Tripartite Chimeras Comprising Functional Domains Derived from the Cytosolic NADPH Oxidase Components p47<sub>phox</sub>, p67<sub>phox</sub>, and Rac1 Elicit Activator-independent Superoxide Production by Phagocyte Membranes

AN ESSENTIAL ROLE FOR ANIONIC MEMBRANE PHOSPHOLIPIDS

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The superoxide-generating NADPH oxidase is converted to an active state by the assembly of a membrane-localized cytochrome<sub>b</sub><sub>559</sub> with three cytosolic components: p47<sub>phox</sub>, p67<sub>phox</sub>, and GTPase Rac1 or Rac2. Assembly involves two sets of protein-protein interactions: among cytosolic components and among cytosolic components and cytochrome<sub>b</sub><sub>559</sub> within its lipid habitat. We circumvented the need for interactions among cytosolic components by constructing a recombinant tripartite chimera (trimera) consisting of the Phox homology (PX) and Src homology 3 (SH3) domains of p47<sub>phox</sub>, the tetratricopeptide repeat and activation domains of p67<sub>phox</sub>, and full-length Rac1. Upon addition to phagocyte membrane, the trimera was capable of oxidase activation in vitro in the presence of an anionic amphiphile. The trimera had a higher affinity (lower EC<sub>50</sub>) for p67<sub>phox</sub> compared with the combined individual components, full-length or truncated. Supplementation of membrane with anionic but not neutral phospholipids made activation by the trimera amphiphile-independent. Mutagenesis, truncations, and domain replacements revealed that oxidase activation by the trimera was dependent on the following interactions: 1) interaction with anionic membrane phospholipids via the polybasic stretch at the C terminus of the Rac1 segment; 2) interaction with anionic membrane phospholipids through the SH3 domain of the p47<sub>phox</sub> segment, supplementing the electrostatic attraction; and 3) an intrachimeric bond among the p67<sub>phox</sub> and Rac1 segments complementary to their physical fusion. The PX domain of the p47<sub>phox</sub> segment and the insert domain of the Rac1 segment made only minor contributions to oxidase assembly.

Phagocytes produce reactive oxygen radicals, part of their microbicidal arsenal, by means of a tightly regulated enzyme complex commonly referred to as NADPH oxidase. At the origin of all oxygen radicals is the superoxide anion (O<sub>2</sub><sup>-</sup>), generated by the NADPH-derived one-electron reduction of molecular oxygen. The O<sub>2</sub><sup>-</sup>-generating NADPH oxidase complex (briefly "oxidase") consists of a membrane-associated flavocytochrome (cytochrome<sub>b</sub><sub>559</sub>) comprising two subunits (gp91<sub>phox</sub> and p22<sub>phox</sub>) and four cytosolic components (p47<sub>phox</sub>, p67<sub>phox</sub>, p40<sub>phox</sub>, and small GTPase Rac1 or Rac2) (reviewed in Refs. 1–3). Electron flow from NADPH to oxygen occurs along three redox stations, all of which are located on gp91<sub>phox</sub>, the NADPH-binding site, FAD, and two non-identical hemes. It is assumed that initiation of electron flow is the consequence of a conformational change in gp91<sub>phox</sub> induced by its interaction with p67<sub>phox</sub>. The region in p67<sub>phox</sub> presumed to be involved in such interaction is known as the "activation domain" and consists of residues 199–210 (4). It has been suggested that the roles of p47<sub>phox</sub> and Rac are to serve as carriers of p67<sub>phox</sub> to the membrane or as membrane anchors for p67<sub>phox</sub> to enable the correct juxtapositioning of the activation domain on p67<sub>phox</sub> at its target site on gp91<sub>phox</sub> (4–7). We have recently proposed that an additional function of p47<sub>phox</sub> is to augment the stability of the assembled complex (8). The carrier/anchor for p67<sub>phox</sub> function of p47<sub>phox</sub> is based on two sets of interactions: a tail-to-tail interaction between a proline-rich region (PRR) at the C terminus of p47<sub>phox</sub> and the C-terminal Src homology 3 (SH3) domain of p67<sub>phox</sub> and between the two SH3 domains of p47<sub>phox</sub> and a PRR of the phox<sub>22</sub> C-terminal Src homology 2 domain (9). Further links of p47<sub>phox</sub> to the membrane environment of cytochrome<sub>b</sub><sub>559</sub> are provided by the binding of the Phox homology (PX) domain at its N terminus to specific phosphoinositides (12) and possibly also by the binding

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of a C-terminal domain to gp91phox (13). The mechanism responsible for the binding of p47phox to p22phox has been worked out in great detail and consists of the exchange of an intramolecular autoinhibitory bond between the SH3 tandem and a polybasic region at the C terminus of p47phox for an intermolecular bond between the same SH3 tandem and a PRR at the C terminus of p22phox. The “opening” of the intramolecular bond is the result of phosphorylation of critical serine residues in the polybasic region, found to occur in the course of neutrophil activation (14, 15). The carrier/anchor function of Rac rests on the interaction between the pre-switch I and switch II regions of Rac1 and the tetratricopeptide repeat (TPR) domain in p67phox at one end (16, 17) and the binding of Rac via its polybasic and prenylated C terminus to negatively charged membrane phospholipids (18) and possibly to gp91phox (19, 20) at the other end. Rac has first to be liberated from its bond with the regulatory protein Rho GDP dissociation inhibitor, which keeps Rac in an inactive, GDP-bound conformation in the cytosol (reviewed in Ref. 21). There is evidence for the involvement of free (22) or membrane-associated (23) anionic phospholipids and possibly a membrane-bound guanine nucleotide exchange factor (24) in causing the dissociation of the Rac-Rho GDP dissociation inhibitor complex.

There is evidence for the need for a conformational change to occur in p67phox to allow a productive interaction with the catalytic subunit of cytochrome b555 gp91phox. This change is thought to be the consequence of the binding of the GTP-bound form of Rac to p67phox (25) or of the relief of autoinhibition by the C terminus of p67phox containing the two SH3 domains (26). It is not clear whether the result of the conformational change in p67phox is to augment the actual binding of p67phox to gp91phox (6) or to endow p67phox with an ability to elicit electron flow in gp91phox (27).

A conceptual and methodological advance in our understanding of oxidase activation was the development of in vitro cell-free systems. In these, phagocyte membranes or purified cytochrome b555 preparations are exposed to total phagocyte cytosol or to purified or recombinant cytosolic components in the presence of an activator, represented by an anionic amphiphile such as arachidonate, SDS, or phosphatidic acid (28–33). The availability of recombinant proteins for all cytosolic components made it possible to identify functionally significant domains by combining mutagenesis with assay of the mutated components in what became known as the semirecombinant approach (34). Cell-free systems reproduce the essential steps in oxidase assembly as they occur in vivo, as demonstrated by the fact that anionic amphiphiles mimic the effect of phosphorylation of p47phox, leading to the relief of autoinhibition (35). A further advance was the design of cell-free systems in which oxidase activation takes place in the absence of an amphiphilic activator. Such systems are based on C-terminal truncation of both p47phox and p67phox (26), on prenylation of Rac (5), or on enrichment of the phagocyte membrane with negatively charged phospholipid (37, 38).

A novel approach to the study of protein-protein interactions in oxidase assembly was initiated by the design of chimeric constructs consisting of selected segments derived from two cytosolic components. The first of these was a fusion of p47phox (residues 1–286) with p67phox (residues 1–210) (37). This was followed by the independent description by two groups of p67phox-(1–210 or 1–212)-Rac1 chimeras (39, 40), which could be prenylated at the C terminus (41). Chimeras of cytosolic components are, in general, characterized by EC50 values lower than those of the non-fused proteins. There is less agreement about the effect of fusion on Vmax values; one group reported higher activities for both p47phox-p67phox and p67phox-Rac1 chimeras in a cell-free system (37, 39), whereas lower activities for p67phox-Rac1 chimeras were found by us (40). Fusion of p67phox and Rac1 reduces the dependence of oxidase activation on p47phox (39–41). Finally, prenylation of p67phox-Rac1 chimeras enables oxidase activation in the absence of amphiphile and further reduces its dependence on p47phox (41).

