Binding of Nicotinamide Adenine Dinucleotide Phosphate to the Tetratricopeptide Repeat Domains at the N-Terminus of p67^{PHOX}, a Subunit of the Leukocyte Nicotinamide Adenine Dinucleotide Phosphate Oxidase†

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Received August 24, 1999; Revised Manuscript Received December 23, 1999

ABSTRACT: The nicotinamide adenine dinucleotide phosphate (NADPH) binding site of the NADPH oxidase complex is believed to be located on the β, subunit of cytochrome b_{558}. However, our previous studies showed that p67^{PHOX} also contains an NADPH binding site that is essential for normal oxidase activity and that p67^{PHOX} is able to mediate a slow electron transfer from a reduced pyridine nucleotide to an artificial electron acceptor. Using both affinity labeling and fluorescence quenching, we have obtained further evidence that p67^{PHOX} is able to bind NADPH. We have used a number of truncated forms of p67^{PHOX}, including p67^{PHOX}(1−243), p67^{PHOX}(1−210), p67^{PHOX}(1−199), and p67^{PHOX}(244−526) (where the numbers represent the initial and final amino acids in the truncated p67^{PHOX}) in order to localize the binding site. We found that NADPH could bind to p67^{PHOX}(1−243), p67^{PHOX}(1−210), and p67^{PHOX}(1−199) but not to p67^{PHOX}(244−526). The p67^{PHOX}(1−199) fragment consists largely of four tetratricopeptide (TPR) domains. We showed further that Rac2-GTPγS and to a lesser extent Rac2-GDPβS could modulate the binding of NADPH to p67^{PHOX}.

The nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is a multicomponent enzyme system found in professional phagocytes and B lymphocytes that catalyzes the one-electron reduction of oxygen to O_2^- at the expense of NADPH:

$$2O_2 + NADPH \rightarrow 2O_2^- + NADP^+ + H^+$$

The O_2^- generated by this enzyme is the precursor of potent oxidants that are used by phagocytes to kill invading microorganisms (*1*−*5*). The importance of the NADPH oxidase in human host defenses is shown by patients with chronic granulomatous disease, an immunodeficiency syndrome characterized clinically by severe recurrent bacterial and fungal infections (*6*, *7*). In resting cells, the NADPH oxidase exists in an inactive state, with its components distributed between the cytosol and membranes. Upon exposure of cells to soluble or particulate stimuli, the enzyme acquires catalytic activity and the cytosolic components (p47^{PHOX}, p67^{PHOX}, p40^{PHOX}, and Rac2) migrate to the membranes, where they associate with the membrane-bound components (gp91^{PHOX} and p22^{PHOX}, which together comprise cytochrome b_{558}) to assemble the catalytically active oxidase (*8*−*12*).

Many studies have been carried out in an attempt to understand the function of the individual components of the enzyme. Cytochrome b_{558} contains two hemes, binds flavin adenine dinucleotide (FAD), and possesses a putative consensus sequence for NADPH (*13*, *14*). It is thought to be the final carrier in the electron transport pathway. Rac was shown to be absolutely required for the activation of the NADPH in a cell-free system (*15*, *16*). Its action probably requires its binding to p67^{PHOX} (*17*, *19*). The cytosolic proteins p47^{PHOX} and p67^{PHOX} are thought to interact with cytochrome b_{558} so as to induce a conformational change that permits the start of the electron flow in the flavocytochrome. More precisely, p47^{PHOX}, which is extensively phosphorylated during the activation of the oxidase (*20*−*23*), probably initiates the assembly of the enzyme, while the function of p67^{PHOX} would be to activate catalysis by cytochrome $b_{558,}$ as suggested by two studies demonstrating that NADPH oxidase could be activated by exposing cytochrome b_{558} to high concentrations of p67^{PHOX} and Rac in the absence of p47^{PHOX} (*24*, *25*). Furthermore, a p67^{PHOX} domain (residues 199−210) that could be involved in the activation of cytochrome b_{558} has been described recently (*26*).

Our previous studies showed that p67^{PHOX} was able to bind NADPH and to catalyze pyridine nucleotide dehydrogenation (*27*), suggesting an active role for this subunit in the regulation of electron transfer. In the present study, we show that NADPH binds to the N-terminal fragments p67^{PHOX}-(1−243), p67^{PHOX}(1−210), and p67^{PHOX}(1−199) but not to the C-terminal fragment p67^{PHOX}(244−526). We also show that the activity of the N-terminal p67^{PHOX}(1−243) fragment can be eliminated by NADPH 2′,3′-dialdehyde, an analogue

† Supported in part by USPHS Grant AI-24227 and RR-00833 and the Stein Endowment Fund.

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‡ Recipient of a postdoctoral fellowship from the Arthritis Foundation; postdoctoral fellow from INSERM U479, CHU Xavier Bichat, Paris, France.

[1] Abbreviations: GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); GDPβS, guanosine 5′-*O*-(3-thiodiphosphate); GST, glutathione-S-transferase.

