Phosphorylated p40^{PHOX} as a Negative Regulator of NADPH Oxidase†

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ABSTRACT: The leukocyte NADPH oxidase catalyzes the production of O_2^- from oxygen at the expense of NADPH. Activation of the enzyme requires interaction of the cytosolic factors p47^{PHOX}, p67^{PHOX}, and Rac2 with the membrane-associated cytochrome b_{558}. Activation of the oxidase in a semirecombinant cell-free system in the absence of an amphiphilic activator can be achieved by phosphorylation of the cytosolic factor p47^{PHOX} by protein kinase C. Another cytosolic factor, p40^{PHOX}, was recently shown to be phosphorylated on serine and threonine residues upon activation of NADPH oxidase, but both stimulatory and inhibitory roles were reported. In the present study, we demonstrate that the addition of phosphorylated p40^{PHOX} to the cell-free system inhibits NADPH oxidase activated by protein kinase C-phosphorylated p47^{PHOX}, an effect not observed with the unphosphorylated p40^{PHOX}. Moreover phosphorylated p40^{PHOX} inhibits the oxidase if added before or after full activation of the enzyme. Direct mutagenesis of protein kinase C consensus sites enables us to conclude that phosphorylation of threonine 154 is required for the inhibitory effect of p40^{PHOX} to occur. Although the phosphorylated mutants and nonphosphorylated mutants are still able to interact with both p47^{PHOX} and p67^{PHOX} in pull-down assays, their proteolysis pattern upon thrombin treatment suggests a difference in conformation between the phosphorylated and nonphosphorylated mutants. We postulate that phosphorylation of p40^{PHOX} on threonine 154 leads to an inhibitory conformation that shifts the balance toward an inhibitory role and blocks oxidase activation.

In response to invasive microorganisms, neutrophils and other phagocytic cells react vigorously to produce O_2^- anion and other microbicidal oxygen derivatives. The production of O_2^- is due to the action of NADPH oxidase, a membrane-associated enzyme that catalyzes the one-electron reduction of oxygen to O_2^- at the expense of NADPH (*1*). The oxidase is a multicomponent enzyme, dormant in resting cells, that becomes activated when the neutrophils are stimulated. Upon activation, a cytosolic complex consisting of the cytosolic components p47^{PHOX}, p67^{PHOX}, and p40^{PHOX}, together with the small GTPase Rac2, migrates to the membrane, where they associate with the membrane bound cytochrome b_{558} to assemble the active oxidase (*2*−*8*).

p40^{PHOX} is complexed to p67^{PHOX} in resting cells (*9*) and translocates to the membrane during activation of the NADPH oxidase (*8*, *10*−*12*). Translocation of p40^{PHOX} appears to be dependent on p47^{PHOX} and is mediated by p67^{PHOX} (*11*). Although p40^{PHOX} interacts with both p47^{PHOX} and p67^{PHOX} (*8*, *10*−*13*), it is not required to reconstitute oxidase activity in recombinant cell-free systems (*14*−*17*).

The phosphorylation of the cytosolic component p47^{PHOX} is a well-known mechanism involved in the activation of the oxidase (*17*−*24*). Recent studies showed that p40^{PHOX} also undergoes phosphorylation on multiple sites upon stimulation of the NADPH oxidase (*25*, *26*). More recently it was demonstrated that protein kinase C is one of the kinases capable of phosphorylating p40^{PHOX} (*27*). Although these studies described the binding domains and identified possible kinases and phosphorylation sites in p40^{PHOX}, the role of this cytosolic component in the activation of the oxidase remains uncertain. Paradoxically there are virtually as many reports on an inhibitory role of p40^{PHOX} (*28*, *29*) as on a stimulatory role (*9*, *30*, *31*). Moreover opposite results were found in the same K562 cells (*28*, *31*).

We have recently shown that NADPH oxidase can be activated by phosphorylated p47^{PHOX} in a partially recombinant system containing membranes, p67^{PHOX}, and Rac2 (*17*). To resolve the discrepancies between the reported effects of p40^{PHOX}, we hypothesized that a difference in the phosphorylation state could be involved. We therefore took advantage of our phosphorylation-dependent system and studied the effect of phosphorylated p40^{PHOX} on the activation of the NADPH oxidase. Our findings show that only phosphorylated p40^{PHOX} inhibits oxidase activity whether added before or after full activation of the enzyme. Directed mutagenesis of the protein kinase C consensus sites enabled us to conclude that phosphorylation of the protein kinase C site threonine 154 is involved in the inhibition of oxidase activation by p40^{PHOX}. Limited proteolysis of the phospho-

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rylated and nonphosphorylated mutant proteins showed a different pattern, suggesting a conformational change directly correlated with the inhibitory effect on NADPH oxidase activation.

EXPERIMENTAL PROCEDURES

Materials. Chemicals and enzymes were obtained from the following sources: dextran, Ficoll-Hypaque, glutathione Sepharose beads, and γ-(^{32}P) ATP from Amersham Biosciences; phosphatidylserine, diacylglycerol, isopropyl-β-D-thiogalactopyranoside (IPTG), NADPH, ATP, guanosine 5-*O*-(3-thiotriphosphate) (GTPγS), cytochrome *c*, diisopropylfluorophosphate, thrombin, and phorbol-myristate-acetate (PMA) from Sigma; rat brain protein kinase C, calyculin A, and GF 109203X (GFX) from Calbiochem; leupeptin from Roche; the Bradford protein assay reagent from Biorad.