In this study, we describe the design and bacterial expression, in the form of soluble proteins, of tripartite chimeras consisting of selected segments of p47phox, p67phox, and Rac1, which we call “trimeras.” The prototype trimer, resulting from the fusion of p47phox-(1–286), p67phox-(1–212), and full-length Rac1 (amino acids 1–192), was found to act as a potent amphiphile-dependent oxidase activator upon addition to phagocyte membrane or purified cytochrome b555. Modifying the phospholipid composition of the membrane by supplementation with anionic phospholipids enabled oxidase activation by the trimer in the absence of an amphiphile. Truncations, domain replacements, and point mutations applied to critical regions in the three segments composing the prototype trimer led to the acquisition of novel information on the participation of specific sequences and residues in oxidase assembly.

**EXPERIMENTAL PROCEDURES**

*Chemicals and Reagents*—The hydrolysis-resistant GTP analog GMPNP (tetralithium salt, 83%, 0.2% GTP) was purchased from Roche Applied Science. The fluorescent hydrolysis-resistant GTP analog mant-GMPPNP (triethylammonium salt) was obtained from Jena Bioscience GmbH. The following chemicals and phospholipids were obtained from Sigma: n-octyl β-D-glucopyranoside (98%), 14–23% L-α-phosphatidylcholine (PC; soybean, type II-S, containing 14–23% phosphatidylcholine and many other lipids, product P5638), L-α-phosphatidylcholine (PC; soybean, 99%, product P7443), L-α-phosphatidic acid (PA; sodium salt, egg yolk, 98%, product P9511), L-α-phosphatidyl-DL-glycerol (PG; β-oleoyl-γ-palmitoyl, ammonium salt, synthetic, 99%, product P6956), L-α-phosphatidyl-DL-serine (PS; soybean, 98%, product P0474), and L-α-phosphatidylinositol (PI; ammonium salt, soybean, 98%, product P9594), 1,2-Dioleoyl-sn-glycero-3-(phospho-rac-1-glycerol) (DOPG; sodium salt, synthetic, >99%, product 840475P), 1,2-dioleoyl-sn-glycero-3-phosphoinositol 3,4-bisphosphate (PtdIns(3,4)P2; trimannoside salt, synthetic, >99%, product 850153), and 1,2-dioleoylsn-glycero-3-phosphoinositol 4,5-bisphosphate (PtdIns(4,5)P2; trimannoside salt, synthetic, >99%, product 850155) were purchased from Avanti Polar Lipids.

*Construction of Chimeric Proteins*—See supplemental “Experimental Procedures.”
Expression and Purification of Recombinant Proteins—All recombinant proteins used in this work, with the exception of full-length p47phox, were expressed in and isolated from *Escherichia coli* BL21-CodonPlus™(DE3)-RIL (Stratagene). Rac1 was produced in *E. coli* as described previously (43). Chimera 3 (p67phox-(1–212)-full-length Rac1) was expressed and purified as described (40). Full-length p67phox, p67phox-(1–212), and p47phox (1–286) were expressed as glutathione S-transferase (GST) fusion proteins and purified by batch affinity chromatography on glutathione-agarose (Sigma), followed by thrombin cleavage (44), as described previously (45). p47phox was prepared in baculovirus-infected S9 cells as described (43). The prototype trimer, the trimer lacking the Rac1 C terminus (Rac1ΔC), the trimer with positive residues 183–188 in the Rac1 segment replaced with six neutral residues (glutamines; Rac1(183Q-188Q)), and the trimer lacking the PX domain of p47phox (p47phoxΔPX) were also expressed as GST fusion proteins and purified by affinity chromatography on glutathione-agarose, followed by cleavage with thrombin. The expression vector pGEX-2T (Amersham Biosciences) carrying cDNAs encoding the above-mentioned trimeras was introduced into *E. coli* BL21-CodonPlus™(DE3)-RIL cells, and bacteria were induced with 0.4 mM isopropyl β-D-thiogalactopyranoside at 18 °C for 14–16 h. The induced cells were suspended in TMN buffer (50 mM Tris-HCl, pH 7.5, 4 mM MgCl$_2$, 150 mM NaCl, and 2 mM diithiothreitol) supplemented with Complete EDTA-free protease inhibitor (Roche Applied Science). The bacteria were disrupted by exposure to lysozyme (Sigma) at a concentration of 0.5 mg/ml for 20 min at 4 °C with stirring and by sonication using a 500-watt sonic disruptor (Vibra-Cell, Sonics & Materials, Inc.) at 20% amplitude for 5 min in the 50% pulse mode in ice-cooled buffers. The resulting material was supplemented with 1% Triton X-100 (Sigma) and stirred on ice for 15 min. The bacterial lysate was subjected to centrifugation at 23,000 × g for 25 min at 4 °C; the cleared cell-free extract was applied to BD Talon™ metal affinity resin beads (Clontech); and binding was performed in the batch mode at room temperature for 60 min. The beads were washed with the sodium phosphate buffer supplemented with 5 mM imidazole, followed by two washes with the same buffer supplemented with 20 mM imidazole. The His$_6$-tagged trimeras were eluted from the resin with the same buffer supplemented with 150 mM imidazole. All purified trimer proteins were supplemented with 30% glycerol and stored in small aliquots at −75 °C.

Characterization of Recombinant Trimeras—The protein concentration of the recombinant fusion proteins was measured by the method of Bradford (46), modified for use with 96-well microplates (81), using Bio-Rad protein assay dye reagent concentrate and bovine γ-globulin as a standard. The level of purity of the recombinant proteins was assessed by SDS-PAGE analysis, and the gels were stained with GelCode™ Blue stain reagent (Pierce). Immunoblot analysis was carried out as described (47). For the detection of p47phox, p67phox, and their corresponding segments in the trimeras, we used goat anti-p47phox and anti-p67phox polyclonal antibodies (gifts from T. L. Leto, National Institutes of Health), both diluted 1:2000. For the detection of Rac1 and the Rac1 segment in the trimeras, we used an affinity-purified rabbit anti-Rac1 C-terminal peptide polyclonal antibody (Santa Cruz Biotechnology, Inc.), diluted 1:2000. The secondary antibodies were affinity-purified alkaline phosphatase-conjugated anti-goat IgG (Sigma) for detection of p47phox and p67phox and affinity-purified alkaline phosphatase-conjugated anti-rabbit IgG (Sigma) for detection of Rac1, both diluted 1:2000. The blots were exposed to the primary and secondary antibodies for 1 h. Alkaline phosphatase activity on the blots was detected as described (48). The identity and amount of nucleotides bound to recombinant proteins were determined by liberating the bound nucleotides from the protein as described previously (50) and identifying the nucleotides by anion exchange chromatography on a Parti sil 10 SAX column as described (49).

Nucleotide Exchange—For use in oxidase activation assays, Rac1, chimera 3, and trimeras were subjected to nucleotide exchange from the native, GDP-bound form to a state in which the protein-bound nucleotide was GMPPNP or mant-GMPPNP. Nucleotide exchange reactions were performed at a free Mg$^{2+}$ concentration of 0.5 μM (attained by addition of 12.5 mM EDTA) at a 10-fold molar excess of nucleotide over protein, followed by incubation for 30 min at 30 °C. The
exchange was stabilized by addition of MgCl₂ to a final concentration of 25 mM.

**Gel Filtration**—The prototype trimera exchanged to mant-GMPPNP was loaded onto a Superose 12 10/300 GL fast protein liquid chromatography gel filtration column (Amersham Biosciences), and chromatography was performed on a Waters HPLC system with TMN buffer at flow rate of 0.2 ml/min at 4 °C. Absorbance at 280 nm was measured continuously by a Jasco Model MD-1510 diode array detector. When the trimera was labeled with mant-GMPPNP, the fluorescence signal (excitation at 361 nm and emission at 440 nm) was also recorded continuously by passing the column eluate through a Jasco Model MPF-750 spectrofluorimeter fitted with a Jasco Model MFC-132 HPLC flow cell. Fractions (0.6 ml) were collected, and the trimera-containing fractions were identified by their ability to support cell-free oxidase activation. The column was standardized with molecular mass markers (range of 12–200 kDa).