10.1021/bi9919807 CCC: $19.00
Published on Web 02/22/2000

of NADPH that acts as an affinity label. In addition, we report that Rac2-GTPγS and to a lesser extent Rac2-GDPβS could modulate the binding of NADPH to p67^{PHOX}. These findings support the idea of a physiological role for NADPH binding to p67^{PHOX}.

MATERIALS AND METHODS

Materials. Cytochrome *c*, NADPH, FAD, GTPγS, GDPβS, DL-lysine, isocitric acid, isocitric dehydrogenase, NADP dialdehyde, and $NaCNBH_3$ were obtained from Sigma Chemical Co. (St Louis, MO). Isopropyl β-D-thiogalactoside was from Gibco−BRL (Grand Island, NY) and SDS was from BioRad (Hercules, CA). Na[^{3}H]BH_4 was obtained from Amersham Life Science Inc. (Arlington, IL). Cell cultures were grown in EX401 medium purchased from JRH Bioscience (Woodland Hills, CA).

Preparation of Plasma Membrane and Recombinant Proteins. Human neutrophils were isolated from peripheral blood from healthy donors as described previously (*11*). Plasma membrane were prepared as described elsewhere (*28*). Recombinant p67^{PHOX} was expressed in Sf9 cells and purified as described before (*27*). p47^{PHOX} and Rac 2 were expressed in *Escherichia coli* as the glutathione-S-transferase fusion protein and purified with glutathione−Sepharose beads (Amersham Pharmacia Biotech, Piscataway, NJ) followed by thrombin cleavage (*27*). p47^{PHOX} was further purified on a HiTrap SP column (Amersham Pharmacia Biotech, Piscataway, NJ). Truncated forms of p47^{PHOX} were expressed in *E. coli* as GST fusion proteins, purified with glutathione−Sepharose beads, and then separated from GST while on the beads by cleavage with PreScission protease (Amersham Pharmacia Biotech, Piscataway, NJ). Protein concentrations were determined with the BioRad assay kit with bovine serum albumin as a standard.

Construction of Truncated p67^{PHOX} Forms. p67^{PHOX}(1−243), p67^{PHOX}(1−210), p67^{PHOX}(1−199), and p67^{PHOX}(244−526) were obtained by PCR with *Pfu* polymerase (Stratagene) and wild-type p67^{PHOX} DNA cloned into the vector pBK-CMV. The cDNA sequence encoding residues 1−243 of p67^{PHOX} was amplified by use of a 5′ primer (GGGGGAATTCATGTCCCTGGTGGAGGCCATCAGCCTCTGGAATGAAGGG) containing a sequence that anneals to nucleotides 1−39 and a 3′ antisense primer (TCCCCTTCCAGAGCCCTGAAGATCTCTGGGG) that annealed to nucleotides 696−728. The PCR product was cloned into the *Sma*I site of pGEX-6P-1 (Pharmacia), a maneuver that regenerated the glutamate residue at position 243. The stop codon is supplied by the pGEX-6P-1 vector downstream from the polylinker.

The cDNA sequences encoding residues 1−210 and 1−199 of p47^{PHOX} were amplified by use of a 5′ primer (CGTGGATCCATGTCCCTGCTGGAGGCC) that contained a *Bam*HI site (underlined) and a sequence that annealed to nucleotides 1−18 and a 3′ antisense primer designed to anneal to the p67^{PHOX} sequence immediately 5′ to the region to be truncated (for 1−210, GATGAATTC**TTA**ATCCACCACAGATGCCAC; for 1−199, GATGAATTC**TTA**TAGGTAATCCTTCTT). The 3′ antisense primer was also designed to introduce the ochre stop codon (boldface type) and an *Eco*RI site (underlined). The PCR products were ligated into the *Bam*HI and *Eco*RI sites of the PGEX-6P1 vector (Amersham Pharmacia Biotech, Piscataway, NJ) and transformed into DH5α, for expression of the protein.

The cDNA sequence encoding residues 244−526 of p67^{PHOX} was amplified by use of a 5′ primer (GGAATTCCGCTCACCGTGTGCTATTT) that contained an *Eco*RI site (underlined) and a sequence that annealed to nucleotides 730−748, and a 3′ antisense primer (TGAGCCTCTCTTCAGATCCGGGCGAGCTCGCC) that contained an *Xho*I site (underlined), an antisense sequence that annealed to nucleotides 1564−1578, and the antisense stop codon. The PCR products were ligated into the *Eco*RI and *Xho*I sites of the PGEX-6P3 vector (Amersham Pharmacia Biotech, Piscataway, NJ) and transformed into DH5α for expression of the protein. The sequences constructs were all confirmed by DNA sequencing in the Scripps Research Institute molecular biology facility.

cDNAs encoding shorter truncated forms of p67^{PHOX}, containing residues 1−155, 1−105, 1−71, and 1−35 were also constructed but we were unable to express the polypeptides in *E. coli*.

Synthesis of NADPH Dialdehyde. NADPH dialdehyde was prepared by reduction of commercial NADP dialdehyde with isocitric acid dehydrogenase followed by the separation of NADPH dialdehyde on a MonoQ column (Amersham Pharmacia Biotech, Piscataway, NJ) with a 0−1 M KCl gradient in 0.02 M Hepes buffer, pH 7.4, according to Smith et al. (*29*). For calculating NADPH dialdehyde concentration, ϵ_{340} was taken as 6.22 mM^{-1}. Aliquots of NADPH dialdehyde were stored at −70 °C until use.

