A Prenylated p67phox-Rac1 Chimera Elicits NADPH-dependent Superoxide Production by Phagocyte Membranes in the Absence of an Activator and of p47phox

CONVERSION OF A PAGAN NADPH OXIDASE TO MONOTHEISM*

Received for publication, March 4, 2002
Published, JBC Papers in Press, March 14, 2002, DOI 10.1074/jbc.M202114200

Yara Gorzalczyńska, Nathalie Alloul‡, Natalia Sigal‡, Carolyn Weinbaum* and Edgar Pick‡

From the §Julius Friedrich Cohnheim-Minerva Center for Phagocyte Research and the Ela Kodesz Institute of Host Defense against Infectious Diseases, Sackler School of Medicine, Tel Aviv University, Tel Aviv 69978, Israel and the §Department of Pharmacology and Cancer Biology, Duke University Medical Center, Durham, North Carolina 27710

Activation of the superoxide-generating NADPH oxidase of phagocytes is the result of the assembly of a membrane-localized flavocytochrome (cytochrome b559) with the cytosolic components p47phox, p67phox, and the small GTPase Rac. Activation can be reproduced in an in vitro system in which cytochrome b559-containing membranes are mixed with cytosolic components in the presence of an anionic amphiphile. We proposed that the essential event in activation is the interaction between p67phox and cytochrome b559 and that Rac and p47phox serve as carriers for p67phox to the membrane. When prenylated, Rac can fulfill the carrier function by itself, supporting oxidase activation by p67phox in the absence of p47phox and amphiphile. We now show that a single chimeric protein, consisting of residues 1–212 of p67phox and full-length Rac1 (residues 1–192), prenylated in vitro and exchanged to GTP, becomes a potent oxidase activator in the absence of any other component or stimulus. Oxidase activation by prenylated chimera p67phox-(1–212)-Rac1 (1–192) is accompanied by its spontaneous association with membranes. Prenylated chimeras p67phox-(1–212)-Rac1 (178–192) and p67phox-(1–212)-Rac1 (189–192), containing specific C-terminal regions of Rac1, are inactive; the activity of the first but not of the second chimera can be rescued by supplementation with exogenous nonprenylated Rac1-GTP. An analysis of prenylated p67phox-Rac1 chimeras suggests that the basic requirements for oxidase activation are: (i) a “two signals” membrane-localizing motif present in Rac, including the prenyl group and a C-terminal polybasic sequence and (ii) an intrachimeric or extrachimeric protein-protein interaction between p67phox and Rac1, causing a conformational change in the “activation domain” in p67phox.

Superoxide (O2•−) is generated by the NADPH-derived one-electron reduction of molecular oxygen by a plasma membrane-localized flavocytochrome (cytochrome b559) composed of two subunits, gp91phox and p22phox (reviewed in Refs. 1 and 2). The catalytic core of the enzyme is gp91phox, which contains all redox stations through which electrons flow from NADPH to oxygen (3–5). Stimuli causing O2•− production activate an intracellular signaling process leading to the initiation of electron transport and O2•− production. The most likely mechanistic model for this is the induction of a conformational change in gp91phox, brought about by interaction of cytochrome b559 with one or more cytosolic components, subsequent to their translocation to the plasma membrane. These components are p47phox, p67phox, p40phox, and the small GTPase Rac (Rac1 or 2). Upon transfer to the membrane, a multi-component complex is assembled, known as the O2•−-forming NADPH oxidase complex (referred to as “oxidase”) (reviewed in Ref. 6). Oxidase assembly can be induced in vitro in a cell-free system consisting of phagocyte membranes and the cytosolic components p47phox, p67phox, and Rac, exposed to an anionic amphiphile serving as activator (7, 8). Identifying the cytosolic component(s) responsible for causing the conformational change in gp91phox is essential for elucidating the molecular basis of this change. The best established fact is the binding of p47phox to p22phox, mediated by the interaction between a Src homology 3 domain in p47phox and a proline-rich region in the C terminus of p22phox, consequent to the opening of intramolecular bonds in p47phox (9–12). Findings from three laboratories demonstrated that, under certain conditions, oxidase activation in vitro is possible in the absence of p47phox but not in the absence of either p67phox or Rac (13–15). This is compatible with a model in which either p67phox or Rac or both components are responsible for the induction of the conformational change in gp91phox. A central role for p67phox is supported by the identification of an “activation domain” in p67phox consisting of residues 199–210 (16) or residues 187–193 and 199–210 (17) that was proposed to interact directly with gp91phox and regulate electron flow from NADPH to FAD (18). Direct evidence for such a role is offered by the recent description of the binding of p67phox to whole cytochrome b559 and to the gp91phox subunit, an interaction enhanced by the presence of Rac (19). A regulatory effect of Rac on cytochrome b559, based on direct interaction between these two components involving the “insert region” of Rac, was also proposed (20) and supported by experimental evidence (21).