Two-Dimensional Gel Electrophoresis and Western Blot Analysis. Neutrophils were isolated from normal subjects by dextran sedimentation and Ficoll-Hypaque fractionation of freshly drawn citrate-anticoagulated blood. Cells were suspended at 10^8 cells/ml in a modified relaxation buffer (0.1 M KCl, 3 mM NaCl, 3.5 mM $MgCl_2$, 10 mM PIPES buffer, pH 7.3) and activated with PMA at 37 °C. In PKC inhibition studies, GFX was added at a final concentration of 5 μM to the cells 10 min before activation. After activation, the cell pellets were treated as described (*25*) and subjected to two-dimensional electrophoresis. The membrane was probed with an anti-p40^{PHOX} antibody (1/1000 dilution). The presence of p40^{PHOX} was detected with protein A coupled to horseradish peroxidase using the enhanced chemiluminescence kit.

Preparation of Neutrophil Subcellular Fractions. Neutrophils were isolated from normal subjects by dextran sedimentation and Ficoll-Hypaque fractionation of freshly drawn citrate-anticoagulated blood. Cells were suspended at 10^8 cells/ml in a modified relaxation buffer (0.1 M KCl, 3 mM NaCl, 3.5 mM $MgCl_2$, 10 mM PIPES buffer, pH 7.3). Neutrophil cytosol and membranes were prepared as described by Borregaard et al. (*32*). Cytosol and membranes were divided into aliquots and stored at −70 °C until use.

Production and Purification of Recombinant p67^{PHOX} from Baculovirus-Infected Sf9 Cells. Purified recombinant p67^{PHOX} was produced by means of the baculovirus system described by Leto et al. (*33*), using a p67^{PHOX}-expressing recombinant virus generously provided by T. L. Leto. Large scale production of pure recombinant p67^{PHOX} was obtained by infecting monolayer cultures of Sf9 cells in 150 cm^2 flasks at a density of (1−2) × 10^6 cells/mL. Recombinant p67^{PHOX} was purified as described previously (*17*).

Preparation of Recombinant GST−p67^{PHOX}, GST−p47^{PHOX}, and GST−Rac2 Fusion Proteins. Recombinant fusion proteins composed of glutathione S-transferase (GST) linked to p67^{PHOX}, p47^{PHOX}, or Rac2 were isolated from *Escherichia coli* transformed with pGEX plasmids containing cDNA inserts encoding the downstream proteins as described by Park et al. (*14*). The proteins were purified by affinity chromatography on glutathione Sepharose beads according to Lopes et al. (*17*) followed by gel filtration on a Superdex 200 column (Amersham Biosciences) equilibrated in relaxation buffer.

p40^{PHOX} cDNA Constructs. The full length clone of p40^{PHOX} beginning 18 base pairs before the start codon was obtained as described previously (*13*) and cloned in the pET-32a plasmid (Novagen) downstream of the thioredoxin (Trx) coding sequence and the poly-His tag. Directed mutagenesis was carried out using the Quick Change mutagenesis kit from Stratagene. Residues T154 and S315 were mutated to Ala using sense and antisense primers containing one mismatch and designed according to Stratagene's specifications. The double mutant (T154A/S315A) was obtained after a second round of directed mutagenesis on the T154A mutant cDNA. After confirmation by sequencing, the pET-32a plasmids carrying the mutated forms of p40^{PHOX} were transformed into competent bacteria and the fusion proteins were expressed and purified as described below.

Expression and Purification of Recombinant Trx−p40^{PHOX} Fusion Protein. The protease-deficient BL-21-DE3 (pLysS) *E. coli* strain was transformed with the pET-32a plasmid carrying the wild-type or mutated forms of p40^{PHOX}. An overnight preculture was diluted 10-fold in fresh "Terrific Broth" containing 100 μg/mL ampicillin and incubated at 37 °C with agitation for 1 h before induction with 1 mM IPTG for 3 h at 30 °C. Bacteria were harvested by centrifugation and lysed by sonication in 20 mM HEPES, pH 7.9, 0.5 M NaCl, and 10 mM imidazole, supplemented with 1 mM diisopropyl fluorophosphate and 10 μg/mL leupeptin. The homogenate was centrifuged at 300 000 × *g* for 15 min, and the supernatant was incubated with ProBond resin (Invitrogen) for 1 h at 4 °C. The resin was packed into a fast protein liquid chromatography column and washed in 20 mM HEPES, pH 7.9, 0.5 M NaCl, and 30 mM imidazole. The fusion protein was eluted with 75 mM imidazole and further purified on a Superdex 200 column equilibrated in relaxation buffer.

Phosphorylation of p47^{PHOX} and p40^{PHOX}. Phosphorylation of recombinant p47^{PHOX} and p40^{PHOX} was carried out using 100−200 μg of fusion protein in a final volume of 200 μL. The reaction mixture contained 1 mM ATP, 10 mM magnesium acetate, 1 mM $CaCl_2$, 10 μg of phosphatidylserine, 1 μg of diolein, and 0.5 units of protein kinase C in 200 μL of relaxation buffer. The lipids were added as mixed liposomes prepared by dissolving 2.5 mg/mL phosphatidylserine and 1 mg/mL diacylglycerol in chloroform, removing the chloroform under a stream of nitrogen and then sonicating the dried lipids for 2 min on ice in 0.8 mL of 20 mM Tris-HCl buffer, pH 7.4. The phosphorylated proteins were separated from the reaction mixture as described elsewhere (*14*). Protein kinase C-phosphorylated p47^{PHOX} is designated p47^{PHOX}P$_6$ because it contains 6 mol of phosphate/mol of p47^{PHOX}.