Labeling of Recombinant p67^{PHOX}, N-Terminal p67^{PHOX} Fragments p67^{PHOX}(1−243), p67^{PHOX}(1−210), and p67^{PHOX}(1−199), and the C-Terminal Fragment p67^{PHOX}(244−526) with NADPH Dialdehyde and Sodium Boro[^{3}H]hydride. Labeling of recombinant p67^{PHOX}, N-terminal p67^{PHOX} fragments p67^{PHOX}(1−243), p67^{PHOX}(1−210), and p67^{PHOX}(1−199), and the C-terminal fragment p67^{PHOX}(244−526) was performed by incubating 1.5 nmol of each protein with 0.1 mM NADPH dialdehyde with or without added NADPH (40 mM final concentration) in 0.1 M sodium borate buffer, pH 9.4, followed by the addition of 200 μCi of Na[^{3}H]BH_4 and 20 mM (final concentration) DL-lysine in a final volume of 0.1 mL. After 3 h of incubation at 4 °C, proteins were precipitated with 0.8 mL of acetone for 1 h at −20 °C. The labeled precipitates were washed extensively with acetone and then resuspended in 100 μL of H_2O. The labeled proteins were then analyzed by SDS−PAGE followed by autoradiography. The radioactivity of tritium was amplified by soaking the gel in a fluorographic reagent (Amplify, Amersham).

The effect of Rac2 on the labeling of p67^{PHOX} by NADPH dialdehyde was determined by preincubating p67^{PHOX} with an equimolar amount of Rac2-GTPγS or Rac2-GDPβS for 30 min at 4 °C prior to adding NADPH dialdehyde and Na[^{3}H]BH_4. Rac2 was preloaded with GTPγS or GDPβS by incubation with 25 mM EDTA and 100 μM GTPγS or GDPβS for 15 min at room temperature, and then $MgCl_2$ was added to an excess of 5 mM.

Inactivation of p67^{PHOX} and the N-Terminal Fragment p67^{PHOX}(1−243) by NADPH Dialdehyde. Each protein (1.5 nmol) was incubated with 200 μM NADPH dialdehyde in the presence of 1 mM $NaCNBH_3$ in 0.03 M triethanolamine buffer (pH 8) in a final volume of 100 μL. Aliquots of 4.75 μL were removed at different times and assayed for their ability to participate in the cell-free oxidase activation system

as described below. Parallel samples that contained all the reagents except the NADPH dialdehyde were incubated simultaneously in order to control for any effect of $NaCNBH_3$ on the activity of the oxidase.

Cell-Free Activation of the Leukocyte NADPH Oxidase. Superoxide generation was measured as superoxide dismutase-inhibitable reduction of cytochrome *c* at 550 nm in a dual beam recording Uvikon 941 spectrophotometer thermostated at 37 °C. Rac2 (50 nmol) was preloaded with 100 μM GTPγS in a final volume of 100 μL for 15 min at room temperature in the absence of $MgCl_2$. Loading was then completed by adding $MgCl_2$ to a final concentration of 5 mM. The reaction mixtures contained 1.5×10^7 cell equivalents of membrane, 55 nM p47^{PHOX}, 70 nM p67^{PHOX} or N-terminal p67^{PHOX} fragments or the C-terminal fragment p67^{PHOX}(244−526) as indicated, 250 nM Rac2-GTPγS, 10 μM GTPγS, 10 μM FAD, and 100 μM SDS in a final volume of 0.5 mL. After 3 min of incubation at room temperature, the reaction was started by the addition of NADPH (200 μM final concentration) and cytochrome *c* (100 μM final concentration).

Fluorescence Titration. Protein fluorescence was measured in quartz cuvettes (3 mL, 1 cm light path) at 10 °C with a Perkin-Elmer 650-40 spectrofluorometer equipped with a 150 watt xenon lamp with an excitation wavelength of 280 nm and a slit width of 5 nm, recording the emission spectra between 300 and 500 nm using a 10 nm slit width. Titrations were carried out by serial addition of 344 μM NADPH to a cuvette containing 0.575 μM p67^{PHOX} and either the N-terminal fragment p67^{PHOX}(1−243) or the C-terminal fragment p67^{PHOX}(244−526) in 50 mM Tris-HCl, pH 7.4. Titration of a standard glycyl-tryptophan solution under identical conditions was used to correct for the nonspecific effects of NADPH and the effects of dilution and self-absorbance. Dissociation constants (K_d) were derived from the observed decrease in protein fluorescence according to the relationship of Stinson and Holbrook (*30*):

$$L(t)/\theta = K_d(1-\theta)^{-1} + pE(t)$$

where $L(t)$ and $E(t)$ are the total concentrations of ligand and enzyme respectively, p is the number of independent and equivalent binding sites on E, and θ is the ratio of the observed fluorescence change to the maximum fluorescence change (extrapolated) ($\theta = \Delta F/\Delta F_{max}$). Graphic analysis according to the procedure outlined by Ward (*31*) affords the dissociation constant from a plot of $1/(1-\theta)$ against $L(t)/\theta$.