We recently put forward a model for oxidase assembly in which a central role is attributed to p67phox as the only component that makes direct contact with and induces a conformational change in gp91phox (22). In the intact cell, p67phox is carried to the membrane habitat of cytochrome b559 by two carriers, p47phox and Rac. The “carrier for p67phox functions of

* This work was supported by the Julius Friedrich Cohnheim-Minerva Center for Phagocyte Research, the Ela Kodesz Institute of Host Defense against Infectious Diseases, and Israel Science Foundation Grant 128/01. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Dept. of Human Microbiology, Sackler School of Medicine, Tel Aviv University, Tel Aviv 69978, Israel. Tel.: 972-3-640-7872; Fax: 972-3-642-9119; E-mail: epick@post.tau.ac.il.
p47phox and Rac are not symmetrical, as shown by the fact that oxidase activation in vitro can be achieved by p67phox + Rac but not by p67phox + p47phox (13–15). To investigate the relative roles of Rac, we made use of the fact that in eukaryotic cells small GTPases undergo posttranslational modification, expressed in part in C-terminal prenylation. Thus, the physiological form of Rac in phagocytes is a protein geranylgeranylated (prenylated) at residue Cys189 (23). Prenylated Rac1 was found capable of eliciting oxidase activation in a cell-free system containing phagocyte membranes and p67phox in the absence of p47phox and of an activating amphiphile (22), demonstrating that membrane association of Rac was not only required (24) but also sufficient for the induction of oxidase activation.

Recently, this issue was approached by the use of a novel experimental tool, the construction of chimeric proteins consisting of segments of p67phox and Rac1 (25, 26). A chimera consisting of p67phox truncated at residue 212, fused to full-length Rac1 and exchanged to GTPγS,1 was found capable of amphiphile-dependent oxidase activation in the absence of p47phox (26). This recombinant chimeric protein was expressed in Escherichia coli and, consequently, nonprenylated. Because Rac in mammalian phagocytes is prenylated and prenylated Rac has a superior ability to associate with membranes, we constructed a series of prenylated p67phox-Rac1 chimeras. We now demonstrate that a p67phox(1–212)-Rac1 chimera enzymatically prenylated in vitro and exchanged to GTPγS elicits NADPH dependent O2 production by phagocyte membranes in the absence of an activator and of any other oxidase component. This is the first demonstration of the ability of a single protein to elicit oxidase activation in membranes from resting phagocytes in the absence of any stimulus.

**EXPERIMENTAL PROCEDURES**

**Preparation of Phagocyte Membranes and Membrane Vesicles—** Membranes were prepared from guinea pig peritoneal macrophages (7) and solubilized by n-octyl-β-D-glucopyranoside, and membrane vesicles were produced by dialysis against buffer without detergent (27).

**Preparation of Recombinant Oxidase Components—** p47phox and p67phox were prepared in baculovirus-infected Sf9 cells, and nonprenylated Rac1 was produced in E. coli, by procedures described before (14). p67phox truncated at residue 212 was produced in E. coli, as described previously (22).

**Generation of Nonprenylated p67phox-Rac1 Chimeras—** Four chimeric constructs were generated, joining residues 1–212 of p67phox with full-length Rac1 (residues 1–192) or parts of it, as recently reported (26). The proteins were expressed in E. coli as glutathione S-transferase fusions proteins and purified by batch affinity chromatography on glutathione-agarose and thrombin cleavage in situ.

