Activation of the Leukocyte NADPH Oxidase by Phorbol Ester Requires the Phosphorylation of p47PHOX on Serine 303 or 304*

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The leukocyte NADPH oxidase is an enzyme in phagocytes and B lymphocytes that when activated catalyzes the production of O_2\textsuperscript{-} from oxygen and NADPH. During oxidase activation, serine residues in the C-terminal quarter of the oxidase component p47PHOX become extensively phosphorylated, the protein acquiring as many as 9 phosphate residues. In a study of 11 p47PHOX mutants, each containing an alanine instead of a serine at a single potential phosphorylation site, we found that all but S379A corrected the defect in O_2\textsuperscript{-} production in Epstein-Barr virus (EBV)-transformed p47PHOX-deficient B cells (Faust, L. P., El Benna, J., Babior, B. M., and Chanock, S. J. (1995) J. Clin. Invest. 96, 1499–1505). In particular, O_2\textsuperscript{-} production was restored to these cells by the mutants S303A and S304A. Therefore, apart from serine 379, whose state of phosphorylation in the activated oxidase is unclear, no single potential phosphorylation site appeared to be essential for oxidase activation. We now report that the double mutant p47PHOX S303A/S304A was almost completely inactive when expressed in EBV-transformed p47PHOX-deficient B cells, even though it was expressed in normal amounts in the transfected cells and was able to translocate to the plasma membrane when the cells were stimulated. In contrast, the double mutant p47PHOX S303E/S304E was able to support high levels of O_2\textsuperscript{-} production by EBV-transformed p47PHOX-deficient B cells. The surprising discovery that the double mutant S303K/S304K was also able to support considerable O_2\textsuperscript{-} production suggests either that the effect of phosphorylation is related to the increase in hydrophilicity around serines 303 and 304 or that activation involves the formation of a metal bridge between the phosphorylated serines and another region of the protein.

The leukocyte NADPH oxidase is a membrane-associated enzyme in phagocytes and B lymphocytes that catalyzes the production of O_2\textsuperscript{-} from oxygen using NADPH as electron donor (1).

2 O_2 + NADPH → 2 O_2\textsuperscript{-} + NADP\textsuperscript{+} + H\textsuperscript{+} (Eq. 1)

Dormant in resting cells, it acquires catalytic activity when the cells are exposed to appropriate stimuli. Activation involves the transfer of cytosolic subunits designated p47PHOX and p67PHOX to the plasma membrane, where they associate with a flavocytochrome known as cytochrome b_558 to assemble the active oxidase (2).

During oxidase activation in whole cells, p47PHOX becomes phosphorylated on numerous serine residues that lie between Ser\textsuperscript{303} and Ser\textsuperscript{379} in the C-terminal quarter of this 390-residue molecule (3–7). Studies conducted to date have identified the phosphorylated serines (7–9), examined their susceptibility to phosphorylation by various protein kinases that occur in neutrophils (9, 10), and shown that in all likelihood no single phosphorylated serine is indispensable for oxidase activity (8). The present report is concerned with the phosphorylation of p47PHOX Ser\textsuperscript{303} and Ser\textsuperscript{304} in relation to the activation of the leukocyte NADPH oxidase.

MATERIALS AND METHODS

The mutants p47PHOX S303A/S304A, S303D/S304D, S303E/S304E, and S315A/S320A were constructed by single strand mutagenesis of the p47PHOX cDNA as described previously (8). Mutagenesis was performed on cDNA cloned into pBluescript II KS\textsuperscript{+}. The mutated cDNAs were then subcloned into the mammalian expression vector EBOpLP. Mutations that altered the sequence of the protein were accompanied by silent mutations of nearby restriction sites, introduced to aid in screening. The mutations were confirmed by sequencing in the departmental facility.

The S303K/S304K, S303A/S304E, and S303E/S304A mutations were constructed by a PCR\textsuperscript{3} strategy. In each case, the 5’ end of the “forward” primer spanned a unique Nar\textsuperscript{I} site 16 bases upstream of the 303 codon, the appropriately altered 303 and 304 codons, and 18 additional bases of native downstream sequence. A 19-base “reverse” primer that spanned an Nae\textsuperscript{I} site about 180 base pairs downstream from the 304 codon was used for all three PCR mutagenesis reactions. PCR was performed with Pfu polymerase (Stratagene) using the wild-type p47PHOX cDNA as template. The PCR products were isolated from agarose gels using QIAEXII (Qiagen) and digested with Nar\textsuperscript{I} and Nae\textsuperscript{I}.