**Preparation of Macrophage Membrane Liposomes**—Phagocyte membranes were prepared from guinea pig macrophages obtained by injection of mineral oil into the peritoneal cavity as described (28). The membranes were solubilized in 40 mM n-octyl β-D-glucopyranoside and then reconstituted into liposomes by dialysis against detergent-free buffer as described previously (51). The specific cytochrome b₅₅₉ heme content of membrane liposomes was measured by the difference spectrum of sodium dithionite-reduced minus oxidized samples (52).

**Purification and Relipidation of Cytochrome b₅₅₉**—Cytochrome b₅₅₉ was purified from solubilized macrophage membranes, relipidated, and reflavinated as described (53). PC 20% was used for relipidation at a ratio of 0.2 mg of lipid (260 nmol) to 360 pmol of cytochrome b₅₅₉ heme.

**Membrane Enrichment with Exogenous Phospholipid**—Native macrophage membranes were supplemented with a number of exogenous phospholipids to generate membrane liposomes with an artificially modified electrical charge. As a first step in this procedure, we dissolved PC 20%, PC, PA, PG, PS, PI, and DOPG at a concentration of 5 mM in a buffer also used to solubilize membranes (51) following a procedure described previously (23). The buffer consisted of 120 mM potassium/sodium phosphate buffer, pH 7.4, 1 mM MgCl₂, 2 mM Na₃, 1 mM EGTA, 1 mM dithiothreitol, 10 μM FAD, 20% glycerol, and 40 mM n-octyl β-D-glucopyranoside. The phospholipids were added to the solubilized macrophage membranes at a constant ratio of 4 volumes of phospholipid (5 mM) to 1 volume of membrane (at a concentration equivalent to 1.2 μM cytochrome b₅₅₉ heme) unless mentioned otherwise. In another set of experiments, membranes were supplemented with DOPG and either PtdIns(3,4)P₂ or PtdIns(4,5)P₂. For this purpose, 4 volumes of DOPG (5 mM) dissolved in the above buffer containing 40 mM n-octyl β-D-glucopyranoside were mixed with 0.5 volumes of either PtdIns(3,4)P₂ or PtdIns(4,5)P₂ (both at a concentration of 100 μM in n-octyl β-D-glucopyranoside-containing buffer) and 0.5 volumes of solubilized macrophage membrane at a concentration equivalent to 2.4 μM cytochrome b₅₅₉ heme. All membrane/phospholipid mixtures were dialyzed against 100–200 volumes of n-octyl β-D-glucopyranoside-free buffer for 18 h at 4 °C to convert them into phospholipid-enriched membrane liposomes. The concentration of cytochrome b₅₅₉ in the modified liposomes was determined and was typically found to be close to 240 pmol/ml. The theoretical final concentration of exogenous phospholipids (PC, PA, PG, PS, PI, and DOPG) was considered to be 4 mM, and that of PtdIns(3,4)P₂ or PtdIns(4,5)P₂ was considered to be 10 μM (representing 0.25 mol % of the total exogenous phospholipid). The total concentration of phospholipids in the native unmodified membranes was determined as described (23).

**Cell-free Oxidase Activation Assay**—Activation of the oxidase in vitro was assessed by measuring the NADPH-dependent O₂ production in a semirecombinant cell-free system in the presence or absence of the amphiphilic activator lithium dodecyl sulfate (LiDS) as described (54). Trimeras exchanged to GMPPNP or in the native (GDP-bound) form were tested for their ability to support oxidase activation at various concentrations (5–300 nM). For comparison with trimeras, mixtures of the individual oxidase components p₄⁷ₕₒₓₙ (full-length or truncated at residue 286), p₆₇ₕₒₓₙ (full-length or truncated at residue 212), and Rac1 (full-length and exchanged to GMPPNP) and mixtures of chimera 3 (exchanged to GMPPNP) and p₄⁷ₕₒₓₙ in the same concentration range were assayed in parallel. Two cell-free oxidase activation systems were utilized: (a) an amphiphile-dependent system consisting of membrane liposomes (equivalent to 5 nM cytochrome b₅₅₉ heme) and the various cytosolic activators in the presence of 130 μM LiDS and (b) an amphiphile-independent system consisting of membrane liposomes supplemented with exogenous phospholipid (5 nM cytochrome b₅₅₉ heme) and cytosolic activators in the absence of LiDS. The assay mixtures were incubated in 96-well microplates in a total volume of 200 μl of assay buffer (55) per well with or without LiDS for 90 s at 24 °C before addition of 240 μM NADPH to initiate O₂ production. This was quantified by the kinetics of cytochrome c reduction as described previously (55).

**Curve Plotting**—Plotting of dose-response curves and calculation of Vₘₐₓ and EC₅₀ values were performed using GraphPad Prism Version 4.03.

**RESULTS**

**The Rationale on Which the Design of Trimeras Was Based**—We have reported in the past that a recombinant chimeric protein consisting of p₆₇ₕₒₓₙ-(1–212) fused with full-length Rac1 (referred to as chimera 3) is capable of eliciting NADPH-dependent O₂ production by preparations of phagocyte membranes in the presence of p₄⁷ₕₒₓₙ and an anionic amphiphilic activator (25, 40) and in the absence of p₄⁷ₕₒₓₙ and amphiphile when the chimera is prenylated (25, 41). We now extend these studies to the generation of a single molecule activator of the oxidase by constructing a tripartite p₄⁷ₕₒₓₙ-p₆₇ₕₒₓₙ-Rac1 fusion protein in which p₄⁷ₕₒₓₙ-(1–286) was fused to chimera 3 by a 10-amino acid spacer (Fig. 1). The basic construct, which we call the “prototype trimera,” is composed of the PX domain and the two SH3 domains of p₄⁷ₕₒₓₙ, the TPR and activation domains of p₆₇ₕₒₓₙ, and full-length Rac1. p₄⁷ₕₒₓₙ was truncated at residue 286, right after the C terminus of the second SH3 domain, to generate an “open” conformation of the p₄⁷ₕₒₓₙ segment (10, 11, 15, 58) and also because the p₄⁷ₕₒₓₙ-(1–286) segment is part of a p₄⁷ₕₒₓₙ-p₆₇ₕₒₓₙ chimera originally described by Ebisu et al. (37). p₆₇ₕₒₓₙ was truncated at residue 212, right
Properties of the Prototype and Mutant Trimeras—The prototype and all mutant trimers were successfully expressed in *E. coli* and recovered in the soluble fraction, and the purified proteins were found to be of the expected molecular mass when analyzed by SDS-PAGE (Fig. 2A). Thus, the molecular mass of the prototype trimer was close to the expected 80 kDa. The reduction in molecular mass of mutant trimers p47phoxΔP (lane 2) and Rac1ΔC (lane 7) is evident. The level of purity of all recombinant trimers exceeded 90%. To assess the integrity of each segment in the tripartite fusion protein, the prototype trimer was subjected to immunoblot analysis with antibodies to p47phox, p67phox, and Rac1. As shown in Fig. 2B, the trimer was recognized by all three antibodies (lanes 1–3). The specificity of the antibodies is demonstrated by their ability to react with the respective individual purified components (lanes 4–6).

To assess the size of the trimer under nondenaturing conditions, the prototype trimer was exchanged to the fluorescent nucleotide mant-GMPPNP and injected into a Superose 12 size exclusion column. The elution of the protein from the column was followed by recording the fluorescence signal in-line and by collecting the eluate into tubes (0.6 ml/tube). Fig. 2C illustrates the elution pattern of the trimer; in three independent experiments, the main fluorescent peak eluted at 61.5 min, corresponding to a molecular mass of 80 kDa, in good agreement with the SDS-PAGE data. The collected fractions were also examined for their ability to support oxidase activity by adding a sample from each fraction to membrane liposomes in the presence of LiDS as described below. As shown in Fig. 2D, oxidase-activating ability was detected in fractions eluting at a volume of 12–12.6 ml (60–63 min) and overlapping the fluorescent peak in Fig. 2C.

The presence of a Rac1 moiety in chimera 3 was found to confer on it the properties of a *bona fide* small GTPase (25, 40, 41). The presence of a full-length Rac1 segment in the trimer was likely to have the same effect. To test this, we determined the type of nucleotide bound to the prototype trimer. We found that, in the native form, it contained only GDP (data not shown), as expected to be the case based on information on recombinant Rac produced in *E. coli*, which exhibits high intrinsic GTPase activity (49, 60, 61).