RESULTS

Activity of the Different Truncated Forms of p67^{PHOX}. The cytosolic protein p67^{PHOX} has as its major structural features a highly acidic C-terminus, two SH3 domains located in the middle (residues 245−295) and C-terminal regions (residues 462−512), and two proline-rich sequences in the midsection of the protein (residues 226−234 and 317−329). To localize the NADPH binding site, we have constructed different truncated forms of p67^{PHOX}: p67^{PHOX}(1−243), p67^{PHOX}(1−210), p67^{PHOX}(1−199), and p67^{PHOX}(244−526). We first tested their activity in a cell-free recombinant system with SDS. We found in accord with earlier studies that the N-terminal region of p67^{PHOX} (residues 1−243), which lacks the two SH3 domains but contains the first proline rich sequence, can partially reconstitute superoxide production in a cell-free system activated by SDS (Figure 1) (*25*, *32*−*34*). The N-terminal region of p47^{PHOX} (residues 1−210) could also reconstitute the respiratory burst oxidase activity (Figure 1), though in our hands it did not support O_2^- generation at the same rate as the wild-type p67^{PHOX}, as previously reported by Han and co-workers (*26*). The shortest N-terminal fragment of p67^{PHOX} [i.e., p67^{PHOX}(1−199)] failed to support O_2^- generation, in accord with previous studies (*25*, *26*), nor did the C-terminal fragment p67^{PHOX}(244−526) show any activity (Figure 1).

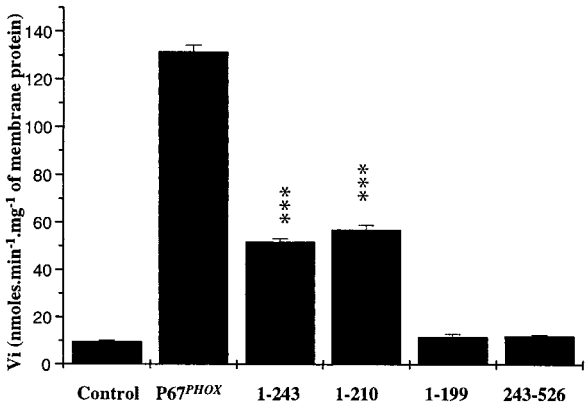

FIGURE 1: Cell-free O_2^- generation from full-length p67^{PHOX} and various truncated forms of p67^{PHOX}. NADPH-dependent O_2^- generation was measured by cytochrome *c* reduction as detailed under Materials and Methods, with 1.5×10^7 cell equiv of membrane as a source of cytochrome b_{558}, 55 nM p47^{PHOX}, 70 nM p67^{PHOX} (full-length or truncated forms) and 250 nM Rac2-GTPγS. (***, $p < 0.001$ as compared with full-length p67^{PHOX}.)

The NADPH Binding Site Is Located in the N-Terminal Region. We have previously demonstrated that p67^{PHOX} contains an NADPH binding site that is essential for oxidase activity (*35*). The different truncated forms of p67^{PHOX} were labeled with NADPH dialdehyde in the presence of tritiated sodium borohydride. Sodium borohydride reduces the Schiff base formed between the dialdehyde derivative and the nearby lysine residues of the NADPH binding site. Using this procedure, we showed that, like the full length p67^{PHOX}, the N-terminal regions p67^{PHOX}(1−243), p67^{PHOX}(1−210), and p67^{PHOX}(1−199) were labeled by NADPH dialdehyde in the presence of tritiated borohydride (Figure 2, left). Furthermore, NADPH prevents this labeling (Figure 2, left), suggesting that labeling is the result of the binding of NADPH to a specific site on the protein. The difference observed in the labeling is not due to a lane-to-lane difference in the amount of protein loaded onto the gel, since the Coomassie-blue stained gel shows a similar amount of protein in each lane (Figure 2, right). The C-terminal fragment of p67^{PHOX}(244−526) was also weakly labeled, but this labeling appeared to be nonspecific, since it was not prevented by excess unlabeled NADPH (data not shown). cDNAs encoding shorter truncated forms [p67^{PHOX}(1−155), p67^{PHOX}(1−105), p67^{PHOX}(1−71), and p67^{PHOX}(1−35)] were also constructed to localize more precisely the NADPH binding site, but we were unable to express them in *E. coli*, a finding previously reported by Diekmann et al. (*18*).