The resulting fragments were ligated into the Nar\textsuperscript{I} site 16 bases upstream of the 303 codon of the appropriately altered S303 and S304 codons, and 18 additional bases of native downstream sequence. A 19-base “reverse” primer that spanned an Nae\textsuperscript{I} site about 180 base pairs downstream from the 304 codon was used for all three PCR mutagenesis reactions. PCR was performed with Pfu polymerase (Stratagene) using the wild-type p47PHOX cDNA as template. The PCR products were isolated from agarose gels using QIAEXII (Qiagen) and digested with Nar\textsuperscript{I} and Nae\textsuperscript{I}. The resulting fragments were ligated into the Nar\textsuperscript{I} and Nae\textsuperscript{I} sites of the p47PHOX S303A/S304A plasmid, a procedure that introduced the desired mutation into the product and destroyed a BseIII site that was introduced during the construction of the p47PHOX S303A/S304A cDNA. All PCR mutations were confirmed and the constructs documented to be error-free by sequencing across the full Nar\textsuperscript{I}-Nae\textsuperscript{I} span. Table I lists the primers and plasmids used in preparing the p47PHOX mutants.

EBV-transformed p47PHOX-deficient B lymphocytes were co-transfected with SV40 plus wild-type or mutant p47PHOX expression vectors as indicated, and expanded under hygromycin selection as described previously (8). Except that the p47PHOX-deficient cells were maintained at 0.5–1.0 × 10\textsuperscript{6} cells/ml after transfection and 10\textsuperscript{6} cells/ml after transfection.

* This work was supported in part by United States Public Health Service Grants AI-24227, AI-28479, AI-33346, DK-41625, and RR-00833 and by the Stein Endowment Fund. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked ‘advertisement’ in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: PCR, polymerase chain reaction; WT, wild-type; EBV, Epstein-Barr virus.
transfection. Transfected cells were assayed only when fewer than 10% of the cells took up trypan blue. The cell line used for these experiments contained an uncharacterized mutation in the p47\(_{PHOX}\) gene that prevented the expression of p47\(_{PHOX}\).

Leukocyte NADPH oxidase activity was measured by chemiluminescence assays using whole cells were carried out as described elsewhere (11), except that 4 \times 10^6 cells and 10 IU of horseradish peroxidase were used in a final volume of 0.35 ml. The cell suspensions were placed in a 96-well microplate, warmed to 37 °C, then activated at the same temperature with phorbol myristate acetate (1 µg/ml). Chemiluminescence was then measured at 1-min intervals using a Luminoskan luminometer ( Labsystems Research, Finland) at 37 °C. For measurement of leukocyte NADPH oxidase activity in a cell-free system, reaction mixtures contained 1.6 \times 10^5 cell equivalents of neutrophil membranes, 9 \times 10^5 cell equivalents of B lymphocyte cytosol, 1 mM luminol, 5 IU of horseradish peroxidase, 90 µM SDS, 160 µM NADPH, and Hanks’ balanced salt solution containing 0.5 mM CaCl\(_2\) and 1 mM MgCl\(_2\), with or without 50 IU of superoxide dismutase, in final volume of 1 ml. Cytosol was prepared by sonicating a suspension of lymphoblasts in Dulbecco’s phosphate-buffered saline for three 10-s intervals at 4 °C, then removing particles by centrifugation for 15 min at the same temperature in an Eppendorf Microfuge. Initially, the assay mixture contained all the components except SDS and NADPH. The oxidase was then activated by adding SDS and incubating for 1 min at room temperature. O\(_2^\bullet\) production was then initiated with NADPH, and chemiluminescence was measured at successive 10-s intervals using a Luminoskan luminometer at room temperature.

The expression of p47\(_{PHOX}\) in the transfected cells and the translocation of p47\(_{PHOX}\) from cytosol to membranes was determined by immunoblotting (8). Fractions were subjected to SDS-polyacrylamide gel electrophoresis on 10% polyacrylamide gels using the Laemmli buffer system. The separated proteins were electrophoretically transferred onto a nitrocellulose membrane, which was blocked with Blotto and probed with a 1:5000 dilution of an antibody against the C-terminal decapetide of WT p47\(_{PHOX}\). In measurements of p47\(_{PHOX}\) expression in whole cells, p47\(_{PHOX}\) on the immunoblots was visualized with alkaline phosphatase-labeled goat anti-rabbit immunoglobulin antibodies followed by visualization with the 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium detection system (12). Expression was measured by densitometry with a Zeinieh laser scanner, determining relative light units (U/mg).