The Prototype Trimer Is a Potent Oxidase Activator in the Presence of an Anionic Amphiphile—Both p47phox, p67phox and p47phox, p67phox, Rac1 chimeras supplemented with the missing cytosolic component are potent oxidase activators in a variety of cell-free systems (25, 37, 39, 40, 41). We thus first examined the ability of the tripartite fusion protein to support amphiphile-dependent oxidase activation in a cell-free system. A critical factor in cell-free oxidase activation by mixtures of individual cytosolic components is optimization of the concentration of the activating amphiphile. This was studied in the past in relation to arachidonate (28), SDS (32), and LiDS (54), and in all cases, the optimal activating concentration was found to be in the 100–150 μM range.

In preliminary experiments, we found that incubation of membrane liposomes (equivalent to 5 nM cytochrome b559 heme) with the prototype trimer at a concentration of 100 nM in the presence of 130 μM LiDS resulted in oxidase activation. We next examined the effect of varying the concentration of

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|----------|-------------|
| Prototype | p47phox, p67phox, Rac1 Chimeras Activate the NADPH Oxidase |

**FIGURE 1. Schematic representation of the prototype and mutant trimers.** The component of origin of the segment incorporated in the trimers appears within the rectangles. Above the rectangles are indicated the residue numbers in the native sequences of p47phox, p67phox, and Rac1 representing the N- and C-terminal limits of the segments forming the trimers. AASTGGSS is a 10-amino acid linker between the p47phox and p67phox segments appearing in all the trimers.

After the end of the activation domain (4), eliminating the PRR and the two SH3 domains. Rac1 was kept full-length because of the requirement for an intact polybasic domain for oxidase activation by Rac1 (18, 56, 57) and p67phox, p67phox, and p67phox, and Rac1 representing

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|--------|-------------|
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| Prototype | p47phox, p67phox, Rac1 Chimeras Activate the NADPH Oxidase |

**FIGURE 1. Schematic representation of the prototype and mutant trimers.** The component of origin of the segment incorporated in the trimers appears within the rectangles. Above the rectangles are indicated the residue numbers in the native sequences of p47phox, p67phox, and Rac1 representing the N- and C-terminal limits of the segments forming the trimers. AASTGGSS is a 10-amino acid linker between the p47phox and p67phox segments appearing in all the trimers.
LiDS from 0 to 300 μM on the level of oxidase activity by the above concentrations of membrane and trimer and compared this with oxidase activation by a mixture of individual full-length and truncated cytosolic components and by a combination of chimera 3 and full-length p47^phox. In all experiments, the trimer, Rac1, and chimera 3 were subjected to nucleotide exchange to GMPPNP. As shown in Fig. 3, when the cytosolic components were present as full-length individual entities, the LiDS optimum lay between 120 and 160 μM, as reported previously (54). Oxidase activation by chimera 3 combined with full-length p47^phox exhibited a similar optimum at 100–160 μM LiDS. In contrast to these values, the trimer was most active at 40–100 μM LiDS. The LiDS dose-response curve of mixtures of C-terminally truncated p47^phox and p67^phox (truncations corresponded to those of the respective segments in the trimer) and full-length Rac1 was flat, with an optimum at 80–140 μM LiDS. This concentration optimum is in a range located between that of the trimer and that of the full-length components. It is also apparent that peak oxidase activities elicited by the trimer were only about half of those obtained with full-length individual cytosolic components and were similar to those obtained with individual truncated p47^phox and p67^phox. No oxidase activation was elicited by the trimer or by any of the combinations of cytosolic activators in the absence LiDS. These results show that, despite the C-terminal truncation of both p47^phox and p67^phox segments, oxidase activation by the trimer remained amphiphile-dependent, albeit the concentration of amphiphile required for maximal activation was lower than that necessary for activation by individual full-length components and by chimera 3 and full-length p47^phox. This finding generated a dilemma as to the concentration of LiDS to be used in experiments in which oxidase activation by the trimer was to be compared with that by non-fused components. We opted for 130 μM LiDS, which is closest to the concentration causing maximal activation by both fused and non-fused components.

Having established the most propitious conditions for activation, we studied the ability of the trimer to activate the oxidase under amphiphile-dependent conditions by performing dose-response experiments in which activation by the prototype trimer...
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FIGURE 3. The concentration of the anionic amphiphile LiDS eliciting maximal oxidase activation varies with the nature of the cytosolic component(s) causing activation. The assay mixtures consisted of membrane liposomes at a concentration equivalent to 5 nM cytochrome b559 heme, to which were added one of the following combinations of cytosolic activators (all at a concentration of 100 nM): (a) chimera 3 exchanged to GMPPNP (▲); (b) chimera 3 exchanged to GMPPNP and full-length p47phox (○); (c) full-length p47phox, full-length p67phox, and Rac1 exchanged to GMPPNP (□); and (d) p47phox-(1–286), p67phox-(1–212), and Rac1 exchanged to GMPPNP (△). The components were incubated for 90 s at room temperature in 200 μl of assay buffer/well in 96-well microplates. The assay buffer contained various concentrations of LiDS (from 0 to 300 μM) in increments of 20 μM as indicated on the x-axis. O2 production was initiated by addition of NADPH (240 μM) and measured as described under "Experimental Procedures." The results represent the means ± S.E. of three experiments for each combination of cytosolic components.

Stability of the Trimera-Cytochrome b559 Complex—The oxidase complex generated in vitro is unstable (reviewed in Ref. 62). The mechanism of deactivation of the assembled complex has been the subject of extensive studies, and the dominant idea is that the stability of the complex is the expression of the balance between assembly and disassembly. The stability of the assembled complex could be improved by the presence of non-hydrolyzable GTP analogs (63), by chemical cross-linkers (64), or by the fusion of p67phox with p47phox (37, 65, 66) and, to a lesser degree, with Rac1 (39, 66). An important role for p47phox in the stabilization of the oxidase complex was reported in the early nineties (64) and reemerged in a recent report (8).

We assessed the stability of the oxidase complex consisting of membrane and the prototype trimera and compared this with the stability of complexes comprising membrane and a mixture of individual cytosolic components (p47phox, p67phox, and Rac1) or a mixture of chimera 3 and p47phox. In these experiments, membrane liposomes (50 nM cytochrome b559 heme) were incubated with (a) prototype trimera-GMPPNP alone at a concentration of 1 μM; (b) a combination of chimera 3-GMPPNP and p47phox (full-length) both at a concentration of 3 μM; (c) a combination of p47phox (full-length), p67phox (full-length), and Rac1-GMPPNP all at a concentration of 1 μM; and (d) a combination of p47phox-(1–286), p67phox-(1–212), and Rac1-GMPPNP all at a concentration of 1 μM. All mixtures were supplemented with 130 μM LiDS and incubated for 90 s at 24 °C to induce the assembly of the oxidase complex. Following assembly, aliquots of the mixture were diluted 10-fold in assay buffer lacking LiDS, resulting in the following final concentrations of reactants: 5 nM membrane cytochrome b559 heme, 100 nM each of the cytosolic activators (with the exception of the combination of chimera 3 and p47phox, for which the final concentration of both components was 300 nM), and 13 μM LiDS (at which concentration no de novo assembly is induced). The mixtures were incubated at 24 °C for incremental time intervals from 0 to 80 min. At each time interval, NADPH (240 μM) was added, and O2 production was measured. Oxidase activities assessed at the various time intervals were expressed as the percentage of initial activities measured at time 0. As shown in Fig. 5, there was a marked loss in activity of mixtures consisting of membrane, p47phox and p67phox (full-length and truncated), and Rac1 and
incorporated in liposomes of PC 20% (50 nM cytochrome \(b_{559}\) heme) were incubated with the trimera (1 \(\mu M\)) in the absence of LiDS for 90 s to induce assembly of the oxidase complex. Following assembly, aliquots of the mixture were diluted 10-fold in buffer lacking LiDS, resulting final concentrations of 5 nM cytochrome \(b_{559}\) heme and 100 nM trimera. The mixtures were incubated at 24 °C for time intervals varying from 0 to 80 min. At each time interval, NADPH (240 \(\mu M\)) was added, and \(O_2^\cdot\) production was measured. Similar to the membrane-trimer complex, the purified and relipidated cytochrome \(b_{559}\)-trimer complex generated in the absence of LiDS was extremely stable, with a calculated half-life of 7.8 h. These results demonstrate that linking all three cytosolic components covalently markedly improves the stability of the assembled oxidase complex in comparison with complexes assembled with non-fused components or with bipartite chimeras.