To confirm that the binding site is localized to the N-terminal region and not the C-terminal region, and also to compare the dissociation constant (K_d) of the N-terminal

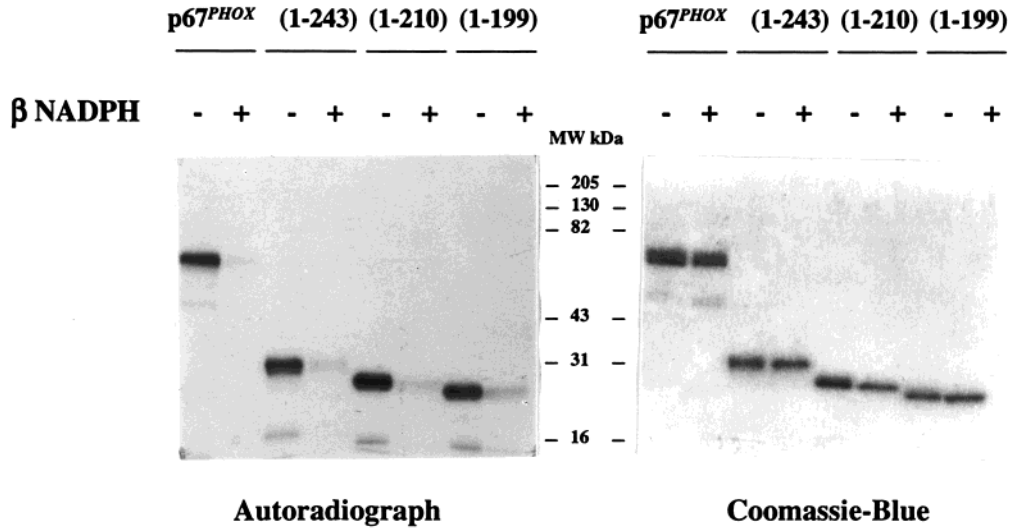

FIGURE 2: NADPH dialdehyde labeling of full-length p67^{PHOX} and p67^{PHOX} truncated forms. Labeling was carried out as described under Materials and Methods in the presence or absence of 40 mM NADPH. The labeled proteins were analyzed by SDS−PAGE and autoradiography. Each lane was loaded with 0.3 nmol of protein. The experiments were performed at least three times; a representative Coomassie blue-stained gel and autoradiograph are shown.

region (1−243) with that of full-length p67^{PHOX}, we used fluorescence spectroscopy. A total of eight tryptophan residues are present in p67^{PHOX}, four of which are in the N-terminal fragment (1−243). The binding of NADPH to p67^{PHOX} or its N-terminal fragment p67^{PHOX}(1−243) would be expected to quench the fluorescence of the tryptophan residues if binding is specific, an effect that can be followed by a decrease in protein fluorescence. As shown in Figure 3A,B, NADPH suppressed the fluorescence of both full-length p67^{PHOX} and the N-terminal fragment p67^{PHOX}(1−243) as compared with their respective guanidine-denatured controls. The fluorescence shown was corrected to take into account the change in volume brought about by the addition of NADPH and the nonspecific effect of NADPH itself, the latter of which was evaluated by the titration of a solution of L-glycyl-tryptophan under the same conditions. The emission peak for tryptophan fluorescence at 340 nm was not shifted by the addition of NADPH (not shown). In contrast to the quenching seen with the full length p67^{PHOX} and the N-terminal fragment p67^{PHOX}(1−243), the C-terminal fragment p67^{PHOX}(244−526) did not show any decrease in fluorescence as compared with its denatured control (Figure 3C).

The K_d can be assessed from the observed decrease in protein fluorescence according to the relationship outlined by Stinson and Holbrook (*30*) as described under Materials and Methods: $L(t)/\theta = K_d (1 - \theta)^{-1} + pE(t)$. In our case, $p = 1$ as determined by Smith et al. (*35*). Graphical analysis according to the procedure outlined by Ward (*31*) affords the K_d from a plot of $1/(1 - \theta)$ against $L(t)/\theta$, where K_d is the slope. From several experiments, we found that the K_d for NADPH of p67^{PHOX} and that of the N-terminal fragment were essentially the same, with values of 7.2 ± 2.0 μM and 6.4 ± 0.7 μM, respectively.

NADPH Dialdehyde Inactivates the N-Terminal Fragment (1−243). It has been known for some time that NADPH dialdehyde inactivates the oxidase. More recently, it was shown that when p67^{PHOX} is preincubated with NADPH dialdehyde, it loses its activity in a cell-free oxidase assay. To see if the N-terminal fragment of p67^{PHOX} was similarly inactivated by NADPH dialdehyde, we performed inactivation experiments by measuring the residual activities of the NADPH oxidase after incubation of full-length p67^{PHOX} or p67^{PHOX}(1−243) with NADPH dialdehyde for defined periods of time. Figure 4 shows that the NADPH oxidase activity of the N-terminal fragment p67^{PHOX} decreased to 35% of its starting activity after incubation for 10 h with NADPH dialdehyde, while the activity of the control without NADPH dialdehyde decreased only to 70% of the starting activity. For full-length p67^{PHOX}, the NADPH oxidase activity decreased to 55% of the initial activity activity upon incubation with NADPH dialdehyde, while the control without NADPH dialdehyde only fell to 90% of the starting activity. The full-length p67^{PHOX} seems to be more stable than the N-terminal fragment since, in the absence of NADPH dialdehyde, its activity decreased to a lesser extent than did the activity of the N-terminal fragment.