Effect of Phosphorylated p40^{PHOX} on the Activation of NADPH Oxidase with p47^{PHOX}P$_6$. Activation of the NADPH oxidase in the cell-free system was directly measured by following the superoxide dismutase-inhibitable reduction of cytochrome *c* at 550 nm in a dual beam recording spectrophotometer. The complete reaction mixture contained 5 × 10^6 cell equivalents of membrane incubated for 10 min at 30 °C with 50 μM GTPγS, 1 mM ATP, 250 nM calyculin A, 105 pmol of Rac2, 104 pmol of p67^{PHOX}, 95 pmol of p47^{PHOX}P$_6$, and 106 pmol of p40^{PHOX} (unphosphorylated or phosphorylated) in a final volume of 200 μL. Reactions were started by adding the detection mixture cytochrome *c* (0.1 mM) and NADPH (0.16 mM). Reduction was followed in a Uvikon 941 dual beam recording spectrophotometer (Kontron

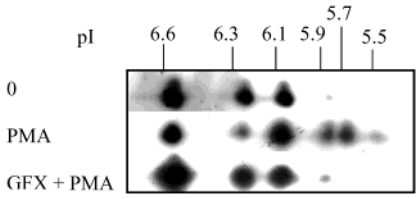

FIGURE 1: Western blot analysis of immunoprecipitated p40^{PHOX} in human neutrophils. After immunoprecipitation, the immunocomplex was subjected to two-dimensional electrophoresis and transferred to a nitrocellulose membrane. p40^{PHOX} was immunodetected with the p40^{PHOX} antibody. The panels show the isoelectric focusing pattern of p40^{PHOX} in resting neutrophils (0), after stimulation of the cells by 1 μg/mL of PMA for 3 min (PMA), and after treatment of the cells with 5 μM GFX for 10 min before the addition of PMA (GFX + PMA).

Instruments, Milan, Italy), reading against a reference containing the same components plus 150 U of superoxide dismutase. Superoxide generation was followed for 20 min at 30 °C, and the rate of O_2^- generation was determined between 2.5 and 7.5 min of recording.

Determination of Phosphorylation of p40^{PHOX} and p40^{PHOX} Mutants. Proteins were phosphorylated in vitro as described in the preceding paragraph, using 1 mM nonradioactive ATP and 10 μCi γ-(^{32}P)-ATP, loaded on SDS−PAGE gel, and phosphorylation was assessed using a PhosphorImager (Biorad) and quantitated using Quantity 1 Software.

Binding of Phosphorylated p40^{PHOX} to p47^{PHOX}, Phosphorylated p47^{PHOX}, and p67^{PHOX}. Phosphorylated GST−p47^{PHOX} was reisolated on glutathione Sepharose beads after phosphorylation. Phosphorylated p40^{PHOX} proteins (and in some cases nonphosphorylated p40^{PHOX} proteins) were incubated on glutathione Sepharose beads with either GST-p47^{PHOX} , phosphorylated GST-p47^{PHOX}, or GST-p67^{PHOX} immobilized for 1 h at 4 °C. After extensive washing in relaxation buffer, the beads were loaded onto a SDS−PAGE. Control was run with GST alone.

Limited Proteolysis by Thrombin. Proteins were diluted once in Tris-HCl, 20 mM, pH 8.0, buffer containing 100 mM NaCl. They were incubated overnight at 4 °C with 0.1 U of thrombin for 20 μg protein. Digested proteins were then submitted to SDS−PAGE and Western blotting using antibodies against p40^{PHOX} as previously described (*25*).

RESULTS

Two-Dimensional Analysis of the Effect of a PKC Inhibitor on the Isoelectric Focusing Pattern of p40^{PHOX} in Isolated Human Neutrophils. In a previous study (*25*), phosphorylation of p40^{PHOX} was demonstrated in differentiated HL60 cells after activation by PMA. To check that the same occurred in human neutrophils, a similar experiment was performed with these cells. As shown in Figure 1, three spots of p*I* 6.6, 6.3, and 6.1 are present in the resting cells and three more acidic spots appear when the cells are treated with PMA. GFX, a specific cell-permeable PKC inhibitor (*34*), suppresses the more acidic spots indicating that they are due to PKC phosphorylation.