**Enrichment of the Phagocyte Membrane with Exogenous Anionic Phospholipid or Its Replacement by Cytochrome \(b_{559}\) Relipidated with Anionic Phospholipid Makes Oxidase Activation by the Trimer Amphiphile-independent**—Assembly of the oxidase complex in the canonical cell-free system requires an anionic amphiphile (28–34). The dominant opinion is that the principal role of amphiphile is to disrupt the autoinhibitory intramolecular bond in \(p47^{phox}\) (35). There is, however, also evidence for an effect of amphiphile on cytochrome \(b_{559}\) (67). C-terminal truncation of \(p47^{phox}\) and \(p67^{phox}\) segments was reported to free oxidase activation from the need for an activating amphiphile by removing the C-terminal partner in the intramolecular bond (26). In view of the fact that, in the prototype trimer, both \(p47^{phox}\) and \(p67^{phox}\) segments are C-terminally truncated, we examined the ability of the trimer to activate the oxidase in the absence of amphiphile. As shown in Fig. 6A, the prototype trimer did not elicit oxidase activity by native membranes in the absence of amphiphile; as expected, a mixture of the three individual components or chimera 3 combined with \(p47^{phox}\) was also inactive. Surprisingly, when phagocyte membrane liposomes were replaced with liposomes of purified cytochrome \(b_{559}\) relipidated with a partially pure preparation of PC (PC 20%, which consists of 14–23% PC and many other lipids and is derived from soybean), the trimer behaved as a potent, dose-dependent oxidase activator in the absence of amphiphile (Fig. 6B). The final concentration of PC 20%, which served for the relipidation of cytochrome \(b_{559}\) in the cell-free assay was 3.5 \(\mu M\). The combined individual cytosolic components or chimera 3 combined with \(p47^{phox}\) was incapable of eliciting oxidase activation by cytochrome \(b_{559}\) liposomes under the same conditions (Fig. 6B). Addition of LiDS (130 \(\mu M\)) to the assay containing the trimer and cytochrome \(b_{559}\) relipidated with PC 20% did not augment \(O_2^\cdot\) production above the level found in the absence of amphiphile (data not shown).

To elucidate the mechanism responsible for the difference in amphiphile dependence between liposomes of native membrane and of purified cytochrome \(b_{559}\) relipidated with PC 20%, we initiated experiments in which the native phospholipid composition of phagocyte membranes was modified by the incorporation of exogenous phospholipids. Supplementation of membrane with PC 20% resulted in a final concentration of exogenous phospholipid in the cell-free assay of 80 \(\mu M\), with
endogenous membrane lipids contributing 12 μM. As evident in Fig. 6C, the membrane supplemented with PC 20% became responsive to activation by the trimer in the absence of amphiphile. Only minor activation was induced by a mixture of the three full-length cytosolic components at a high concentration of components, whereas the combination of chimera 3 and p47phox failed to activate. To ascertain that the ability of the trimer to elicit amphiphile-independent oxidase activation was the result of enrichment of the native membrane with the particular preparation of partially pure PC (PC 20%), we performed a series of control experiments in which phagocyte membrane was enriched with an identical amount of a highly purified (99%) preparation of the neutral phospholipid PC. The final concentration of PC (99%) in the cell-free assay was, as before, 80 μM, with endogenous membrane lipids contributing 12 μM. As shown in Fig. 6D, neither the trimer nor a combination of individual components or of chimera 3 and p47phox was capable of eliciting amphiphile-independent oxidase activation. We hypothesized that the reason for this difference lies with the fact that a major component of PC 20% preparations is anionic phosphatidylinositols, and it thus appears likely that an increase in the proportion of anionic phospholipids in the membrane relieves oxidase activation by the trimer of its dependence on exogenous anionic amphiphile.

As before, the final concentration of anionic phospholipid in the cell-free assay was 80 μM, with endogenous membrane lipids contributing 12 μM. As shown in Fig. 7, there were considerable differences between the effects of the various anionic phospholipids. In all cases, with the exception of membranes supplemented with PI, maximal activation was induced by a mixture of individual p47phox, p67phox, and Rac1, followed by the trimer, with the combination of chimera 3 and p47phox being the least effective. When activation by the trimer was used as a criterion for comparing the effectiveness of the various phospholipids, on the basis of kinetic characteristics, we found that the \( V_{max} \) values were quite similar, with the exception of the membrane supplemented with PA, which exhibited a higher \( V_{max} \) value (Fig. 7, table). \( EC_{50} \) values were, however, markedly different, following the order PA < PG < PS < PI, indicating that PA was the most effective and PI the least effective phospholipid. It should be noted that supplementation of macrophage membrane with exogenous anionic phospholipids did not influence the spectral characteristics of cytochrome \( b_{559} \) in the membrane (data not shown).

**Amphiphile-independent Oxidase Activation in Phagocyte Membranes Enriched with Synthetic PG by the Trimer and Individual Cytosolic Components**—For an in-depth study of the effect of membrane enrichment on responsiveness to the
trimera, we used a well characterized and highly purified anionic phospholipid. Because supplementation of phagocyte membrane with PA led to a low level of spontaneous oxidase activation in the absence of cytosolic components (Fig. 7A), we chose the next most potent anionic phospholipid, PG. Whereas the experiments illustrated in Fig. 7B were performed with β-oleoyl-γ-palmitoyl-PG, we now chose a preparation of β,γ-dioleoyl-PG (DOPG) of >99% purity. The final concentration of DOPG incorporated in membrane liposomes in the cell-free assay was kept at 80 μM.

As shown in Fig. 8, we set up experiments similar to those in Fig. 4, but this time, oxidase activation was performed in the absence of amphiphile. It is evident that membrane enriched with DOPG enabled amphiphile-independent oxidase activation by trimera-GMPPNP and by a combination of full-length p47phox, p67phox, and Rac1-GMPPNP, confirming the results obtained with β-oleoyl-γ-palmitoyl-PG (Fig. 7B). DOPG-supplemented membranes also responded to a mixture of p47phox(1–286), p67phox(1–212), and full-length Rac1-GMPPNP by amphiphile-independent oxidase activation. This response was similar to that obtained with unmodified membranes in the presence of LiDS (Fig. 4), as evident in the sigmoidal dose-response curve as opposed to the hyperbolic curve found with the trimera and with full-length individual components. The V_max value for oxidase activation by trimera-GMPPNP was 1.8 times lower than that achieved with individual full-length components, but within the range of that measured with truncated components. However, the EC_{50} value measured with the trimera was 3 and 8 times lower than the values measured with individual full-length components and individual truncated components, respectively. The trimera in the GDP-bound form exhibited significant activity, particularly at high concentrations. In general, the kinetic characteristics of oxidase activation by the trimera with DOPG-supplemented membranes in the absence of an exogenous amphiphile were similar to those found with unmodified membranes in the presence of amphiphile, suggesting that negatively charged phospholipid incorporated into the phagocyte membrane functions as a substitute for a soluble anionic amphiphilic activator.