**Enzymatic Prenylation of p67phox-Rac1 Chimeras and Rac1 in Vitro—** Nonprenylated chimeras and Rac1 were geranylgeranylated in vitro by recombinant mammalian geranylgeranyltransferase type I. Recombinant human geranylgeranylated type I was produced in Sf9 cells cotransfected with baculoviruses encoding the two subunits of the enzyme and purified as described (28). Enzymatic prenylation was performed essentially as described in Ref. 29. A typical reaction mixture contained 10 nmol of recombinant p67phox-Rac1 chimera, 20 nmol of geranylgeranyl pyrophosphate (Sigma), and 10 units of geranylgeranyltransferase type I (one unit transfers 1 nmol of geranylgeranyl to protein/h) in 1 ml of 50 mM Tris-HCl, pH 7.7, 5 mM MgCl2, 50 mM NaCl, 2 mM dithioerythritol, and 10 µM GDP. The reaction mixture was incubated for 45 min at 37 °C on an orbital shaker at 600 rpm, whereupon n-octyl-β-D-glucopyranoside was added to a final concentration of 4.375 mM, and the reaction continued for additional 45 min. The mixture was ice-cooled and subjected to sonic disruption in an ultrasonic processor (Vibra Cell, 400 w; Sonics and Materials). At this stage, an aliquot was removed for assessing the degree of prenylation by Triton X-114 partition. To the rest of the reaction mixture, glycerol was added to a final concentration of 20% (v/v), bringing the final concentration of n-octyl-β-D-glucopyranoside to 3.5 mM.

Assessing the Degree of Prenylation by Phase Separation in Triton X-114—Each preparation of prenylated protein was examined for the success of prenylation by assessing the relative amounts of chimera partitioning in the detergent and aqueous phases upon temperature-dependent phase separation in Triton X-114, as described in Ref. 30. Aliquots were taken from the two phases, brought to equal volumes, were subjected to SDS-PAGE, and the degree of prenylation was estimated from the relative intensities of the bands present in the two phases. The correlation of prenylation with partitioning in the detergent phase was proven by the absence of material in the detergent phase when chimeras were mock-prenylated in the absence of geranylgeranylation.

**Nudix Exchange—** Chimeras or Rac1 were subjected to nucleotide exchange to GTPγS or GDPβS (Roche Molecular Biochemicals) as described before (22).

**Activation of NADPH Oxidase in Vitro in the Absence of Amphiphile—** Prenylated chimeras were tested for the ability to elicit NADPH-dependent O2 production upon addition to phagocyte membrane vesicles, essentially as described for nonprenylated chimeras (26) but in the absence of an amphiphilic activator. In all experiments, the membranes were present at an equivalent of 5 nM cytochrome b555 heme, and chimeras were added at concentrations that varied from 50 to 800 nM. When p47phox was added, it was present at a concentration of 300 nM. This concentration was chosen for the sake of simplicity, based on preliminary experiments showing that active prenylated p67phox-Rac1 was different from that using equimolar concentrations of chimera and p47phox (50–800 nM, each). The activity of chimeras was compared with that of a combination of isolated p67phox (1–212) and prenylated Rac1 at concentrations that varied from 50 to 800 nM, each.

**Binding of Chimeras to Phagocyte Membrane Vesicles—** Aliquots of 2 nM of prenylated chimeras were mixed with membrane vesicles (300 pmol equivalent of cytochrome b555, heme) in a volume of 0.6 ml. The mixtures were kept for 10 min at 4 °C and injected in a Superose 12 HR 10/30 fast protein liquid chromatography gel filtration column (Amer sham Biosciences). The chimeras were quantified by assessing the functional competence of the p67phox segment shared by all three chimeras. Thus, membrane-bound and free chimeras were measured by assaying lutein dodecyl sulfate- and NADPH-dependent O2 production by 50–150 aliquots of column fractions upon supplementation with p47phox and Rac1-GTPγS (both at 300 nM), in the absence (membrane-bound chimera) or presence (free chimera) of membrane vesicles (equivalent of 5 nM cytochrome b555 heme). The membrane vesicles were quantified by measuring the amount of cytochrome b555, as described in Ref. 22.

