in the presence of nonprenylated Rac1. Co-translocation of prenylated Rac1 and p67phox to the membrane results in the formation of an assembled oxidase complex, which produces O$_2^-$ upon the addition of NADPH, in the absence of free cytosolic components and amphiphile. The sequence of events in this form of oxidase assembly cannot yet be established with certainty. However, it is likely that translocation of prenylated Rac1 to the membrane precedes that of p67phox. Support for this possibility is provided by the ability of exogenous p67phox to join membrane vesicles that bound prenylated Rac1 to generate a fully assembled O$_2^-$-generating complex, as opposed to the inability of achieving the same result when binding of p67phox to the membrane precedes that of Rac1.

A seemingly unusual feature of the system described in this report is that the GDP-bound form of prenylated Rac1 is only
slightly less active than the GTP-bound form in supporting oxidase activation, whether in the presence of membrane or purified cytochrome b_{559}. In fact, this is quite similar to our earlier finding that the GDP-bound form of prenylated Rac1, in complex with Rho GDI (σ₁), is a potent activator of oxidase in the amphiphile-activated cell-free system (33). We also showed, recently, that the relative abilities of the GTP- and GDP-bound forms of nonprenylated Rac1 to support amphiphile-dependent oxidase activation are related to the concentrations of p47^{phox} and p67^{phox} present; at a 200 nM concentration of these components, the difference in V_{max} between Rac1-GTP•S and Rac1-GDP•S was marginal (25). Indeed, it might be of significance that, in the present work, the activities of prenylated Rac1-GTP•S and Rac1-GDP•S were compared at a concentration of 300 nM p67^{phox}.

Oxidase activation by prenylated Rac1, in the absence of amphiphile and p47^{phox}, is prevented by agents, such as Rho GDI and PC vesicles, that compete with the membrane for binding of Rac1 via its geranylgeranyl tail. These results and the lack of inhibition by a polybasic Rac1 peptide point to the key importance of hydrophobic bonds in the binding of Rac1 to the membrane. Both Rho GDI and PC are inhibitory only when added before the completion of oxidase assembly. The fact that they are ineffective after assembly indicates either that, once membrane-bound, Rac cannot be dislodged or that, on the contrary, the continuous presence of Rac in the complex is not required for O_{2} \textsuperscript{−} generation.

A remarkable characteristic of oxidase activation by prenylated Rac1 is the lack of requirement for activating amphiphile and p47^{phox}, contrasting with the absolute dependence of the canonical cell-free system on both components. The preponderant view is that amphiphiles cause a conformational change in p47^{phox}, leading to the conversion of intramolecular bonds in p47^{phox} to intermolecular bonds between p47^{phox} and the p22^{phox} subunit of cytochrome b_{559} (34). This is expected to lead to the secondary translocation to the membrane of p67^{phox}, in complex with p47^{phox}. Amphiphile-independent oxidase activation was described in the past but required the presence of both p47^{phox} and p67^{phox} and was conditional on either C-terminal truncation of both components (35) or on phosphorylation of p47^{phox} by protein kinase C (36). On the other hand, p47^{phox} was shown not to be required for oxidase activation in vitro, provided that p67^{phox} and Rac were present at high (>1 μM) concentrations (37, 38), and a recent report mentions that, under these conditions, activation also occurred in the absence of amphiphile (39). All this points to prenylated Rac1 being capable of taking over the combined actions of amphiphile and p47^{phox}, to serve as a carrier for the translocation of p67^{phox} to the membrane.

Our results support the following model of oxidase assembly. Binding of p67^{phox} to cytochrome b_{559}, via an interaction with either p91^{phox} or p22^{phox} or with both subunits, is responsible for the induction of a conformational change in gp91^{phox} and the consequent initiation of electron transport leading to O_{2} \textsuperscript{−} generation. The establishment of p67^{phox}-cytochrome b_{559} contact(s) is dependent on translocation of p67^{phox} from cytosol to the plasma membrane environment; this first, essentially non-specific, step is followed by movement of p67^{phox} in the plane of the membrane, culminating in the formation of specific protein-protein interactions with the cytochrome. Unlike p47^{phox}, p67^{phox} is incapable of unassisted translocation to the membrane and, in the intact cell, requires the participation of both p47^{phox} and Rac to act as carrier proteins. It is not known whether the interaction of Rac with p67^{phox} takes place in the cytosol or only following the insertion of Rac into the membrane. Another important question is whether the affinity of Rac for p67^{phox} is enhanced by prenylation and/or by membrane attachment of Rac. A recent example of such a situation is the correlation between C-terminal processing of Rac1 and its ability to stimulate phospholipase C-β₂ (40). Since preny-
lated Rac exists in the cytosol exclusively as a dimer with Rho GDI, membrane attachment of Rac, in vitro, must be preceded by a process resulting in its complete or at least partial dissociation from Rho GDI. We also have to conclude that the carrier of p47phox and Rac, for p67phox, are not symmetrical and not mutually interchangeable. This is shown by the ability of (Rac + p67phox) to fully support oxidase activation in vitro, whereas (p47phox + p67phox) is unable to do so, both in the presence and absence of amphiphiles.

The cell-free system described in this report is clearly not an accurate reflection of the in vivo reality, since p47phox is required for O2 production in intact phagocytes. It does, however, provide new information on the molecular mechanisms of oxidase assembly. Thus, the principal, if not the only, role of Rac appears to be to recruit p67phox to the membrane in a manner that leads to a “productive” interaction with cytochrome b556. It is suggested that this recruitment is based exclusively on the presence and absence of amphiphile.

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