Effect of Deletions and Sequence Replacements Focused on the C Terminus of the Rac1 Segment in the Prototype Trimera on Oxidase-activating Ability—The realization of the seminal importance of membrane phospholipid charge in oxidase activation prompted the construction of deletion and domain replacement mutants focused on a region likely to have a major effect on the electrostatic interaction of the trimera with anionic membrane phospholipids. This region is the polybasic stretch at the C terminus of Rac1, shown in several earlier studies to mediate charge-based interaction with the membrane (18, 23, 25, 41, 56, 57, 68, 69).
We subjected the prototype trimera to the following changes: replacement of residues 183–188 (KKRKRK) in Rac1 with residues 183–188 (RQQKRA) in Rac2 (Rac1→Rac2), deletion of the C terminus of Rac1 (residues 179–192; Rac1/H9004C), and replacement of the positive residues 183–188 in Rac1 with six neutral residues (glutamines; Rac1(183Q-188Q)). These modifications were expected to result in a reduction in the positive charge at the C terminus of the trimera. In mutant Rac1→Rac2, the number of positive residues was reduced from six to three, whereas in the two other mutants (Rac1/H9004C and Rac1(183Q-188Q)), the number of clustered positive residues was reduced from six to zero. The mutants were assayed in two types of cell-free assays: (a) on native membrane in the presence of amphiphile and (b) on DOPG-supplemented membrane in the absence of amphiphile. As shown in Fig. 9, mutants Rac1→Rac2 completely lost their oxidase-activating ability, as expressed in marked decreases in \( V_{\text{max}} \) and the inability to calculate EC50 values, whether assayed on native membranes (panel A) or on DOPG-supplemented membranes (panel B). The Rac1→Rac2 trimera expressed a moderate reduction in \( V_{\text{max}} \) but a marked increase in EC50 on both native and DOPG-supplemented membranes. The pronounced changes in EC50 apparent with all three mutant trimeras demonstrate the centrality of the Rac1 segment-associated polybasic C terminus in determining the affinity of the trimera for its most likely membrane target, anionic phospholipids.
Effect of Deleting the PX Domain of the p47phox Segment and the Insert Domain of the Rac1 Segment on the Oxidase-activating Ability of the Prototype Trimera—We next examined the role of two additional regions possessing a net positive charge on the oxidase-activating ability of the trimera. These regions are the PX domain in the p47phox segment (residues 4–125 in the p47phox monomer) and the insert domain in the Rac1 segment (residues 124–135 in the Rac1 monomer) of the trimera. An additional reason for addressing the role of the Rac1 insert domain was the proposal that this region mediates the direct interaction of Rac with gp91phox (19, 20). We thus introduced the following two changes in the structure of the prototype trimera: deletion of residues 1–150 in the p47phox segment, which includes the PX domain (p47phox/H9004PX), and deletion of residues 124–135 in the Rac1 segment (Rac1Δinsert). The mutants were assayed on unmodified membrane in the presence of amphiphile and on DOPG-supplemented membrane in the absence of amphiphile. As shown in Fig. 10 (A and B), removal of either the PX or Rac1 insert domain did not significantly affect the \( V_{\text{max}} \) for oxidase activation by the mutant trimeras and caused only a minor increase in \( EC_{50} \) values in both amphiphile-dependent and -independent systems. The only exception was a more pronounced increase in \( EC_{50} \) upon deletion of the insert domain in the Rac1 segment when assayed on native membrane in the presence of LiDS.

We reasoned that our failure to detect anything more than a minor effect of removing the PX and Rac1 insert domains on the ability of the trimera to activate the oxidase might be due to the fact that the membrane lacked phospholipids known to serve as specific targets for the deleted domains. Thus, the PX domain of p47phox possesses a high affinity for PtdIns(3,4)P₂ (12), and the insert domain of Rac1 binds preferentially to PtdIns(3,4)P₂ and phosphatidylinositol 3,4,5-trisphosphate (59). We consequently prepared two more forms of phospholipid-enriched membrane liposomes: the first contained, in addition to DOPG, PtdIns(3,4)P₂, and the second contained DOPG and PtdIns(4,5)P₂ and served as a negative control. Both phosphoinositides were present at a final concentration of 10 \( \mu \text{M} \); in a distinct set of experiments, this concentration was found to be optimal in promoting the dissociation of Rac1-Rho GDP dissociation inhibitor complexes by negatively charged liposomes and in enhancing amphiphile-independent oxidase activation.

![FIGURE 10. Effect of deleting the PX domain of the p47phox segment and the insert domain of the Rac1 segment](image)

**Effect of Deleting the PX Domain of the p47phox Segment and the Insert Domain of the Rac1 Segment on the Oxidase-activating Ability of the Prototype Trimera**—We next examined the role of two additional regions possessing a net positive charge on the oxidase-activating ability of the trimera. These regions are the PX domain in the p47phox segment (residues 4–125 in the p47phox monomer) and the insert domain in the Rac1 segment (residues 124–135 in the Rac1 monomer) of the trimera. An additional reason for addressing the role of the Rac1 insert domain was the proposal that this region mediates the direct interaction of Rac with gp91phox (19, 20). We thus introduced the following two changes in the structure of the prototype trimera: deletion of residues 1–150 in the p47phox segment, which includes the PX domain (p47phox/H9004PX), and deletion of residues 124–135 in the Rac1 segment (Rac1Δinsert). The mutants were assayed on unmodified membrane in the presence of amphiphile and on DOPG-supplemented membrane in the absence of amphiphile. As shown in Fig. 10 (A and B), removal of either the PX or Rac1 insert domain did not significantly affect the \( V_{\text{max}} \) for oxidase activation by the mutant trimeras and caused only a minor increase in \( EC_{50} \) values in both amphiphile-dependent and -independent systems. The only exception was a more pronounced increase in \( EC_{50} \) upon deletion of the insert domain in the Rac1 segment when assayed on native membrane in the presence of LiDS.

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by such complexes. The prototype trimera and mutants p47\textsuperscript{phox}ΔPX and Rac1Δinsert were tested for the ability to activate the oxidase in membranes enriched either in DOPG and PtdIns(3,4)P\textsubscript{2} or in DOPG and PtdIns(4,5)P\textsubscript{2} in the absence of amphiphile. It was expected that the specific affinity of PX domains and of the insert domain of Rac1 for PtdIns(3,4)P\textsubscript{2} would be expressed in enhanced oxidase activation by the prototype trimera acting on membrane enriched in DOPG and PtdIns(3,4)P\textsubscript{2} and in the lack of such enhancement by the deletion mutants. The experimental results did not fulfill this prediction. Neither the V\textsubscript{max} nor EC\textsubscript{50} values measured with the prototype chimera acting on membrane supplemented with DOPG and PtdIns(3,4)P\textsubscript{2} (Fig. 10C) were different from those measured with control membrane supplemented with only DOPG (Fig. 10B) or membrane supplemented with DOPG and PtdIns(4,5)P\textsubscript{2} (Fig. 10D). Also, the kinetic characteristics of the PX and Rac1 insert domain deletion mutants assayed on membrane supplemented with DOPG and PtdIns(3,4)P\textsubscript{2} were undistinguishable from those derived by assay of the mutants on membrane supplemented with DOPG only or on membrane supplemented with DOPG and PtdIns(4,5)P\textsubscript{2}.

Effect of Point Mutations Focused on Regions in the Prototype Trimera Involved in Interaction with the p22\textsuperscript{phox} Subunit of Cytochrome b\textsubscript{559} and in Intrachimeric Interaction between the p67\textsuperscript{phox} and Rac1 Segments on Oxidase-activating Ability—Trp\textsuperscript{193} in p47\textsuperscript{phox} is an essential residue in the interaction between the SH3 domains in p47\textsuperscript{phox} and the PRR in p22\textsuperscript{phox} (15, 58, 70). To influence the interaction of the trimera with p22\textsuperscript{phox}, we generated a mutant in which Trp\textsuperscript{193} in the p47\textsuperscript{phox} segment was replaced with Arg. The p47\textsuperscript{phox}(W193R) trimera was found to be incapable of activating the oxidase on native membrane in the presence of amphiphile (Fig. 11A) and exhibited only weak oxidase-activating ability when assayed on DOPG-supplemented membrane in its absence (Fig. 11B). The functional impairment was most evident in the unquantifiable or markedly increased EC\textsubscript{50} values, a reflection of reduced affinity for its target protein in the membrane, p22\textsuperscript{phox}. The lesser effect of the W193R mutation on amphiphile-independent activation of DOPG-supplemented membranes suggests that, in the presence of a charge-related bond, the dependence on the p47\textsuperscript{phox}, p22\textsuperscript{phox} interaction is less pronounced. These results indicate that, although interaction modules connecting the trimera to the phospholipid milieu of cytochrome b\textsubscript{559} based on protein-lipid electrostatic attraction are essential for oxidase activation, there is an additional requirement for protein-protein interaction involving the N-terminal SH3 domain of the p47\textsuperscript{phox} segment of the trimera and the PRR of the p22\textsuperscript{phox} subunit of cytochrome b\textsubscript{559}.