**RESULTS AND DISCUSSION**

**Generation of Prenylated p67phox-Rac1 Chimeras—** Four nonprenylated p67phox-Rac1 chimeras were generated in E. coli, as recently described (26), and were subsequently prenylated enzymatically in vitro. The structure and nomenclature of the prenylated chimeras are identical to those of the nonprenylated counterparts and are illustrated in Fig. 1A. Prenylation was confirmed by the partition of over 90% of the product in the detergent layer upon phase separation in Triton X-114 at 37 °C (Fig. 1B). The prenylated chimeras had the expected Mr, with only minor proteolytic degradation.

**Oxidase Activation by Prenylated p67phox-Rac1 Chimeras—** We and others have recently reported that a chimeric protein consisting of the N-terminal residues 1–212 of p67phox and full-length Rac1 (our chimera 3) is capable of oxidase activation in the absence of an anionic amphiphile, even in the absence of p47phox (25, 26). These chimeras were expressed in E. coli and were therefore nonprenylated. We now demonstrate that the addition of a geranylgeranyl group to the C terminus of chimera 3 results in the formation of a molecule that when added to phagocyte membranes, elicits dose-dependent NADPH-dependent O2 generation in the absence of any activator (Fig. 2). Aliquots from the reaction mixture were used to evaluate the activating ability of prenylated chimera 3 exchanged to the nonhydrolyzable nucleotide analog GTPγS or left in the native state. As seen in Fig. 2A, chimera 3-GTPγS had a clearly superior activating ability, and the dose-response curve was hyperbolic. The presence of p47phox added little to the activating capacity of the chimera; Vmax was 111.6 ± 5.6

1 The abbreviations used are: GTPγS, guanosine 5′-3′-(thio)triphosphate; GDPβS, guanosine 5′-2′-(thio)diophosphate.
mol O$_2$/s/mol cytochrome b$_{559}$ heme in the presence of p47$^{phox}$ and 101.9 ± 5.9, in its absence (means ± S.E. of three experiments, for each group). The corresponding EC$_{50}$ values were 145.7 ± 23.9 and 111.7 ± 24.0 nm chimera 3, respectively. The oxidase-activating ability of prenylated chimera 3 in the absence of amphiphile was superior to that of its nonprenylated equivalent assayed in the presence of the amphiphile lithium dodecyl sulfate (26). It also differed from nonprenylated chimera 3 in native form (open circles) or absence (open circles) of p47$^{phox}$ (300 nM). Also shown are the effects of supplementing chimera 1 with equimolar amounts of p47$^{phox}$ (open squares) or Rac1-GDP$^p$S (open triangles). C, oxidase activation by prenylated chimera 1 in native form (open circles) or absence (open circles) of p47$^{phox}$ (300 nM). Also shown are the effects of supplementing chimera 1 with equimolar amounts of nonprenylated Rac1-GTP$^p$yS (open squares) or Rac1-GDP$^p$S (open triangles). D, oxidase activation by prenylated chimera 8 exchanged to GTP$^p$S (0–800 nM), in the presence (filled circles) or absence (open circles) of p47$^{phox}$ (300 nM). Also shown are the effects of supplementing chimera 1 with equimolar amounts of nonprenylated Rac1-GTP$^p$yS (open squares) or Rac1-GDP$^p$S (open triangles). The assay conditions in B–D were as described for A. The results in all panels are the means ± S.E. of three experiments.

Fig. 1. Prenylated p67$^{phox}$-Rac1 chimeras used in the present report. A, schematic representation of prenylated p67$^{phox}$-Rac1 chimeras. The numbers in the rectangles indicate the residues in the native sequences of p67$^{phox}$ and Rac1, representing the N- and C-terminal limits of the segments incorporated in the chimeras. The geranylgeranyl tail is drawn schematically. B, SDS-PAGE analysis of chimeras prenylated in vitro. Following prenylation, the chimeras were subjected to phase partition in Triton X-114. Aliquots from the aqueous phase (P), containing the prenylated fraction (the sums of the amounts of protein applied to lanes labeled NP and P, for each chimera, were 3–7 μg), were subjected to SDS-PAGE. The first lane shows molecular weight markers (Dalton Mark VII-L, Sigma) with $M_r$ × 10$^3$ values indicated to the left of the lane.