Phosphorylated p40^{PHOX} Inhibits NADPH Oxidase Activation by p47^{PHOX}P$_6$. Recently, we demonstrated that the oxidase can be activated by p47^{PHOX} phosphorylated by protein kinase C in a partially recombinant system containing membranes and recombinant p47^{PHOX}, p67^{PHOX}, and Rac2

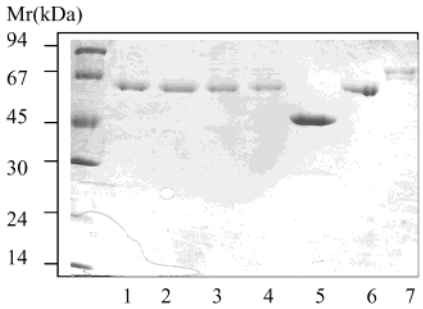

FIGURE 2: SDS−PAGE of the purified recombinant proteins. Proteins were purified as described under experimental procedures and separated on a 13% polyacrylamide gel stained with Coomassie Blue. The molecular weights of the markers are indicated on the left: lane 1, WT p40^{PHOX}; lane 2, p40^{PHOX} S315A; lane 3, p40^{PHOX} T154A; lane 4, p40^{PHOX} S315A + T154A; lane 5, GST-Rac2, lane 6, p67^{PHOX}, lane 7, GST−p47^{PHOX}.

Table 1: Concentration Dependence Inhibition of p47^{PHOX}P$_6$ NADPH Oxidase Activation by Phosphorylated and Unphosphorylated p40^{PHOX} [a]

	O_2^- production (% control)	
p40^{PHOX} (pmol)	phosphorylated	unphosphorylated
0	100	100
53	50 ± 11	96 ± 6
106	36 ± 3	88 ± 5
212	13 ± 0.3	94 ± 3

[a] The activation reactions were carried out as described under Experimental Procedures with increasing concentrations of phosphorylated or unphosphorylated p40^{PHOX} (pmol). O_2^- production was determined (mean ± SE) for five or more separate experiments, and the results are expressed as percent of the maximal rate of superoxide production (obtained in the absence of p40^{PHOX}).

(*17*). Proteins used in this study are shown in Figure 2. O_2^- production was measured using this cell-free system in the presence of phosphorylated or unphosphorylated p40^{PHOX} (Figure 3A,B). The maximal rate of O_2^- generation elicited by p47^{PHOX} phosphorylated by protein kinase C was 1.46 ± 0.22 nmol O_2^- /min/10^7 cell equivalents of membranes (mean ± SE, n = 5). The addition of phosphorylated p40^{PHOX} to the incubation mixture reduced by 87% the activity of the oxidase as compared with the addition of the unphosphorylated protein, which did not have a significant effect. The omission of any of the three cytosolic components of the oxidase abolished oxidase activity suggesting that p40^{PHOX} cannot substitute for another component. Furthermore, oxidase activation decreased with increasing amounts of phosphorylated p40^{PHOX}, whereas no effect of unphosphorylated p40^{PHOX} was observed, even at high amounts (Table 1).

Phosphorylated and Unphosphorylated p40^{PHOX} Inhibit NADPH Oxidase Activation by SDS. It has been shown (*29*) that p40^{PHOX} inhibits the activation of the oxidase by arachidonic acid in a cell-free system. We tested the effect of phosphorylated p40^{PHOX} in the recombinant cell-free system using SDS as the activating stimulus. The activity of the uninhibited oxidase was 7.4 ± 1.5 nmol O_2^-/min/10^7 cell equivalents of membranes. The addition of p40^{PHOX} decreased it to 56% ± 12% and 62% ± 13% for the phosphorylated and unphosphorylated p40^{PHOX}, respectively. Thererefore, when SDS is the stimulus, both phosphorylated and unphosphorylated p40^{PHOX} inhibit the activation of the NADPH oxidase in a similar manner (Table 2).

