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17. Consistent with other reports (*3*), nucleotide exchange activity was recovered more in the cytosol fraction than in the membrane fraction when phosphatase inhibitors of serine-threonine phosphatases or tyrosine phosphatases were added. The activities in both fractions were indistinguishable from each other in the elution profile on either the anion-exchange or gel filtration column. These activities may represent an identical exchange factor (M. J. Subuleski, H.-f. Kung, T. Kamata, unpublished data).
18. PC-12 or Trk–PC-12 cells were incubated without serum for 48 hours and treated with or without NGF (50 ng/ml) for 10 min. The cells were sonicated in 0.5 ml of Buffer A [20 mM 3-(*N*-morpholino)propanesulfonic acid) (pH 7.5), 1 mM $MgCl_2$, 0.1 mM EDTA, 1 mM dithiothreitol (DTT), 200 mM sucrose, 10 μM phenylmethylsulfonyl fluoride (PMSF), leupeptin (10 μg/ml), and pepstatin (1.7 μg/ml)] that contained phosphatase inhibitors as described (*3*). The homogenates were centrifuged at 100,000*g* for 20 min at 4°C, and the supernatants were used. Normal H-Ras expressed in *Escherichia coli* was purified (*26*), and Ras (0.25 μg) was incubated with 2 μM [^{3}H]GDP (10 to 15 Ci/mmol; Amersham) in 50 μl of the reaction buffer [25 mM tris-HCl (pH 7.5), 5 mM EDTA, 1 mM DTT, and bovine serum albumin (100 μg/ml)] for 30 min at 37°C. Cell extracts (3 μg) were added to the Ras-[^{3}H]GDP complex in the presence of 2 mM unlabeled GTP and 18 mM $MgCl_2$ at 37°C for 10 min. Ras was immunoprecipitated with the Y13-259 antibody, and the immune complexes were washed three times with washing buffer. Nucleotides were eluted, and the radioactivity was quantitated as described (Fig. 1). Activity is given as a percentage of bound [^{3}H]GDP dissociated. [^{3}H]GDP (15,000 cpm) was bound to Ras incubated with the control homogenization buffer. Data represent means ± SD (n = 3).
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21. We fractionated PC-12 cell homogenates by spinning them at 100,000*g*. By immunoblotting using the antibody to a peptide derived from NF1 (NF1 GRD, serum 31) (provided by D. R. Lowy), we detected NF1 protein in the particulate (P100) fraction but not in the cytoplasmic (S100) fraction, as reported (*27*). Because we used the cytoplasmic fraction (S100) for the GAP assay (Fig. 4), this indicates that our cytoplasmic extract preparations do not contain NF1. The extracts contained Ras-GAP as detected by immunoblotting. In another set of experiments, the particulate fractions (P100) from PC-12 cells were solubilized in lysis buffer and immunoprecipitated by the NF1 antibody. Although the immunoprecipitated NF1 exhibited the activity to stimulate Ras GTPase as reported (*27*), treatment of PC-12 cells by NGF did not enhance the GTPase-stimulating activity of NF1.
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28. We starved the cells of serum in medium that contained 0.2% horse serum and 0.1% fetal bovine serum to minimize potential effects of the various serum components on NGF action, which may have complicated the interpretation of the results.
29. We thank D. Longo for support, H. Young and G. Varesio for critical review, M. Subuleski for the Ras preparations, S. Rabin for assistance with GAP immunoprecipitation, and C. Schaff for the preparation of this manuscript. This project has been funded in part by the Department of Health and Human Services under contract N01-CO-74102 with Program Resources, Inc./DynCorp and under contract N01-CO-74101 with Advanced Bioscience Laboratory–Basic Research Program (D.K.).