Effect of Rac2 on NADPH Binding. Several studies have demonstrated that Rac2 is able to bind p67^{PHOX} (*36*) although it does not contain a CRIB motif, an established target of Rac. Furthermore, the interaction is considered to be important for oxidase activation, since mutations in Rac that lead to defective interactions are unable to activate the oxidase (*18*, *36*). To determine if Rac could influence the binding of NADPH to p67^{PHOX}, we preincubated p67^{PHOX} with an equimolar amount of Rac2-GTPγS or Rac2-GDPβS for 30 min at 4 °C prior to adding NADPH dialdehyde and Na[^{3}H]BH$_4$. As shown in Figure 5A and 5B, the binding of NADPH to p67^{PHOX} decreased significantly in the presence of Rac2-GTPγS and to a lesser extent in the presence of Rac2-GDPβS. The decrease in NADPH binding was not due to GTPγS or GDPβS, which could in principle compete with the NADPH binding site, since excess of GTPγS or GDPβS 5 mM alone in the absence of Rac2, did not change the binding of NADPH dialdhyde to p67^{PHOX} (data not shown). Rac2-GTPγS or Rac2-GDPβS that were added to the assay were not labeled by NADPH dialdehyde in the presence of Na[^{3}H]BH$_4$, giving further evidence of specificity of the labeling.

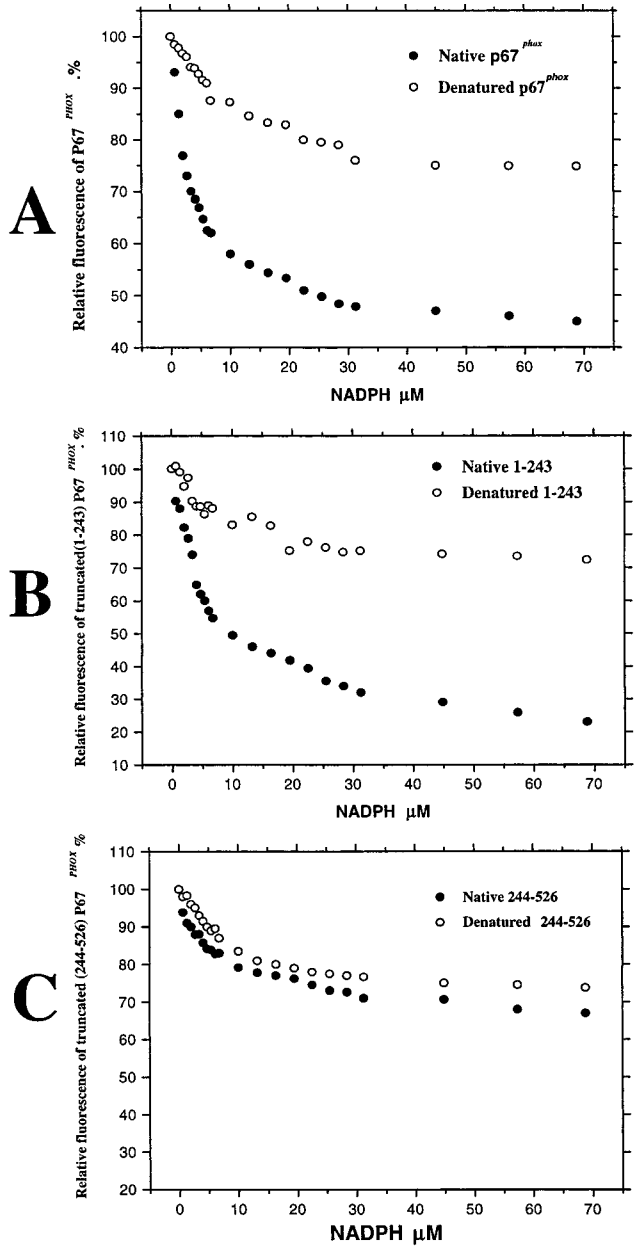

FIGURE 3: Fluorescence quenching by NADPH of full length p67^{PHOX} (A), the N-terminal fragment p67^{PHOX}(1−243) (B) and the C-terminal fragment p67^{PHOX}(244−526) (C). Proteins were used at a concentration of 0.575 μM. The excitation wavelength was 280 nM, and emission spectra were recorded between 300 and 500 nm. The proteins were denatured by 6 M guanidine hydrochloride. The results shown are the fluorescence at 340 and 350 nm for the native and denatured polypeptides, respectively. These results are corrected values that take into consideration both the change in volume brought about by the addition of NADPH and nonspecific quenching by NADPH, the latter of which was evaluated by the titration of a solution of L-glycyl-tryptophan with NADPH under the same conditions. The experiments were performed three times; a representative quenching curve for each protein is shown.