Intrachimeric interactions between the p67\textsuperscript{phox} and Rac1 segments were shown by us to be essential for oxidase activation by chimera 3 (8, 25, 40, 41), and it was of interest to find out whether this also applied to the trimera. Mutating Arg\textsuperscript{102} in the p67\textsuperscript{phox} moiety or Ala\textsuperscript{27} in the Rac1 moiety was found to reduce oxidase activation by chimera 3 (25). We designed mutants p67\textsuperscript{phox}(R102E) and Rac1(A27K) to prevent an intrachimeric interaction between the p67\textsuperscript{phox} and Rac1 segments. The ability of the prototype trimera and of the mutant trimeras p47\textsuperscript{phox}(W193R) (○), p67\textsuperscript{phox}(R102E) (●), and Rac1(A27K) (▲) to activate the oxidase was tested in two cell-free systems: amphiphile-dependent, in which native membrane liposomes were used in the presence of LiDS (130 μM) (A); and amphiphile-independent, in which membrane liposomes supplemented with DOPG were used in the absence of LiDS (B). In both systems, the concentration of the membrane was equivalent to 5 nm cytochrome b\textsubscript{559} heme, and the concentration of the trimera was varied from 0 to 300 nm. All trimera were exchanged to GMPNP. Following incubation for 90 s in the presence or absence of LiDS, O2\textsuperscript{−} production was initiated by addition of NADPH (240 μM) and measured as described under “Experimental Procedures.” The table displays a comparison of the kinetic data (V\textsubscript{max} and EC\textsubscript{50}) derived from the displayed curves. nd, not determinable. The results represent the means ± S.E. of three to nine experiments for each combination of membrane preparation and trimera (prototype or mutant).
interaction between the p67phox and Rac1 segments of the trimera. The Rac1(A27K) mutant was found to be defective in its ability to activate the oxidase on both native and DOPG-supplemented membranes in the presence and absence of amphiphile, respectively, with a more pronounced impairment being apparent with native membrane (Fig. 11). The impairment was most evident in the marked increases in EC50 values, reflecting the reduced affinity for the most likely target, cytochrome b559. The counterpart mutant, p47phox(R102E), exhibited only a minor reduction in oxidase-activating ability on both types of membranes, but the existence of some functional impairment was suggested by a significant increase in EC50 on native membrane and a lesser increase on DOPG-supplemented membrane (Fig. 11). These results demonstrate that, despite the physical fusion between the p67phox and Rac1 segments in the trimera, an intrachimeric interaction between the TPR domain of the p67phox segment and the pre-switch I region of the Rac1 segment is required for oxidase activation. The mild effect of mutation R102E in the p67phox segment as opposed to the pronounced effect of mutation A27K in the Rac1 segment remains unexplained, although a similar, although less marked, difference in the effects of mutating these two residues was described in chimera 3 (25). Arg102 was shown to make direct hydrogen-bonding interactions with four residues in Rac1, but not with Ala27 (17). It is possible that, because of steric hindrance, such bonds cannot be established in the trimera and that other residues in the TPR domain are involved in interaction with the Rac1 segment.

DISCUSSION

In this study, we have described the design, construction at the DNA level, and successful bacterial expression of a soluble fusion protein consisting of functionally important segments derived from the three cytosolic components essential for activating the O2-generating oxidase in vitro. The segments were chosen based on the accumulated information concerning the involvement of specific domains in p47phox, p67phox, and Rac in oxidase assembly (reviewed in Refs. 1–3) and on previous work with p47phox, p67phox (37) and p67phox, Rac1 (Refs. 39, 40, 41, and 25; reviewed in Ref. 8) chimeras. We hypothesized that physically fusing crucial parts of the three cytosolic components would bypass the need for protein-protein interaction between p47phox and p67phox and between p67phox and Rac, resulting in a single protein, the interaction of which with cytochrome b559 and its membrane environment can be studied. p47phox-p67phox and p67phox-Rac1 chimeras fulfill this goal only partially because they require supplementation with Rac1 or p47phox, respectively, for optimal functioning. The trimera was designed to incorporate in its structure the prototype p67phox-Rac1 chimera, described by us in the past and known as chimera 3 (40). A spacer was introduced between the p47phox and p67phox segments to ensure a more flexible conformation and to mimic the spacer present in the p47phox-p67phox chimera described by Ebisu et al. (37). The trimera possesses several advantageous characteristics: (a) the p47phox segment (and possibly the p67phox segment, too) is free of an autoinhibitory region; (b) the presence of Rac makes the molecule act as a bona fide small GTPase, with a GTP/GDP switch; (c) the C terminus has the potential to be prenylated; and (d) a single molecule activator should be ideal for high throughput screening of pharmacological agents affecting oxidase function.

The prototype trimera in the GMPPNP-bound form was found to be a potent activator of the oxidase in native phagocyte membrane in the absence of any additional component, with the exception of an anionic amphiphile. The dependence on amphiphile seemed surprising, at first, because of the absence of the autoinhibitory region in the p47phox segment and because of the finding that p47phox-p67phox chimeras supplemented with Rac1-GTP are active with a cytochrome b559 preparation in the absence of amphiphile (37). The explanation for the latter finding lies with the fact that the particular cytochrome b559 preparation was relipidated with a mixture rich in anionic phospholipids, a procedure found by us in the experiments described in this study to enable amphiphile-independent oxidase activation by the trimera. The persistence of a requirement for amphiphile despite the “built-into-the-trimera” truncation of the p47phox segment provides some support for the idea of a direct effect of amphiphile on cytochrome b559 (67) and is in agreement with the requirement for a lower concentration of LiDS for trimeria-elicited oxidase activation compared with that required for activation by components including full-length p47phox.

The trimera forms an exceedingly stable complex with cytochrome b559 in marked contrast to the lability of complexes formed with three individual components, whether full-length or truncated, or with chimera 3 combined with p47phox. Stable oxidase complexes were generated in the past by chemical cross-linking (64) or with mixtures of a p47phox-p67phox-chimera and Rac1-GTP (37, 65, 66). By using a methodology similar to that used by us, complexes with half-lives of 3.5 and 4.2 h were obtained with p47phox-p67phox chimeras supplemented with Rac1-GTP activated in the presence and absence of amphiphile, respectively (66). The stability of the complexes was increased to half-lives of 4.5 and 6.6 h, respectively, by treatment with a chemical cross-linker. Ours appears to be the first description of a stable oxidase complex comprising a single cytosolic activator molecule without the need for artificial cross-linking. The half-lives achieved with the trimera (~8 h) exceeded those obtained with bipartite chimeras combined with a third component, even when the latter combinations were treated with a cross-linker. This result supports the contention that, under physiological conditions, the stability of the catalytically active assembled complex is limited and that there is a continuous exchange of cytochrome b559-bound cytosolic components for fresh components translocating from the cytosol (Refs. 71 and 72; reviewed in Ref. 62). In the case of the trimera, the p47phox and Rac1 segments bind to the membrane and possibly to cytochrome b559 and, by being fused to the p67phox segment, ensure a long-lasting association of p67phox with gp91phox, which stabilizes an “activated” conformation in gp91phox.

When compared with the kinetic features of activation by individual components, those of oxidase activation by the trimera are lower EC50 values, suggesting a higher affinity for cytochrome b559, together with a seemingly paradoxical lowering of Vmax values, indicating less efficient activation. It is pos-
sible that the high stability of the bond between the trimera and cytochrome \( b_{559} \) as opposed to the continuous recruitment dynamics seen with individual cytosolic components, is not ideal for maximal oxidase activity and might offer an explanation for the lower \( V_{\text{max}} \) values.

In the course of work meant to test the ability of the trimera to activate purified cytochrome \( b_{559} \) we used cytochrome \( b_{559} \) preparations relipidated with non-purified PC (PC 20%) as described previously (53). This led us to the finding that cytochrome \( b_{559} \) relipidated with a low purity preparation of PC responded to the trimera by NADPH-dependent \( O_2^- \) production in the absence of LiDS. The finding that native membrane and membrane supplemented with a neutral phospholipid such as pure PC did not respond to the trimera, whereas membrane supplemented with PC 20% (rich in anionic phosphatidylinositols) did, points to a role for negatively charged phospholipids. The more vigorous response of cytochrome \( b_{559} \) liposomes compared with that of supplemented membrane is most likely related to the presence in the membrane, but not in relipidated cytochrome \( b_{559} \) of endogenous lipid, which is predominantly neutral (73, 74).