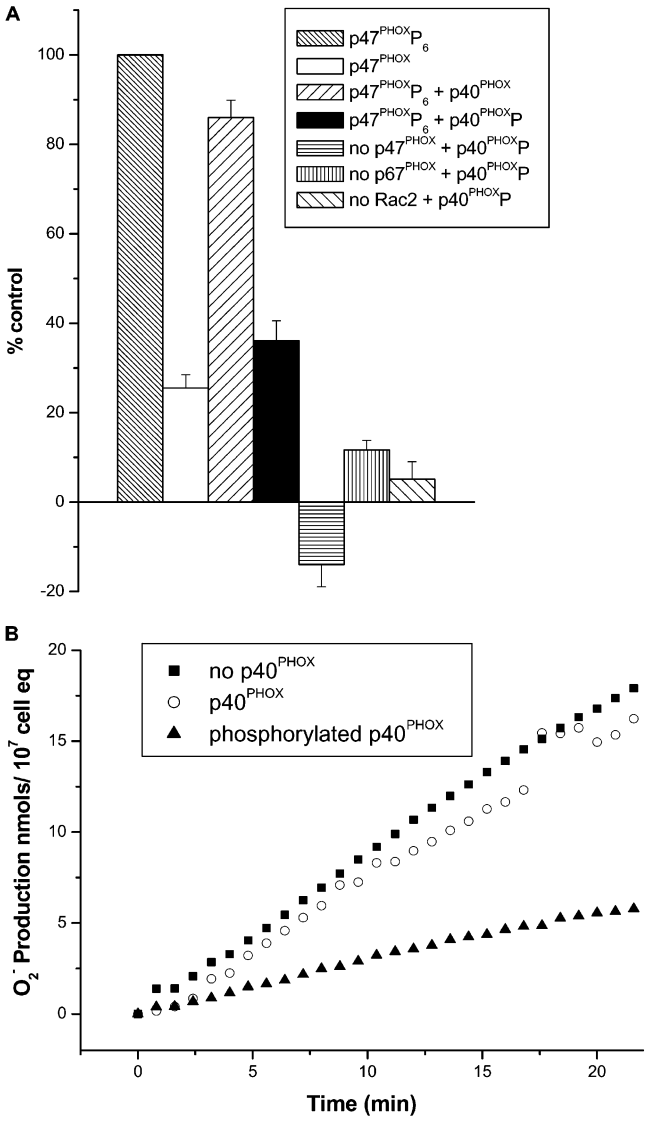

FIGURE 3: Phosphorylated p40*PHOX* inhibits NADPH oxidase activation by p47*PHOX*P_6. In panel A, the SOD-sensitive cytochrome *c* reduction assay was conducted as described in Experimental Procedures. Results are shown as percent of p47*PHOX*P_6 activation of NADPH oxidase and expressed as mean ± SE of six or more experiments. Components were omitted from the assays as indicated. Difference between O_2^- production by p47*PHOX*P_6 and p47*PHOX*P_6 + phosphorylated p40*PHOX* was significant at $p < 0.01$ and between p47*PHOX*P_6 + phosphorylated p40*PHOX* and p47*PHOX*P_6 + unphosphorylated p40*PHOX* was significant at $p < 0.05$. Panel B records O_2^- production as a function of time during assay of NADPH oxidase in the absence of p40PHOX (no p40PHOX), in the presence of p47*PHOX*P_6 + phosphorylated p40*PHOX* (phosphorylated p40PHOX) or unphosphorylated p40*PHOX* (p40PHOX). The curves are representative of six or more separate experiments.

Phosphorylated p40^{PHOX} *Inhibits p47*$^{PHOX}P_6$*-activated NADPH Oxidase*. We then wanted to check whether phosphorylated p40*PHOX* inhibited activity or activation of the oxidase and, therefore, decided to add phosphorylated p40*PHOX* to the p47*PHOX*P_6-dependent system, after the enzyme had been activated which, according to our previous work, occurs immediately after the addition of NADPH (*17*). Therefore, phosphorylated p40PHOX was added between 2.5 and 3 min after the addition of NADPH, when the oxidase had reached full activation (linear part of the curve). Phosphorylated p40*PHOX* inhibited the oxidase when added before or after (Figure 4) activation of the enzyme.

Table 2: Effect of Phosphorylated and Unphosphorylated p40*PHOX* on NADPH Oxidase Activation by SDS[a]

	O_2^- production (% control)
no p40*PHOX*	100
unphosphorylated p40*PHOX*	62 ± 13
phosphorylated p40*PHOX*	56 ± 12

[a] The incubations were carried out as described under Experimental Procedures assaying O_2^- production by cytochrome *c* reduction. The reaction mixtures contained nonphosphorylated p47*PHOX* and 90 μM SDS as the activating stimulus. Results were taken from the mean ± SE of five or more separate experiments and expressed as percentage of the maximal value (obtained in the absence of p40*PHOX*). Differences in O_2^- production between no p40PHOX and phosphorylated p40*PHOX* or between no p40PHOX and unphosphorylated p40*PHOX* were both significant at $p < 0.05$.

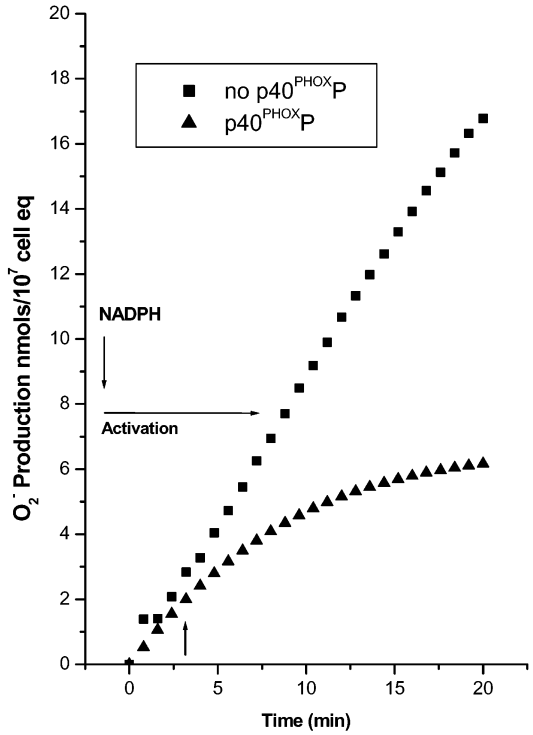

FIGURE 4: Phosphorylated p40*PHOX* inhibits p47*PHOX*P_6-activated NADPH oxidase. The incubations were carried out as described under Experimental Procedures. The effect of buffer only (no p40*PHOX*P), or phosphorylated p40*PHOX* (p40*PHOX*P) added after activation of the oxidase by p47*PHOX*P_6 is shown. The curves are representative of three or more separate experiments. The arrow indicates the moment when buffer only or p40*PHOX*P was added. The horizontal arrow indicates the time elapsed since the addition of NADPH (activation of the oxidase).