DISCUSSION

The binding of NADPH to p67^{PHOX} was first suggested by Umei et al. (*37*), who showed that NADPH dialdehyde specifically labeled a 66 kDa protein in a preparation of pig neutrophils. Later Smith et al. (*35*) found that p67^{PHOX} was specifically labeled by NADPH dialdehyde at a mole for mole stoichiometry and that this labeling destroyed the ability of p67^{PHOX} to support oxidase activity in a cell-free system

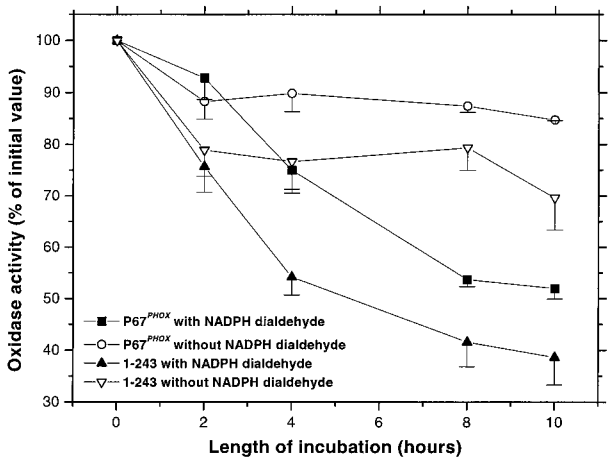

FIGURE 4: Inactivation of p67^{PHOX} and N-terminal fragment p67^{PHOX}(1−243) by NADPH dialdehyde as a function of time. Full-length p67^{PHOX} or the N-terminal fragment (1.5 nmol) was incubated at 4 °C with 200 μM NADPH dialdehyde and 1 mM $NaCNBH_3$ in 0.03 M triethanolamine buffer (pH 8) in a final volume of 100 μL. Aliquots of 4.75 μL were removed at different times and assayed for their ability to participate in the cell-free oxidase activation system. The results are the mean ± SE of three experiments. Initial rates averaged 127.0 ± 9.2 and 63.4 ± 5.2 nmoles min^{-1} mg^{-1} for full-length p47^{PHOX} and the N-terminal fragment, respectively.

(*35*, *38*). We recently obtained further evidence supporting this idea, showing that p67^{PHOX} possesses a weak but significant NADPH dehydrogenase activity (*27*). In the present study we used affinity labeling and fluorescence spectroscopy to provide further evidence that p67^{PHOX} is able to bind NADPH. NADPH can bind to N-terminal regions p67^{PHOX}(1−243), p67^{PHOX}(1−210), and p67^{PHOX}(1−199) but not to the C-terminal region p67^{PHOX}(244−526). These results show that residues between 199 and 243 are not necessary for binding. This region was shown to contain an activation domain, which was mapped to the stretch between residues 199 and 210 (*26*). Thus the activation domain of p67^{PHOX} is not involved in the binding of NADPH. However, it is interesting to note that the region 1−199 of p67^{PHOX} contains four tetratricopeptide repeats (TPR), three of which were found to be important for Rac binding (*39*). Rac2-GTPγS and to a lesser extent Rac2-GDPβS were found to decrease the binding of NADPH to p67^{PHOX}, suggesting that Rac is able to change the conformation of p67^{PHOX}, rendering the binding site for NADPH less accessible. Another possibility is that the NADPH binding site and the Rac2 binding site overlap. It is surprising that Rac2-GDPβS decreased the labeling of p67^{PHOX} (although to a lesser extent than Rac2-GTPγS) because Rac2-GDP is thought not to bind p67^{PHOX} (*18*). On the other hand, Rac1-GDP was found to be a potent activator of the O_2^--forming NADPH oxidase of macrophages (*40*). Furthermore, the dissociation constants of the full-length p67^{PHOX} and the N-terminal fragment were found to be the same, suggesting that in the intact protein the C-terminal fragment does not influence the affinity of p67^{PHOX} for NADPH.

In agreement with previous studies (*25*, *32*−*34*), we found that the N-terminal fragments 1−243 and 1−210 of p67^{PHOX} were sufficient to reconstitute the activity of the oxidase in our semirecombinant system, although the initial rate is only about 40% of the initial rate with the full-length protein. It has been shown, however, that the N-terminal fragment