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Cytochrome b_{558}: The Flavin-Binding Component of the Phagocyte NADPH Oxidase

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The phagocyte respiratory burst oxidase is a flavin-adenine dinucleotide (FAD)–dependent dehydrogenase and an electron transferase that reduces molecular oxygen to superoxide anion, a precursor of microbicidal oxidants. Several proteins required for assembly of the oxidase have been characterized, but the identity of its flavin-binding component has been unclear. Oxidase activity was reconstituted in vitro with only the purified oxidase proteins $p47^{phox}$, $p67^{phox}$, Rac-related guanine nucleotide (GTP)–binding proteins, and membrane-bound cytochrome b_{558}. The reconstituted oxidase required added FAD, and FAD binding was localized to cytochrome b_{558}. Alignment of the amino acid sequence of the β subunit of cytochrome b_{558} ($gp91^{phox}$) with other flavoproteins revealed similarities to the nicotinamide adenine dinucleotide phosphate (reduced) (NADPH)–binding domains. Thus flavocytochrome b_{558} is the only obligate electron transporting component of the NADPH oxidase.

Phagocytic white blood cells contain a multicomponent electron transfer chain that is responsible for production of microbicidal oxidants. To identify its proximal flavin and NADPH-binding component we explored the superoxide generating capacity of a cell-free system comprised solely of purified proteins. In this cell-free system, complete reconstitution of the oxidase required the presence of the cytosolic factors $p47^{phox}$, $p67^{phox}$, and Rac-related GTP-binding proteins (*1–3*), and the membrane component cytochrome b_{558} (*4–7*) (Fig. 1). In the optimally reconstituted purified protein system (*8*) the concentration of NADPH required for half maximal activity (30 to 40 μM, n = 2) was identical to that observed with the enzyme reconstituted from crude cytosol and membranes (30 to 40 μM, n = 3). This purified protein system produced superoxide only in the presence of added FAD (Fig. 2), the concentration of FAD required for half-maximal superoxide production was from 1 to 20 nM (n = 4). Neither $p47^{phox}$ nor $p67^{phox}$ bind flavin (*2*) and, on the basis of structural data from the Ras superfamily of low molecular weight GTP-binding proteins, binding of flavin to members of the Rac/G25K family also seemed unlikely. Accordingly, we focused our attention on cytochrome b_{558}.

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Relipidated cytochrome b_{558} was incubated with FAD and subjected to gel filtration to separate bound from free flavin (Fig. 3A). A small peak of flavin fluorescence coeluted with the purified cytochrome b_{558}, which was well resolved from the major peak of free flavin. The early eluting peak of flavin was not detected when mixtures of the purified cytosolic components plus FAD or phospholipid vesicles, alone, plus FAD were subjected to gel filtration.

A functional comparison of flavin-reconstituted or unflavinated cytochrome b_{558} was made in the cell-free assay with the purified cytosolic components in the pres-

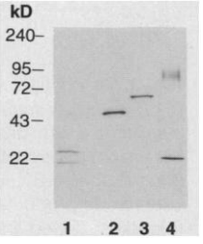

Fig. 1. Protein components of the NADPH oxidase [SDS–polyacrylamide gradient gel (8 to 16%) stained with Coomassie Blue R-250]. Lane 1, Superdex-75 peak fraction (4 μl) showing the 26-kD and 21-kD bands identified by microsequencing as rhoGDI and Rac-related GTP-binding proteins, respectively (*3*); lane 2, recombinant $p47^{phox}$ (1.5 μg); lane 3, recombinant $p67^{phox}$ (1.5 μg); and lane 4, cytochrome b_{558} (2.7 μg; 74 pmol). The specific content of peak cytochrome b_{558}–containing fractions (18 to 33 nmol of heme per milligram of protein) and the molar ratio of FAD to heme (<1:100) in three preparations of purified cytochrome b_{558} were comparable to reported values (*4–6*).

ence or absence of excess FAD added to the final assay mix (Fig. 3A). Activity of the FAD-incubated and gel-filtered cytochrome b_{558} ranged from 20 to 40% (n = 3) of the activity of the identical column fraction measured in the presence of excess FAD added to the final assay mix. In contrast, cytochrome b_{558} preparations gel-filtered without prior exposure to FAD had an activity <10% of that seen when excess FAD was added to the final assay mix (Fig. 3B). Thus, "reflavination" of cytochrome b_{558} restored, at most, only a third of the full potential of activity.