P40^{PHOX} *S315A Inhibits NADPH Oxidase Activation by p47*$^{PHOX}P_6$. A previous report (*27*) identified two in vivo protein kinase C phosphorylation sites on p40*PHOX*: threonine 154, localized 20 residues upstream of the SH3 domain, and serine 315, near the C-terminus of p40*PHOX*, the region that interacts with the inter-SH3 domain of p67*PHOX* (*35*−*37*). To determine which sites of p40*PHOX* phosphorylated by protein kinase C are necessary for the inhibition of the oxidase, p40PHOX mutants in which residues Thr154 (p40*PHOX* T154A) or Ser315 (p40*PHOX* S315A) or both (p40*PHOX* T154A/S315A) had been changed to alanine were added to the p47*PHOX*P_6-dependent system. Phosphorylation of the mutants was investigated in the presence of radioactive ATP. As shown in Figure 5A, p40*PHOX* S315A mutant and WT p40*PHOX*

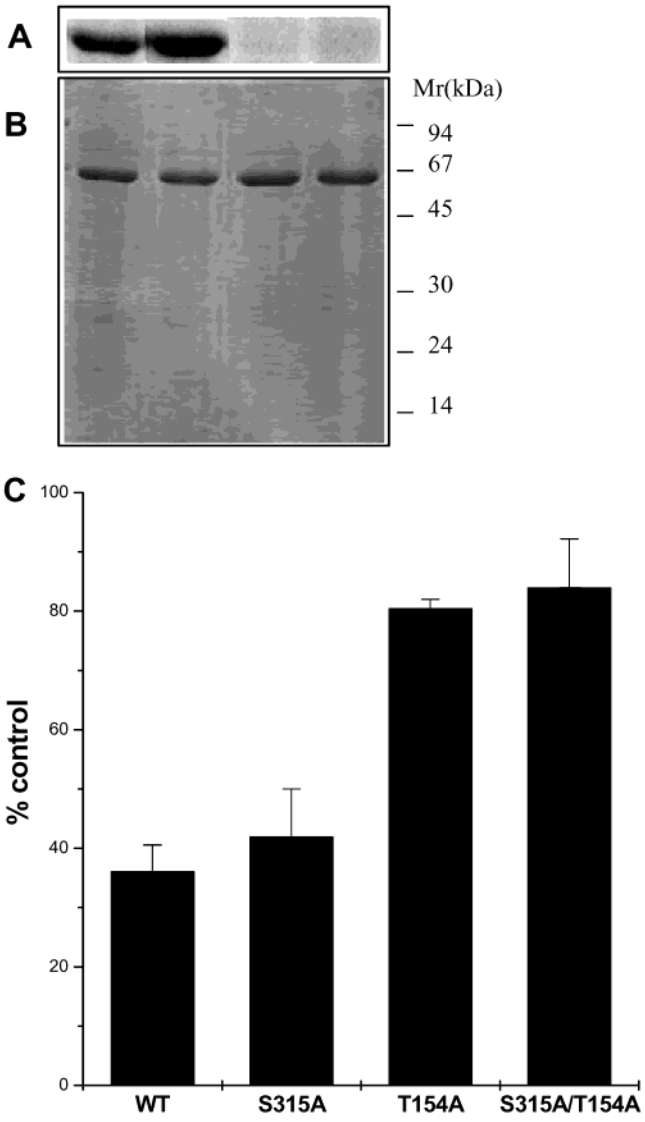

FIGURE 5: Phosphorylation of Thr154 is required for p40^{PHOX} inhibition of NADPH oxidase activation by p47^{PHOX}P$_6$. In panel A, for analysis of phosphorylation, p40^{PHOX}WT and p40^{PHOX} mutants were phosphorylated using γ-(^{32}P)-ATP as described in Experimental Procedures. Aliquots were loaded onto a SDS−PAGE, and phosphorylation was analyzed using a PhosphorImager. In panel B, the presence and integrity of the p40^{PHOX} WT and p40^{PHOX} mutants proteins was checked directly on the gel that was used for phosphorylation analysis after Coomassie Blue staining. In panel C, for NADPH oxidase activity, the incubations were carried out as described under Experimental Procedures assaying O_2^- production by cytochrome *c* reduction. The reaction mixtures contained 106 pmols of phosphorylated WT or mutant forms of recombinant p40^{PHOX}. Results are expressed as mean ± SE of three or more separate experiments and shown as percent of p47^{PHOX}P$_6$ activation of NADPH oxidase. Differences in the production of O_2^- between p40^{PHOX} WT and the p40^{PHOX} T154A mutant were significant at $p < 0.05$ and at $p < 0.01$ between p40^{PHOX} WT and p40^{PHOX} S315A/T154A.

are phosphorylated, whereas p40^{PHOX} T154A and the double mutant p40^{PHOX} T154A/S315A are not phosphorylated. We checked that absence of phosphorylated species was not due to disappearance of the protein (Figure 5B). The results of the cell-free assay are in good agreement with these findings (Figure 5C). Even though they have been incubated with protein kinase C, the p40^{PHOX} T154A mutant and the double mutant are not phosphorylated and behaved like unphosphorylated wild-type p40^{PHOX} in the oxidase assay. In contrast, phosphorylation of p40^{PHOX} S315A mutant (likely to occur on Thr154) appears to be sufficient for p40^{PHOX} to inhibit the oxidase as well as the phosphorylated wild-type protein. Absence of phosphorylation of p40^{PHOX}T154A mutant suggests a hierarchical mechanism of p40^{PHOX} phosphorylation, Thr154 being a primary site of phosphorylation by protein kinase C. As expected the double mutant p40^{PHOX} S315A/T154A behaved like p40^{PHOX}T154A and failed to inhibit the activation of the oxidase. Altogether these results suggest that the phosphorylation of Thr154 is necessary for the inhibition of superoxide production by p40^{PHOX}.