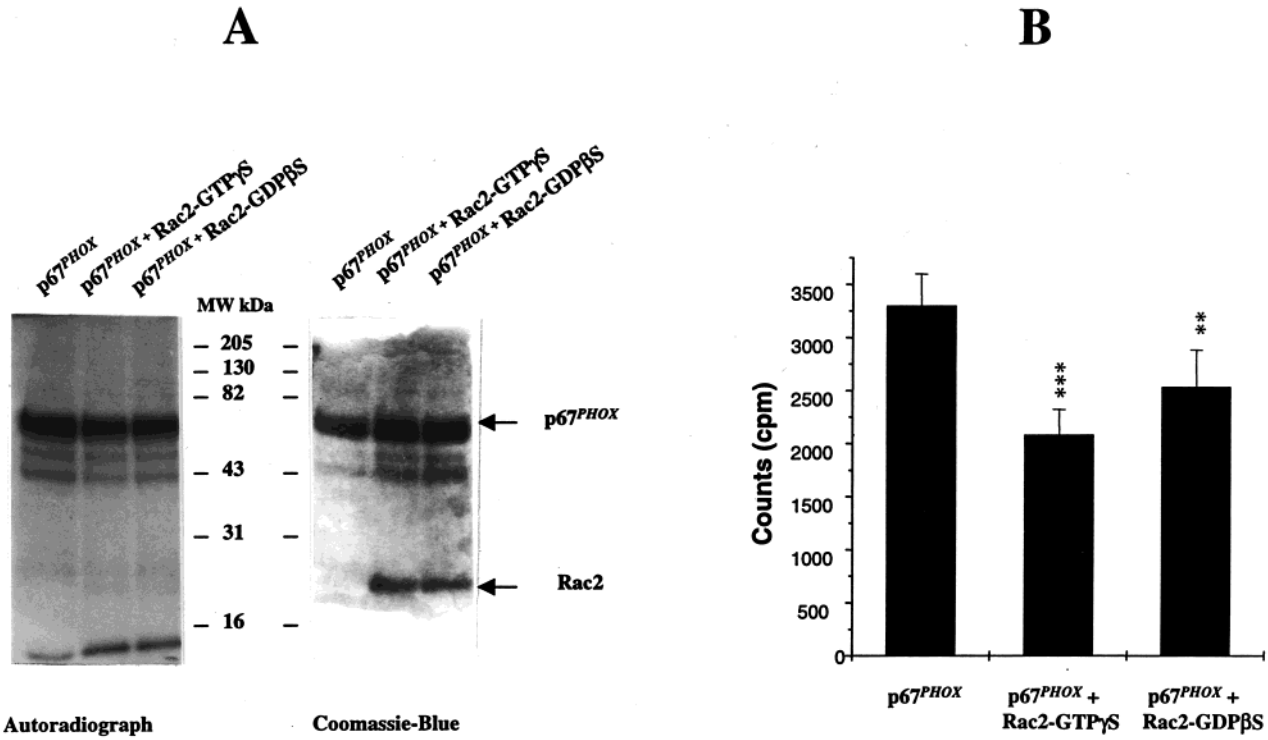

FIGURE 5: Effect of Rac2-GTPγS or Rac2-GDPβS on NADPH binding by p67^{PHOX}. The effect of Rac2 on the labeling of p67^{PHOX} by NADPH dialdehyde was determined by preincubating p67^{PHOX} with an equimolar amount of Rac2-GTPγS or Rac2-GDPβS for 30 min at 4 °C prior to addition NADPH dialdehyde and Na[^{3}H]BH_4 as described under Materials and Methods. (A) Decrease of binding as shown by autoradiography. The figure shown is representative of three experiments. (B) Decrease of binding as shown by counting. p67^{PHOX} bands were excised and their radioactivity was measured by liquid scintillation counting. The results are the mean ± SE of three experiments. **, $p < 0.01$; ***, $p < 0.001$.

p67^{PHOX}(1−246) is not able to restore oxidase activity nor to translocate to the plasma membrane in p67^{PHOX}-deficient Epstein−Barr virus-transformed B cells (*34*). Thus, in contrast to the situation that prevails in the cell-free studies, the C-terminal domain is essential for the assembly and activity of the oxidase in whole stimulated cells.

Pick and associates have provided very strong evidence that, under appropriate conditions, the oxidase component cytochrome b_{558} can by itself catalyze the production of O_2^- from oxygen and NADPH at rates close to those seen with the complete oxidase (*41*, *42*). Earlier, it was shown that gp91^{PHOX}, one of the subunits of cytochrome b_{558}, contains regions of homology to the NADPH binding sites of a number of other enzymes, the highest degree of homology being seen with ferrodoxin-NADP$^+$ reductase (*13*, *14*), and that the cytochrome contains an NADPH binding site as determined by affinity labeling (*14*, *43*). It is clear that cytochrome b_{558} contains all the equipment required to catalyze O_2^- production by the complete leukocyte NADPH oxidase. It therefore seems likely that the entire O_2^--forming electron transport chain of the leukocyte NADPH oxidase resides solely in the 91 kDa subunit of cytochrome b_{558}. At the same time, however, it is clear that p67^{PHOX} possesses a catalytically significant binding site for NADPH and indeed can catalyze the transfer of electrons from NADPH to an artificial electron acceptor, albeit at very low rates. What is the purpose of this feature of the leukocyte NADPH oxidase?

One possibility is suggested by analogy with catalase (*44*). Catalase is a heme enzyme whose iron atom cycles between Fe^{III} and a (nominal) Fe^V state as the enzyme alternately reduces and oxidizes its substrate, H_2O_2. Catalase also has a binding site for NADPH. From time to time the iron in Fe^V catalase is reduced to Fe^{IV} either by ambient O_2^- or by an enigmatic "endogenous donor". Fe^{IV} catalase is catalytically inactive, but the enzyme is able to use the NADPH to restore its activity by returning the iron to its Fe^{III} state. It may be that, like catalase, the activity of the leukocyte NADPH oxidase depends on the redox state of one of its cofactors; that this cofactor is converted in a side reaction to a redox state in which the enzyme is inactive; and that p67^{PHOX} is able to reactivate the enzyme by restoring the cofactor to a catalytically functional redox state. The flavin, for example, may cycle between $FADH_2$ and $FADH^\bullet$ as it transfers electrons to its acceptor but may occasionally be oxidized to FAD, which would have to be converted to $FADH_2$ or $FADH^\bullet$ in order to reactivate the enzyme. This hypothetical reduction can be imagined as one of the possible functions of p67^{PHOX}.

ACKNOWLEDGMENT

We thank Dr. John Whiteley for helpful suggestions regarding fluorescence spectroscopy.

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BI9919807