The near absolute requirement for added FAD in the purified protein system contrasted with the marginal increase in activity when FAD was added to a cell-free system containing purified cytosolic components and neutrophil membranes (Fig. 2B), suggesting that flavin was tightly bound to the native cytochrome b_{558}, but was released during purification. Alternatively, the native membrane could carry free flavin, which could become available to a soluble apoprotein during activation of the oxidase. The latter possibility was excluded by mixing experiments. Neutrophils of individuals with X-linked cytochrome b_{558}–deficient chronic granulomatous disease (CGD) lack a normal respiratory burst response. Membranes from these neutrophils were added, as a potential source of flavin, to a cell-free system that contained purified protein components, including normal cytochrome b_{558}. Inclusion of membranes from three such patients did not eliminate the requirement for exogenous FAD (Fig. 2B).

The initial steps in the purification of cytochrome b_{558} use detergents that effect a separation of flavin and heme (*9, 10*). Thus, weak binding of flavin to the purified cytochrome b_{558}, relative to its counterpart in native membranes, could reflect conformational changes in the cytochrome b_{558} that persist despite relipidation. Because full activity was seen when flavin was added to the assay during activation, we anticipated that binding of flavin might be stabilized by an induced fit between a cytosolic component and the relipidated cytochrome b_{558}. Support for such a mechanism comes from experiments in which assembly of the oxidase from purified protein components was initiated by arachidonate in the presence of excess FAD, followed by gel filtration to remove free flavin. Under these conditions, there was no requirement for additional FAD in the final assay mix (Fig. 3C).

Biological redox chains generally employ a flavoprotein intermediate to couple a reduced nicotinamide, a two-electron donor, to the strictly one-electron–carrying heme or iron-sulfur proteins (*11*). The crystal structures of several flavoproteins are known and the regions of their polypeptide chains that participate in binding flavin and NADP have been characterized. Regions of the β subunit of cytochrome b_{558} were aligned with five peptide segments that participate in nucleotide-binding in spinach ferredoxin-$NADP^+$ reductase (FNR), nitric oxide synthase (NOS), and cytochrome P-450 reductase (CPR) (*12–15*) (Fig. 4A). The basis for the conserved order and relative spacing of these peptide segments can be understood from the published crystal structure of the nucleotide-binding domains of FNR (*12, 13*). NADP is coordinated by hydrogen bonding to short peptide loops that emerge from the COOH-termini of five strands of a parallel β-sheet core. Thus, it is the loop segment amino acid composition and the spacing and coordinated alignment of the loops relative to each other that are important for binding NADP.

Alignment of the amino acid sequence

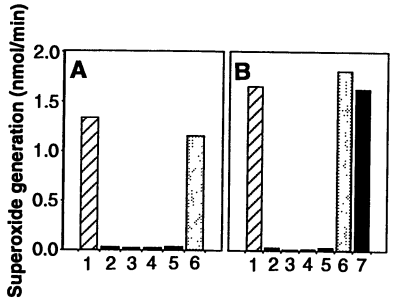

Fig. 2. Protein and FAD requirements of the cell-free superoxide generating system. (**A**) Reactions contained: lane 1, recombinant $p47^{phox}$ (1.5 μg), recombinant $p67^{phox}$ (1.5 μg), rhoGDI/Rac–related GTP-binding proteins (6 μl of Superdex-75 peak column fraction), and relipidated cytochrome b_{558} (0.5 pmol) in buffer containing 10 μM FAD. In lanes 2 to 5 individual protein components were omitted from the reaction: lane 2, without $p47^{phox}$; lane 3, without $p67^{phox}$; lane 4, without rhoGDI/Rac–related GTP-binding proteins; and lane 5, without cytochrome b_{558}. In lane 6 relipidated cytochrome b_{558} was replaced with 2.5×10^5 cell equivalents of deoxycholate-solubilized neutrophil membranes (*8, 20*) containing 0.4 pmol of cytochrome b_{558}. The oxidase was activated by addition of 30 μM arachidonate and superoxide generation was measured (*20*). (**B**) Reactions contained: lane 1, recombinant $p47^{phox}$ (1.5 μg), recombinant $p67^{phox}$ (1.5 μg), and Rac-related proteins (6 μl of Superdex-75 peak column fraction) and relipidated cytochrome b_{558} (0.5 pmol) in buffer containing 10 μM FAD. In lanes 6 and 7 relipidated cytochrome b_{558} was replaced with 2.5×10^5 cell equivalents of deoxycholate-solubilized neutrophil membranes containing 0.4 pmol of cytochrome b_{558}. FAD was omitted from lanes 2 to 5 and 7; in lanes 3 to 5 cytochrome b_{558}–deficient neutrophil membranes from three patients with X-linked CGD (2.5×10^5 cell equivalents per well) were added as a potential source of free flavin. Data in (A) and (B) are means of triplicate wells in a single experiment representative of two and three similar experiments, respectively. Standard deviations were <10% of the mean values shown.