### RESULTS

O\(_2^\bullet\) Production—In prior studies, we have shown that the activation of the leukocyte NADPH oxidase in neutrophils and EBV-transformed B lymphocytes is accompanied by the phosphorylation of serines 303 and 304 in the cytosolic oxidase component p47\(_{PHOX}\) (7). Consistent with the possibility that the phosphorylation of these serines is involved in oxidase activation, we reported that the conversion of either Ser303 or Ser304 to alanine resulted in a 50% decrease in O\(_2^\bullet\) production by EBV-transformed lymphocytes expressing the mutant p47\(_{PHOX}\) proteins (8). For the individual serines these decreases were not statistically significant (p > 0.05), but a level of significance of p < 0.004 was achieved when the results were reanalyzed under the hypothesis that the loss of either one of these serines resulted in a decrease in O\(_2^\bullet\) production (Wilcoxon rank sum test). This analysis suggested that the phosphorylation of at least one of these serines was necessary for oxidase activation in EBV-transformed B lymphocytes.

To test this possibility, O\(_2^\bullet\) production was measured in p47\(_{PHOX}\)-deficient EBV-transformed B lymphocytes expressing p47\(_{PHOX}\) with a number of double mutations at positions Ser303 and Ser304. Time courses for O\(_2^\bullet\) production by untransformed B lymphocytes and by cells expressing wild-type or mutant forms of p47\(_{PHOX}\) are illustrated in Fig. 1; peak chemiluminescence values for the same cells are shown in Fig. 2. Inspection of the results obtained with the mutant in which serines 303 and 304 were converted to alanines showed that O\(_2^\bullet\) production was nearly abolished by these mutations, supporting the idea that the phosphorylation of at least one of these serines is required for oxidase activation. A second double Ser → Ala mutant, p47\(_{PHOX}\) S310A/S315A, was fully active, indicating that the mere replacement with alanines of 2 serines in the region of the phosphorylation targets was not enough to abolish the activity of p47\(_{PHOX}\).

Further support for this idea was obtained when these two serines were replaced, not with alanines, but with negatively...
Expression and Translocation of the p47PHOX Mutants—One possible explanation for the inability of cells transfected with the S303A/S304A mutant to generate O$_2^*$ is that the EBV-transformed p47PHOX-deficient B cells were unable to express p47PHOX S303A/S304A, the mutant that failed to support O$_2^*$ production. The findings presented in Fig. 3 and Table II, however, show that all the mutant forms of p47PHOX examined in these experiments were expressed to approximately the same extent as the WT protein. The inability of p47PHOX S303A/S304A to support O$_2^*$ production therefore could not be attributed to a failure of expression by the transfected cells. A second possible explanation is that, although fully expressed, p47PHOX S303A/S304A could not fold into a native conformation, and was therefore unable to participate in the activation of the leukocyte NADPH oxidase. To investigate this possibility, we examined the ability of the mutant forms of p47PHOX to support O$_2^*$ production in a cell-free system in which activation was accomplished by an anionic detergent (in this case, SDS), not phosphorylation. Fig. 4 shows that there was little difference in O$_2^*$ production among cell-free systems containing the various forms of p47PHOX. In particular, the cell-free system that contained p47PHOX S303A/S304A produced O$_2^*$ at the same rate as the system that contained WT p47PHOX. Therefore the conformation of p47PHOX S303A/S304A was sufficiently similar to the native conformation that the mutant protein functioned normally in the cell-free oxidase activating system, even though it was inactive in whole cells.