Fig. 2. Amphiphile-independent NADPH oxidase activation by prenylated p67$^{phox}$-Rac1 chimeras. A, oxidase activation in a cell-free system consisting of membrane (equivalent to 5 nm cytochrome b$_{559}$ heme) and varying concentrations (0–800 nm) of prenylated chimera 3 exchanged to GTP$^p$S (circles), prenylated chimera 3 in the native form (squares), or nonprenylated chimera 3 (triangles). For comparison, we measured oxidase activation by mixtures of equimolar amounts of p67$^{phox}$ (1–212) and Rac1 prenylated in vitro, exchanged to GTP$^p$S (diamonds). The assays were in the absence of amphiphile and in the presence (filled symbols) or absence (open symbols) of p47$^{phox}$ (300 nM). B, oxidase activation by prenylated chimera 2 in native form (0–800 nm), in the presence (filled circles) or absence (open circles) of p47$^{phox}$ (300 nm). Also shown are the effects of supplementing chimera 2 with equimolar amounts of nonprenylated Rac1-GTP$^p$yS (open squares) or Rac1-GDP$^p$S (open triangles). C, oxidase activation by prenylated chimera 1 in native form (0–800 nm), in the presence (filled circles) or absence (open circles) of p47$^{phox}$ (300 nm). Also shown are the effects of supplementing chimera 1 with equimolar amounts of nonprenylated Rac1-GTP$^p$yS (open squares) or Rac1-GDP$^p$S (open triangles). D, oxidase activation by prenylated chimera 8 exchanged to GTP$^p$S (0–800 nm), in the presence (filled circles) or absence (open circles) of p47$^{phox}$ (300 nm). The assay conditions in B–D were as described for A. The results in all panels are the means ± S.E. of three experiments.
unable to activate the oxidase under conditions in which chimera 3 was markedly active. These results can be interpreted as indicating that the role of Rac is more than just a carrier for p67phox and that a region upstream of residue 178, missing in chimeras 1 and 2, is required for oxidase activation. The insert region of Rac1 was proposed to play such a role by direct interaction with cytochrome b559 (20, 21). To test this hypothesis, we examined the oxidase-activating ability of a mutant of chimera 3 (chimera 8), in which the insert region of Rac1 was deleted. The nonprenylated form of chimera 3 (chimera 8), in which the insert region of Rac1 was proposed to play such a role by direct interaction with cytochrome b559, was equal in activity to chimera 3, when the Ras segment was in GTP-bound form (32). Intra-chimeric bonds were also reported to exist in Ras-Raf1 chimera 3-GTPS, exchanged to GTP-H9253, Rac1-GTPS, and Rac1-GTPS + nonprenylated Rac1-GTPS, and recently direct evidence for this, based on mutating residues involved in p67phox-Rac1 interaction (31), emerged. 2 Intramolecular bonds were also reported to exist in Ras-Raf1 chimeras, when the Ras segment was in GTP-bound form (32). To test this possibility, we supplemented prenylated chimeras 1 and 2 with equimolar concentrations of nonprenylated Rac1, exchanged to GTPS, or prenylated chimeras 2 and 1, and the mixtures were injected in a Superose 12 fast protein liquid chromatography gel filtration column. We measured the recruitment of chimeras to the membrane (A) and the ability of membrane-bound chimeras to activate the oxidase (B). A, the elution volume of membrane vesicles, indicated by the presence of cytochrome b559, and binding of chimeras to these vesicles were determined as described under “Experimental Procedures.” The curves represent the membrane marker cytochrome b559 (open diamonds) and membrane-bound chimeras 3 (open circles), 2 (open squares), and 1 (open triangles). B, oxidase activation by membrane-bound chimeras was measured by the ability of 50-μl aliquots of column fractions to produce O2 upon the addition of 240 μM NADPH in the absence of amphiphile. The curves represent oxidase activation by chimeras 3 (filled circles), 2 (filled squares), and 1 (filled triangles). The figure illustrates one representative experiment of three performed. The table at the bottom summarizes the relationship between characteristics of the chimeras and oxidase-activating ability.