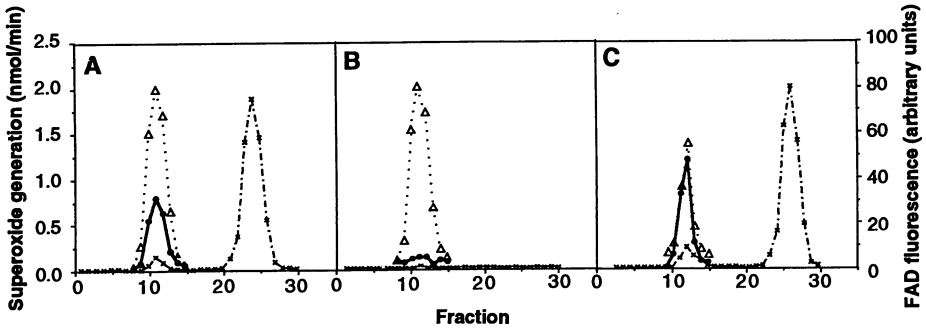

Fig. 3. Reconstitution of flavin binding to cytochrome b_{558}. (**A** and **B**) Relipidated cytochrome b_{558} (*5*) (50 μl, 20 pmol) was incubated (30 min, 4°C) with 1 μM FAD (A) or with buffer alone (B) and applied to a 2-ml desalting column (excellulose GF-5, Pierce Chemical Co., equilibrated with 75 mM potassium phosphate, pH 7, 4 mM $MgCl_2$, 1 mM EGTA) to separate bound from free flavin. Fractions (four drops, ≈80 μl) were collected and aliquots (20 μl) of each fraction were assayed for superoxide production by complementation with purified cytosolic components (*8*) in the presence (triangles, dotted line) or absence (circles, solid line) of 10 μM FAD added to the final assay mix. An aliquot (20 μl) of each fraction was diluted with two volumes of 0.15% Triton X-100 in 0.15N HCl, heated for 3 min in a boiling water bath, and assayed for FAD (x's, dashed line) by fluorescence spectroscopy (*28*). The threshold for detection of flavin was <1 nM. (**C**) Relipidated cytochrome b_{558} (10 μl, 2 pmol) was mixed with $p47^{phox}$ (1.5 μg), $p67^{phox}$ (1.5 μg), and rhoGDI/Rac–related GTP-binding proteins (6 μl of peak Superdex-75 column fraction) and brought to a final volume of 50 μl with 75 mM potassium phosphate, pH 7, 4 mM $MgCl_2$, 1 mM EGTA +/− 1 μM FAD. Assembly of the oxidase was initiated by addition of arachidonate (80 μM) and the mixture was immediately applied to a 2-ml desalting column and fractionated, as above. Aliquots (20 μl) of each fraction were assayed for FAD (x's, dashed line) and for superoxide production initiated by addition of 200 μM NADPH in the presence (triangles, dotted line) or absence (circles, solid line) of 10 μM FAD added to the final assay mix.

of gp91^{phox} reveals similarities to these loop regions in the FNR family. The glycine-rich loop of FNR (beginning at Met^{167}) hydrogen bonds to the pyrophosphate moiety of NADP and contains the sequence motif, Gly-Xaa-Gly-Xaa-Xaa-Pro, which is structurally similar to the canonical dinucleotide binding helix, Gly-Xaa-Gly-Xaa-Xaa-Gly (*16, 17*). In FNR, the conserved Pro^{176} lies in the cleft between the NADP and flavin-binding domains, where it approaches the flavin ring involved in electron transfer. The corresponding region of gp91^{phox} (beginning at Leu^{406}) contains the identical sequence motif; the equivalent residue of gp91^{phox}, Pro^{415}, is replaced by His^{415} in an individual with an atypical form of chronic granulomatous disease due to a nonfunctional cytochrome b_{558} (*18*).