Effect of p40^{PHOX} Mutations on the Interaction of p40^{PHOX} with p47^{PHOX} and p67^{PHOX}. To understand the inhibitory effect of phosphorylated p40^{PHOX}, the interaction of the various phosphorylated mutants with the other cytosolic factors was investigated. Using pull-down assays, we see no difference in binding of the phosphorylated p40^{PHOX} mutants to GST−p47^{PHOX} or GST−p67^{PHOX}. We also compared the binding of phosphorylated p40^{PHOX} and nonphosphorylated p40^{PHOX} to phosphorylated GST−p47^{PHOX}, and we did not see a difference either (data not shown).

Protease Protection Assay of p40^{PHOX} and Mutants. Phosphorylated p40^{PHOX} (wild-type and mutants) were submitted to limited digestion by thrombin, and the proteolytic pattern after SDS−PAGE and Western blotting was examined. Control experiment consisted in overnight incubation in the absence of thrombin (Figure 6A). As shown in Figure 6B, the proteolysis pattern differs significantly between wild-type p40^{PHOX} and p40^{PHOX} S315A mutant (which inhibit the oxidase) on one hand, and p40^{PHOX} T154A and p40^{PHOX} S315A/T154A (which do not inhibit the oxidase) on the other hand. Nonphosphorylated proteins were completely degraded (Figure 6C). Surprisingly p40^{PHOX} T154A and p40^{PHOX} S315A/T154A, although not phosphorylated, showed a resistant pattern to thrombin. This effect was not observed in the presence of PKC activators only (data not shown), which indicates that protection is due to interaction with the kinase. Nevertheless, results clearly show that the two types of proteins have a different conformation pattern, which can be directly related to their inhibitory effect.

DISCUSSION

p40^{PHOX} is not an essential component of the NADPH oxidase complex in vitro (*15*, *16*). Although its interactions with both p67^{PHOX} and p47^{PHOX} (*8*, *9*, *13*, *25*, *35*, *37*, *38*, *39*) were extensively studied, the role of p40^{PHOX} in the activity or activation of NADPH oxidase remains controversial. p40^{PHOX} was shown to inhibit oxidase activation, whether added to the other cytosolic components in a cell-free assay activated by arachidonic acid or expressed by cotransfection into cells lacking the endogenous protein (*28*, *29*). On the other hand, a recent study by Kuribayashi (*31*) found a stimulatory role of p40^{PHOX} in virtually the same cells. In a fully purified cell-free system, Cross (*30*) observed an inhibition at high concentrations of p40^{PHOX} but a stimulation was found in a semipurified system when p47^{PHOX} was the limiting component. Along the same lines, antibodies against p40^{PHOX} were shown to inhibit NADPH oxidase in vitro (*9*), suggesting that p40^{PHOX} might be an activator of the NADPH oxidase, but nonspecific inhibition by addition of antibodies to the cell-free system could occur, especially in the presence of amphiphiles.

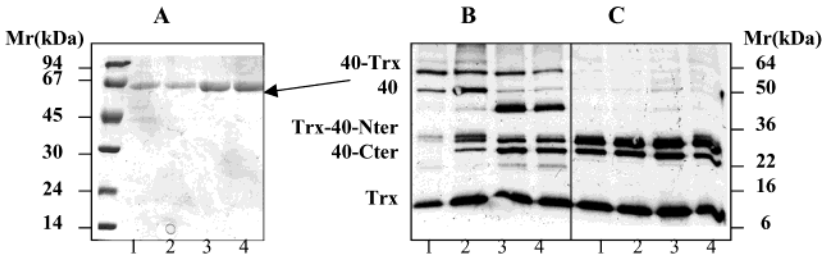

FIGURE 6: Limited proteolysis of unphosphorylated and phosphorylated p40^{PHOX} and mutants. Panel A shows SDS−PAGE of the p40^{PHOX} proteins fused to thioredoxin (Trx) after overnight incubation at 4 °C in Tris-HCl, 20 mM, pH 8.0, NaCl, 100 mM, in the absence of thrombin: lane 1, WT p40^{PHOX}; lane 2, p40^{PHOX} S315A; lane 3, p40^{PHOX} T154A; lane 4, p40^{PHOX} S315A + T154A. In panel B, phosphorylated proteins were submitted to limited proteolysis by thrombin, using 0.1 U of thrombin/20 μg of protein overnight at 4 °C in Tris-HCl, 20 mM, pH 8.0, NaCl, 100 mM. p40^{PHOX} fragments were analyzed by Western blotting using anti-p40^{PHOX} antibody: lane 1, WT p40^{PHOX}; lane 2, p40^{PHOX} S315A; lane 3, p40^{PHOX} T154A; lane 4, p40^{PHOX} S315A + T154A. The molecular weight (kDa) markers are indicated by arrows on the right, and the bands are labeled on the left. The experiment presented is representative of three separate experiments. In panel C, phosphorylated proteins were treated by thrombin under the same conditions: lane 1, WT p40^{PHOX}; lane 2, p40^{PHOX} S315A; lane 3, p40^{PHOX} T154A; lane 4, p40^{PHOX} S315A + T154A.