2 R. Sarfstein, Y. Gorzalczany, and E. Pick, manuscript in preparation.
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propose that Rac1-GTP/S, but not Rac1-GDP/S, interacts with the p67phox (1-212) segment of chimeras 1 or 2, forming tripartite complexes (Fig. 3, B and C). Extrachimeric interaction of chimeras 1 and 2 with Rac1-GTPYS mimics the intrachimeric bonds between the Rac1 and p67phox segments in prenylated chimera 3 (Fig. 3A). The binding of exogenous Rac1-GTP/S induces a conformational change in the activation domain of the p67phox segment, which is essential for a productive interaction with cytochrome b550. The Rac-induced conformational change in p67phox is, so far, hypothetical; no difference in the conformation of free (17) and Rac-associated (31) p67phox was detected in structural studies, but the latter employed p67phox truncated at residue 203 and therefore lacked most of the activation domain, including the crucial residue 204 (16). A further condition for oxidative activation is the association of the chimeras with the membrane. The success of chimera 2, as opposed to the failure of chimera 1, to activate in the presence of exogenous Rac1 is best explained by the requirement for two signals for recruitment to the membrane: prenylation and a C-terminal polybasic domain. A “two signals” requirement for membrane localization of prenylated Rac was proposed (24) and demonstrated recently by the use of a Rac1 mutant lacking residues 183–188 (33). The particular roles of Rac and p67phox in oxidative activation, as revealed by this and an earlier study (26), bear a striking resemblance to those of Ras and Raf1.

Prenylated p67phox-Rac1 Chimeras Associate Spontaneously with Phagocyte Membranes—The ability of prenylated chimera 3 to elicit oxidative activation upon addition to phagocyte membranes in the absence of p47phox and amphiphil is most readily explained by its spontaneous association with the membrane and the formation of a complex with cytochrome b550. We tested this hypothesis directly by quantifying the binding of prenylated chimera 3-GTP-S and of native chimeras 2 and 1 to phagocyte membrane vesicles by gel filtration on Superose 12. This was based on the finding that membrane vesicles elute in the exclusion volume of the column (7.5 ml), whereas free chimeras elute at 12.9–14.1 ml.3 As seen in Fig. 4A, chimeras 3 and 2 bound to membrane vesicles with similar affinity, as shown by their detection in close correlation with the cytochrome b550 marker; a small amount of chimera 1 was also found associated with the membrane. The mere addition of NADPH to fractions containing membrane-associated chimera 3 led to O2 production (Fig. 4B); only minimal O2 production by fractions containing membrane-bound chimera 2 and no O2 production by fractions containing the small amount of membrane-bound chimera 1 were detected. These findings are in agreement with the direct assessment of the oxidase-activating ability of the chimeras (Fig. 2), with one exception: membrane-bound chimera 2 did exhibit a low level of activity in the gel filtration assay that was not detectable in the regular cell-free assay. The results provide further support for our proposal that oxidative activation by prenylated p67phox-Rac1 chimeras depends on the fulfillment of two conditions: high affinity membrane association resting on two signals and the presence of intrachimeric bonds between the p67phox and Rac segments of the chimera (Fig. 4, bottom panel).

This is, to the best of our knowledge, the first report of a single recombinant protein capable of NADPH oxidase activation in vitro, in the absence of any other oxidase component

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A Prenylated p67<sub>phox</sub> -Rac1 Chimera Elicits NADPH-dependent Superoxide Production by Phagocyte Membranes in the Absence of an Activator and of p47<sub>phox</sub>: CONVERSION OF A PAGAN NADPH OXIDASE TO MONOTHEISM
Yara Gorzalczany, Nathalie Alloul, Natalia Sigal, Carolyn Weinbaum and Edgar Pick

J. Biol. Chem. 2002, 277:18605-18610.
doi: 10.1074/jbc.M202114200 originally published online March 14, 2002

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