In FNR the nearby Val^{204} hydrogen bonds to the NADP ribose moiety and the amino acid composition of the corresponding region of the cytochrome resembles that of the FNR family members, particularly CPR. In the next loop region of FNR, Tyr^{246} hydrogen bonds to the nucleotide ribose 2′-phosphate and the spacing between this invariant tyrosine and the downstream cysteine-glycine couplet (Cys^{272}-Gly^{273} of FNR) is conserved in FNR family members and in gp91^{phox}. In FNR, Cys^{272} approaches the nicotinamide C4 atom that may participate in hydride transfer to the flavin. It is likely that these spatial relationships are perturbed in an unusual patient with chronic granulomatous disease due to a nonfunctional cytochrome b_{558} (*19*). This patient has an in-frame deletion that predicts a protein lacking ten amino acids (Ala^{488}-Glu^{497}) in an adjacent region of gp91^{phox}.

An aromatic amino acid is the final or penultimate residue in COOH-termini of the FNR family members. Tyr^{314} of FNR may occupy the nicotinamide binding pocket in the absence of NADP, maintaining the enzyme structure and protecting the flavin (*12*). If the equivalent residue of gp91^{phox}, Phe^{570}, is similarly positioned, then it could regulate the oxidase by serving as a pseudosubstrate; p47^{phox} probably has a functional interaction with the COOH-terminus of gp91^{phox} during activation of the burst (*20*).