Activation of the leukocyte NADPH oxidase is accompanied by the translocation of the oxidase components p47PHOX and p67PHOX from the cytosol to the plasma membrane, where they associate with the flavocytochrome b$_558$ (2, 14). This association is thought to result at least in part from the appearance of a membrane-binding site on p47PHOX when the oxidase is activated (12, 15). We found that when p47PHOX-deficient cells expressing the double mutants p47PHOX S303A/S304A, p47PHOX S303D/S304D, or p47PHOX S303E/S304E were activated with phorbol ester, the mutant p47PHOX polypeptides were transferred to the plasma membrane as efficiently as the WT protein (Fig. 5). The finding that p47PHOX S303A/S304A translocates normally suggests that a negative electrostatic potential in the vicinity of residues 303–304 is not needed for the binding of p47PHOX to the flavocytochrome, although it is required for the enzyme to express its catalytic activity. The phosphorylation of serines 303 and 304 causes a large increase in the polarity of the polypeptide in the vicinity of those residues. To determine whether the effect of phosphorylation on oxidase activity was due to the negative electrostatic potential or the increase in...
cells. Consequently, it was not possible to be absolutely certain from those results whether or not the phosphorylation of p47PHOX, either at Ser^{379} or at any other potential phosphorylation site, was required for the activation of the oxidase. Unlike Ser^{379}, serines 303 and 304 are extensively phosphorylated during oxidase activation (7, 9). In earlier work, we found that the oxidase could still be activated if either serines 303 or 304 were converted to alanine (8). The present results, however, show that both of these serines are required for the activation of the oxidase. Unlike Ser^{379}, serines 303 and 304 are not negatively charged carboxymethyl groups for the two hydroxymethyl groups known to be negatively charged in the S303K/S304K mutant. Various protein kinase inhibitors can diminish or prevent O_2^- production (16–18) while protein phosphatase inhibitors augment O_2^- production (19–23); and that recombinant p47PHOX phosphorylated by protein kinase C can activate the oxidase in the cell-free system in the absence of added detergents (24–26).

The effect of the serine-to-alanine mutations is to substitute methyl groups for two of the hydroxymethyl groups known to be phosphorylated during oxidase activation in whole cells (7). The conversion of these serines to alanines destroyed the activity of p47PHOX. In contrast, the conversion of the same serine to glutamate, a mutation that substituted negatively charged carboxymethyl groups for two of the hydroxymethyl groups known to be negatively charged in the S303K/S304K mutant, gave rise to mutant protein with considerable activity. The conversion of these serines to alanines destroyed the activity of p47PHOX. Therefore, apart from serine 379, no single phosphorylation site appeared to be essential for oxidase activation. As to Ser^{379}, its state of phosphorylation in the activated oxidase is unclear: it became phosphorylated only to a very limited extent when neutrophils were activated, and p47PHOX S379D was no more active than p47PHOX S379A in the p47PHOX-deficient oxidase activation system.

In an earlier study of 11 p47PHOX mutants containing an alanine instead of a serine at a single potential phosphorylation site, we found that all but p47PHOX S379A were active when expressed in EBV-transformed p47PHOX-deficient B cells (8). Therefore, apart from serine 379, no single phosphorylation site appeared to be essential for oxidase activation. As to Ser^{379}, its state of phosphorylation in the activated oxidase is unclear: it became phosphorylated only to a very limited extent when neutrophils were activated, and p47PHOX S379D was no more active than p47PHOX S379A in the p47PHOX-deficient oxidase activation system.

FIG. 5. Translocation of WT and mutant p47PHOX during oxidase activation. Experiments were carried out as described previously (8), except for changes in the immunoblotting procedure as described under "Materials and Methods." Results are representative of two experiments, each carried out in duplicate with a separate transfection. The track labeled CTRL contained 1.5 x 10^6 cell eq of cytosol. The remaining tracks each contained 1.25 x 10^6 cell eq of membrane. Plus (+) and minus (−) indicate cells activated with phorbol or resting cells, respectively.

FIG. 6. Translocation of p47PHOX S303K/S304K during oxidase activation. Experiments were carried out as described previously (8), except for changes in the immunoblotting procedure as described under "Materials and Methods." Results are representative of two experiments, each carried out in duplicate with a separate transfection. The track labeled CTRL contained 1.5 x 10^6 cell eq of cytosol. The remaining tracks each contained 1.25 x 10^6 cell eq of membrane. Plus (+) and minus (−) indicate cells activated with phorbol or resting cells, respectively.

p47PHOX

S303,304

CTRL
WT A D E

2 L. P. Faust and B. M. Babior, unpublished observation.
How can these local effects promote the activity of the leukocyte NADPH oxidase? Because the mutants all translocate normally, it is clear that phosphorylation at Ser\textsuperscript{303}-Ser\textsuperscript{304} is not an obligatory precursor of translocation. In principle, then, phosphorylation at these positions can take place either before or after the translocation of p47\textsuperscript{PHOX} to the membrane. Earlier evidence showing that the final 1 or 2 phosphorylations take place after translocation (6) suggests that at least some p47\textsuperscript{PHOX} is phosphorylated after it is transferred from the cytosol to the membrane. It seems likely that the phosphorylation of Ser\textsuperscript{303} and Ser\textsuperscript{304} converts the membrane-bound subunit into a form that is able to render the oxidase catalytically active.

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\textit{J. Biol. Chem.} 1998, 273:9539-9543.
doi: 10.1074/jbc.273.16.9539

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