The difficulty of drawing definite conclusions from work with uncharacterized phospholipids motivated the performance of experiments with highly purified anionic phospholipids. These experiments (summarized in Fig. 7) clearly establish that modifying the lipid composition of the phagocyte membrane, leading to an increase in negative charge, causes a major change in the response of the oxidase to cytosolic activators. This is expressed in an ability to be activated by the trimera in the absence of amphiphile, but also allows activation by a mixture of individual cytosolic components and by chimera 3 combined with \( p47^{\text{phox}} \).

We propose that this represents a novel mechanism of oxidase assembly and activation in vitro, which is likely to have its equivalent in vivo. Whereas in the canonical cell-free system, the anionic amphiphilic activator is free in solution, in this system, the anionic phospholipid is in the membrane. The main role of membrane-bound anionic phospholipid is likely to be the provision of an electrostatic anchor to the principal positively charged region in the trimera, namely the polybasic stretch at the C terminus of the Rac1 segment (Fig. 12).

The capacity of membrane-incorporated phospholipid to enable amphiphile-independent oxidase activation was described before for \( p47^{\text{phox}} \)-\( p67^{\text{phox}} \)-Rac1 chimeras supplemented with Rac (37) and for mixtures of individual cytosolic components comprising \( p47^{\text{phox}} \) mutants in which the autoinhibition of SH3 domains was relieved (38). Both groups reported that, under the same conditions, no amphiphile-independent activation was seen with full-length non-mutated components. We found that membrane enriched with certain anionic phospholipids also responds to full-length cytosolic components in the absence of amphiphile. This is most likely the result of the high concentration of anionic phospholipid achieved when purified phospholipids are used for membrane enrichment. Indeed, the final concentration of anionic phospholipid present in the reaction (80 \( \mu \)M) is similar to that of soluble anionic amphiphile in the canonical cell-free system. The theoretical pI of the prototype trimera is 6.94, and thus, charge-mediated interactions must be centered on particular regions in the trimera. We have demonstrated that the principal, if not the only such, region is the polybasic C terminus of the Rac1 segment. Binding of Rac1 to anionic phospholipids exhibits little specificity; interaction of Rac1 with PA, PG, PS, and PI and with a number of phosphoinositides was described (23, 59, 68, 69), although a preference for PS over PG and PI was reported recently (75).

We found no good evidence for a major role of the PX domain in the \( p47^{\text{phox}} \) segment and the insert domain in the Rac1 segment in oxidase activation by the trimera. Domain deletion studies indicated only minor decreases in the affinity for the membrane, reflected by increases in \( EC_{50} \) values. No specificity for PtdIns(3,4)P2 could be shown, leading to the conclusion that binding of the PX and Rac1 insert domains to phospholipids is based exclusively on charge. A similar lack of specificity for phosphoinositides was found in a recent study on amphiphile-independent oxidase activation by individual cytosolic components (38).

The design of the trimera also allowed us to examine whether interaction with \( p22^{\text{phox}} \) forms part of the activation process. The marked inhibitory effect of the W193R mutation on the activity of the trimera was surprising in light of the many occasions in which oxidase activation by individual components (5, 8, 76, 77) or by \( p67^{\text{phox}} \)-Rac1 chimeras (25, 41) was found to be \( p47^{\text{phox}} \)-independent. A survey of the situations in which oxidase activation took place in the absence of \( p47^{\text{phox}} \) reveals that this requires the presence of an additional membrane localization signal such as prenylation of the C terminus of Rac (5, 25, 41) or supplementation of membranes with anionic phospholipid. Indeed, we found that supplementation of membranes with certain anionic phospholipids made amphiphile-independent oxidase activation by \( p67^{\text{phox}} \) and Rac1 or by chimera 3 in the absence of \( p47^{\text{phox}} \) possible (in the following order of efficiency: PA > PG > PS > PI) (data not shown).

Finally, we found that, despite the physical fusion between segments within the trimera, there is a requirement for intra-chimeric protein-protein interaction between the \( p67^{\text{phox}} \) and Rac1 segments, similar to that described in the bipartite \( p67^{\text{phox}} \)-Rac1 chimeras (25, 40, 41). Fig. 12 presents an idealized rendering of the overall structure of the prototype trimera in the GMPPNP-bound form and of its sites of contact with membrane phospholipids and cytochrome \( b_{559} \). The approximate locations of the residues, the mutation of which causes functional changes, are indicated, as well as the intrachimeric interaction and the hypothetical point of contact between the activation domain in the \( p67^{\text{phox}} \) segment and the cytosolic tail of gp91phox.

Trимера-elicited oxidase activation provides support for a model in which the process of oxidase assembly is seen as a two-stage process. In the first stage, \( p47^{\text{phox}} \) and Rac-GTP (acting as “organizers”) establish nonspecific, electrostatic, and hydrophobic bonds with negatively charged membrane phospholipid facing the cytosol. Before, simultaneously with, or following binding to the membrane, \( p47^{\text{phox}} \) and Rac-GTP interact with \( p67^{\text{phox}} \) (which lacks intrinsic membrane tropism). In the second stage, protein–protein bonds are established between \( p67^{\text{phox}} \) (acting as “activator”) and gp91phox, the end
result of which is the induction of a conformational change in gp91phox expressed in the promotion of electron flow from NADPH to oxygen and the generation of O2. There are good arguments for the contention that the binding of p67phox to gp91phox is the consequence of a Rac-GTP-induced conformational change in p67phox, which is propagated to gp91phox. A parallel pathway assisting the binding of p67phox to gp91phox, but not leading to the induction of a conformational change in gp91phox, involves the binding of p47phox to p22phox.

Our results are also in agreement with a model in which oxidase activation is initiated by the generation of anionic phospholipid “patches” facing the cytosol and providing a novel microenvironment for cytochrome b559. In the experiments described in this study, the concentration of anionic phospholipid in supplemented membrane liposomes was 4 mM, and its distribution was probably random. It is unlikely that such a concentration is ever achieved in membranes in vivo, and it is thus likely that lipids of higher negative charge are involved and concentrated in microdomains. The obvious candidates for such a function are phosphoinositides, as exemplified by the fact that PtdIns(4,5)P2 has a valence of —4 compared with —1 for PS (78). Phosphatidylinositol 3,4,5-trisphosphate and PtdIns(4,5)P2 were indeed found to target small GTPases, possessing a C-terminal polybasic cluster, to the plasma membrane (79). Another possible candidate is PA, generated by phospholipase D, by virtue of it being the membrane supplement supporting the most vigorous response to the trimera and individual cytosolic components. Both phosphoinositides (reviewed in Ref. 80) and PA (36) were proposed as intermediates in the signal transduction path leading from membrane receptors to oxidase assembly.

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REFERENCES
1. Nauseef, W. M. (2004) Histochem. Cell Biol. 122, 277–291
2. Quinn, M. T., and Gauss, K. A. (2004) J. Leukocyte Biol. 76, 760–781
(1993) J. Biol. Chem. 268, 20983–20987
72. Van Bruggen, R., Anthony, E., Fernandez-Borja, M., and Roos, D. (2004) J. Biol. Chem. 279, 9097–9102
73. Mason, R. J., Stossel, T. P., and Vaughan, M. (1972) J. Clin. Invest. 51, 2399–2407
74. Henriques, C., Atella, G. C., Bonilha, V. L., and de Souza, W. (2003) Parasitol. Res. 89, 123–133
75. Finkelstein, C. V., Overduin, M., and Capelluto, D. G. S. (2006) J. Biol. Chem. 281, 27317–27326
76. Freeman, J. L., and Lambeth, J. D. (1996) J. Biol. Chem. 271, 22578–22582
77. Koshkin, V., Lotan, O., and Pick, E. (1996) J. Biol. Chem. 271, 30326–30329
78. McLaughlin, S., and Murray, D. (2005) Nature 438, 605–611
79. Heo, W. D., Inoue, T., Park, W. S., Kim, M. L., Park, B. O., Wandless, T. J., and Mayer, T. (2006) Science 314, 1458–1461
80. Perisic, O., Wilson, M. I., Karathanassis, D., Bravo, J., Pacold, M. E., Ellson, C. D., Hawkins, P. T., Stephens, L., and Williams, R. L. (2004) Adv. Enzyme Regul. 44, 279–298
81. Bio-Rad Laboratories (1984) Bio-Rad Technical Bulletin 1177EG, Richmond, CA