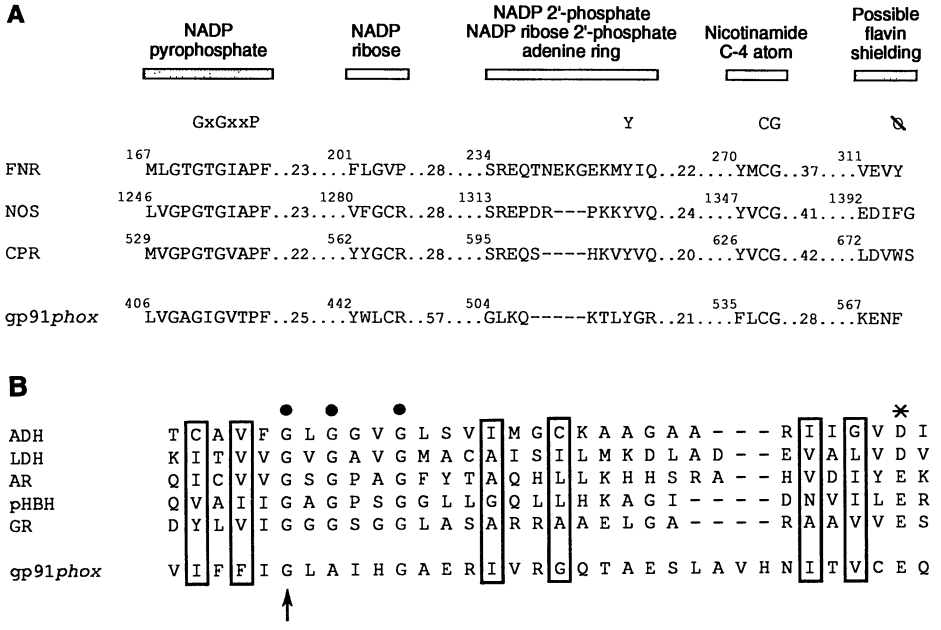

Fig. 4. (A) Comparison of the amino acid sequence of gp91^{phox} and NADP-binding domains of spinach ferrodoxin NADP$^+$ reductase (FNR) and related flavoproteins. Peptide sequences corresponding to nucleotide-binding loops identified in the crystal structure of FNR (*12, 13*) are aligned with the corresponding regions of the FNR family members nitric oxide synthase (NOS) and cytochrome P-450 reductase (CPR). The integers between peptide segments indicate the number of intervening amino acid residues not shown; uppercase numbers refer to the positions of the residues at the NH_2-terminus of each peptide segment. In FNR, residues in the loop regions hydrogen bond to or approach the nucleotide at the positions on the nucleotide indicated above the horizontal bars. Residues or sequence motifs that are important in nucleotide binding in FNR are indicated above the FNR sequence. Regions of gp91^{phox} that share a similar spacing and many of the structural elements that are responsible for nucleotide binding to FNR are discussed in the text. **(B)** Alignment of gp91^{phox} residues Val^{213} to Gln^{246} with amino acid residues comprising the NAD- and FAD-binding folds of alcohol dehydrogenase (ADH), lactate dehydrogenase (LDH), adrenodoxin reductase (AR), *p*-hydroxybenzoate hydroxylase (*p*HBH), and glutathione reductase (GR) (*17, 26*). Features that characterize the "fingerprint sequence" of the prototypical nucleotide binding βαβ-fold (*17*) are boxed or indicated by symbols and discussed in the text.

Thus, there are significant sequence correlations between the NADP binding domains of FNR family members and candidate regions of gp91^{phox}. Regions equivalent to the FAD binding domain of FNR are not apparent in gp91^{phox}. However, the region of gp91^{phox} at Gly^{218}-Leu-Ala-Ile-His-Gly (Fig. 4B, arrow) resembles the canonical dinucleotide binding helix, Gly-Xaa-Gly-Xaa-Xaa-Gly, which forms hydrogen bonds to the dinucleotide pyrophosphate in the context of a compact βαβ-fold (*16, 17*). Models of secondary structure according to Chou-Fasman and Garnier-Osguthorpe-Robson predict a βαβ-fold in this region of gp91^{phox} (*21*). Structural features are conserved in these compact loop regions and a characteristic "fingerprint" for the amino acid sequence of the dinucleotide binding βαβ-fold has been deduced (*16, 17*). Three glycines (Fig. 4B, solid circles) within the sequence motif, Gly-Xaa-Gly-Xaa-Xaa-Gly connect the first β-strand and the α-helix. Only the first of these glycines is strictly conserved, as the absence of a side chain at this position is essential for close approach of the dinucleotide. Substitution of alanine for either of the two remaining glycines, as occurs in gp91^{phox}, has been noted infrequently in other members of this family (*17, 22*). Six conservatively spaced, predominantly hydrophobic residues (boxed in Fig. 4B) that form the core of the βαβ-unit are present in this region of gp91^{phox}. A conserved acidic residue (glutamic acid in FAD-binding folds and aspartic acid in NAD-binding folds) that hydrogen bonds to the adenine ribose 2′-hydroxyl (*16*) is located near the COOH-terminus of the second β-strand (Fig. 4B, asterisk).

Taken together, our functional studies obviate a requirement for any of several candidate flavoproteins that were thought to be components of the oxidase based on inhibitor and affinity labeling studies (*23*) or based on their ability to transfer electrons from NADPH to artificial acceptors in the absence of cytochrome b_{558} (*24*). Our findings are, however, entirely consistent with the close correlation between the flavin and cytochrome b_{558} content of the neutrophil-specific granule-enriched fraction and the virtual absence of flavin from this subcellular fraction in cytochrome b_{558}–deficient chronic granulomatous disease (*10*). Our data define the essential elements of a model system of the oxidase in which the α- and β-subunits of cytochrome b_{558} together incorporate the NADP-binding site and both the FAD and heme electron transfer moieties.

Note added in proof: Cytochrome b_{558} was found by Segal (*25*) to bind FAD and analogs of NADPH.