In cell-free systems activated with amphiphiles, the role of phosphorylation is difficult to observe because the negative charges brought by amphiphiles mimic phosphorylation. In the present study, we present evidence indicating that the addition of phosphorylated p40^{PHOX} to the recombinant system activated by PKC-phosphorylated p47^{PHOX} inhibited the oxidase. We also compared phosphorylated and nonphosphorylated p40^{PHOX} in a SDS-activated recombinant cell-free system and found no difference between the two forms. Altogether the present study corroborates previous studies (*28*, *29*) in demonstrating that unphosphorylated p40^{PHOX} is able to inhibit the amphiphilic activation of the oxidase. However, the present study shows that unphosphorylated p40^{PHOX} does not inhibit the oxidase when p47^{PHOX}P$_6$ is used instead of the amphiphile.

Interaction between p47^{PHOX} and p67^{PHOX} is considered to play a crucial role in forming an active NADPH oxidase complex (*3*). In resting cells, p40^{PHOX} is complexed to p67^{PHOX} (*8*), but during oxidase activation, p40^{PHOX} dissociates from p67^{PHOX} (*12*) after translocating to the membrane (*10*−*12*). Our findings that phosphorylated p40^{PHOX} but not the unphosphorylated protein inhibited the activation of the oxidase by protein kinase C phosphorylated p47^{PHOX} suggest a possible role for p40^{PHOX} in modulating the interactions between the *PHOX* components. However, we found no difference between binding to p47^{PHOX}, phosphorylated p47^{PHOX}, and p67^{PHOX} of phosphorylated and unphosphorylated p40^{PHOX} mutants. Moreover, phosphorylated p40^{PHOX} inhibits the NADPH oxidase even when added after full activation of the enzyme. It is noteworthy that in the classical systems, only small molecules had access to the oxidase when it was activated. In our recombinant p47^{PHOX}P$_6$-activated cell-free system, phosphorylated p40^{PHOX} apparently inhibits both activation and activity of the NADPH oxidase.

We observed that elimination of Thr154 abrogated phosphorylation of p40^{PHOX} and abolished the inhibitory effect of the protein in the p47^{PHOX}P$_6$-activated cell-free system. This is consistent with a hierarchical phosphorylation of protein kinase C consensus sites. Thr154 is located close to the SH3 domain of p40^{PHOX}, in a region extremely rich in basic residues. It is tempting to speculate that addition of a negative charge to this region would release an intramolecular interaction, thereby allowing subsequent phosphorylation of Ser315 or another yet uncharacterized protein kinase C site. In fact, phosphorylation of p40^{PHOX} changes the conformation of the protein as evidenced by a differential thrombin proteolysis pattern between the S315A mutant, which is phosphorylated on Thr154, and the T154A and T154A/S315A double mutants, which are not. The fact that p40^{PHOX} T154A and p40^{PHOX} T154A/S315A also change their conformation when they are incubated with PKC suggests that they might act as substrate traps for the kinase.

Our results are in contrast to a stimulatory effect of p40^{PHOX} described previously by Cross (*30*). While Cross used limited concentrations of p47^{PHOX} , in the present study, equimolar ratios of p47^{PHOX} and p67^{PHOX} were used in the present study. These ratios reflect the translocation of these subunits to the membrane as described by Quinn (*40*). In addition, our results confirm an inhibitory role for p40^{PHOX} in vivo described by Sathyamoorthy (*28*) who reported that transient expression of p40^{PHOX} leads to inhibition of NADPH oxidase activation when cells are stimulated with PMA. In fact, these authors demonstrated that the expression of the SH3 domain of p40^{PHOX} by itself inhibited oxidase activation more efficiently than the full length p40^{PHOX}. The SH3 domain of p40^{PHOX} is capable of binding to p47^{PHOX} at the same region targeted by p67^{PHOX} and therefore inhibits the binding of p67^{PHOX} to p47^{PHOX} (*37*). Activation of the oxidase by protein kinase C is known to change the conformation of p47^{PHOX} leading to the exposure of the SH3 domain enabling it to bind to p22^{PHOX} (*41*, *42*). One possible explanation for the inhibition of NADPH oxidase activity by p40^{PHOX} would be that in its phophorylated form p40^{PHOX} shifts to a conformation in which the SH3 domain of the protein is exposed and now inhibits, probably by competition, the binding of p67^{PHOX} to p47^{PHOX}, thereby shutting down the oxidase.

In conclusion, we propose a model in which p40^{PHOX} modulates NADPH oxidase and has two mechanisms, one inhibitory and one stimulatory. Phosphorylation affects the balance between the two effects in favor of an inhibition. One should also keep in mind that three spots of p40^{PHOX} are already present in the resting state of either human neutrophils or HL60 cells. Therefore another possibility would be that phosphorylated p40^{PHOX} prevents activation of the oxidase in the resting state.

Altogether results show that the phosphorylation of p40^{PHOX} induces a conformational change of the protein leading to an inhibition of the NADPH oxidase. Further studies are necessary to uncover the signaling pathways that control phosphorylation of p40^{PHOX}, but the present study

suggests that the regulatory effect of the p40^{PHOX} component involves phosphorylation of Thr154 by protein kinase C.

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