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3. Recombinant $p47^{phox}$ and recombinant $p67^{phox}$ were produced in Sf9 insect cells (*2*). A third cytosolic factor required for reconstitution of the oxidase was purified by fast protein liquid chromatography (FPLC) based on the ability of column fractions to support neutrophil membrane- and arachidonate-dependent oxidase activity in the presence of $rp47^{phox}$ and $rp67^{phox}$. Neutrophil cytosol was subjected to four purification steps: fractionation by ammonium sulfate precipitation, hydrophobic interaction chromatography on phenyl 5PW, ion exchange on DEAE Sepharose, and gel filtration on Superdex-75. The purified factor consisted of bands of 26 kD and 21 kD on SDS–polyacrylamide gel electrophoresis (PAGE). Tryptic digests of the resolved bands yielded peptides matching the cDNA predicted sequences of rho guanine nucleotide dissociation inhibitor (rhoGDI), and Rac-related low molecular weight GTP-binding proteins (C. Kwong *et al.*, manuscript in preparation).
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7. Cytochrome b_{558} was purified from Triton N-101–solubilized human neutrophil membranes (*20*) essentially as described (*4*). The purified cytochrome b_{558} was diluted in 20% glycerol containing 1% Triton N-101, readsorbed to heparin agarose for detergent exchange into 40 mM octylglucoside, and eluted with a 0 to 1 M NaCl gradient in the 20-fold diluted buffer A (*4*) containing octylglucoside. Aliquots were reserved for SDS-PAGE and for determination of protein (BCA assay, using bovine serum albumin as standard), cytochrome b_{558} content [based on an extinction coefficient (559 to 540 nm) of 21.6 mM^{-1} cm^{-1} (*27*) for the dithionite-reduced minus oxidized spectrum], and FAD content by fluorescence spectroscopy (*28*). The remaining cytochrome b_{558} (≈1 to 2 μM) was relipidated (*5*) by addition of soybean phosphatidylcholine to a final concentration of 1 mg/ml from a tenfold concentrated stock solution prepared in 40 mM octylglucoside. The cytochrome-phosphatidylcholine mixture was diluted ≥fivefold with 120 mM Na phosphate (pH 7.4), 1 mM $MgCl_2$, 1 mM EGTA, 1 mM dithiothreitol, 20% glycerol, maintained on ice for ≥30 min, and stored at −70°C. The only protein contaminant consistently seen in over ten preparations of purified cytochrome b_{558} was a 59- to 60-kD doublet that eluted at a higher salt concentration than the single fraction that contained the peak oxidase activity and peak cytochrome b_{558} content. The cytochrome b_{558} in peak fractions was >98% pure based on titrations of bovine serum albumin and carbonic anhydrase standards to estimate the threshold for visualization of contaminant bands by SDS-PAGE. The low molecular weight GTP-binding protein Rap1a did not copurify with cytochrome b_{558} under these conditions as determined by immunoblotting with monoclonal antibody 142/24E5, which detects 3 ng of recombinant Rap1a (D. Rotrosen, unpublished data).
8. Cell-free superoxide generation assays were performed in a 96-well microtiter plate in a final volume of 100 μl (*20*). The standard reaction contained 10^6 cell equivalents of neutrophil cytosol that was replaced, as indicated, with a mixture of recombinant $p47^{phox}$ (1.5 μg), recombinant $p67^{phox}$ (1.5 μg), and Rac-related proteins (2 to 6 μl of Superdex-75 peak column fraction). These represent saturating amounts of the recombinant proteins and a near-saturating amount of Rac-related proteins. Deoxycholate-solubilized neutrophil membranes [2.5 to 5.0 × 10^5 cell equivalents per well (*20*)] were replaced, as indicated, with relipidated cytochrome b_{558} (0.5 to 2.0 pmol/well, within the linear range of the dose-response for the cytochrome). The reaction mixture contained 4 mM $MgCl_2$, 1.25 mM EGTA, 200 μM NADPH, 10 μM GTPγS, 30 to 40 μM arachidonic acid, 200 μM acetylated ferricytochrome c, and 10 μM FAD in 75 mM potassium phosphate, pH 7. In Figs. 2B and 3, FAD was omitted, as indicated. Superoxide generation was measured by following the rate of superoxide dismutase inhibitable reduction of acetylated ferricytochrome c at 550 nm in a kinetic microplate reader (*20*).
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