The Phosphorylation Targets of p47^{phox}, a Subunit of the Respiratory Burst Oxidase

Functions of the Individual Target Serines as Evaluated by Site-directed Mutagenesis

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Abstract

The respiratory burst oxidase of phagocytes and B lymphocytes catalyzes the reduction of oxygen to O_2^- at the expense of NADPH. Dormant in resting cells, the oxidase is activated by exposing the cells to appropriate stimuli. During activation, p47^{phox}, a cytosolic oxidase subunit, becomes extensively phosphorylated on a number of serines located between S303–S379. To determine whether this phosphorylation is necessary for oxidase activation, we examined phorbol-elicited oxidase activity in EBV-transformed B lymphoblasts deficient in p47^{phox} after transfection with plasmids expressing various S → A mutants of p47^{phox}. The mutant containing S → A mutations involving all serines between S303 and S379 [S(303–379)A] was not phosphorylated, did not translocate to plasma membrane during activation and was almost devoid of function. As to individual serines, S379 was of special interest because (*a*) p47^{phox} S379 was phosphorylated in phorbol-activated lymphoblasts expressing wild-type p47^{phox}, and (*b*) p47^{phox} S379A failed to translocate to the membrane, and was as functionless as p47^{phox} S(303–379)A; other single S → A mutations had little effect on oxidase activity. These findings suggest that the phosphorylation of S379 may be important for oxidase activation in whole cells. (*J. Clin. Invest.* 1995. 96:1499–1505.) Key words: superoxides • phagocytes • phosphorylation • B lymphocytes • respiratory burst

Introduction

The respiratory burst oxidase is a membrane-bound enzyme of phagocytes and B lymphocytes that catalyzes the reduction of oxygen to O_2^- at the expense of NADPH (1):

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

In resting cells the enzyme is dormant, and its components are distributed between the cytosol and the plasma membrane. When the cells are activated, however, the cytosolic components migrate to the plasma membrane, where they associate with the membrane-bound components to assemble the catalytically active oxidase (2).

Among the cytosolic oxidase components is p47^{phox} (3–6), a basic protein that becomes extensively phosphorylated when the oxidase is activated (7–10). The phosphorylation targets are a group of serines (S303 through S379) in the highly basic carboxy-terminal quarter of the polypeptide (11). The relationship between the phosphorylation of p47^{phox} and the initiation of O_2^- production by neutrophils exposed to an activating agent suggested that the phosphorylation of this protein was an essential step in the mechanism of activation of the oxidase. To date, however, this hypothesis has been supported only by the indirect evidence that has been provided by inhibitor studies (12–16), temporal correlations between phosphorylation and oxidase activation (17, 18), and demonstrations that ATP is required for oxidase activation (19, 20).

Phagocytes and B lymphocytes from patients with p47^{phox} deficiency are unable to manufacture O_2^- in response to agents that elicit O_2^- production from normal cells. We recently demonstrated that the respiratory burst in EBV-transformed B lymphoblasts (hereafter called lymphoblasts) deficient in p47^{phox} could be restored by transfection with a plasmid that caused the lymphoblasts to express p47^{phox} (21). We have used this system to obtain direct evidence regarding the role of phosphorylated p47^{phox} in the activation of the respiratory burst oxidase.

Methods

EBV-transformed p47^{phox}-deficient B lymphoblasts were co-transfected with SV40 and EBOpLPP-derived p47^{phox} expression vectors (wild-type or mutant pEBOp47fx$^+$ or the antisense vector pEBOp47fx$^-$) and expanded under hygromycin selection as described elsewhere (21).

^{32}P-labeled p47^{phox} from transfected cells. Transfected p47^{phox}-deficient B lymphoblasts expressing wild-type p47^{phox} or p47^{phox}S(303–379)A were incubated overnight at 37°C in DMEM/10 mM Hepes (pH 7.5)/2 mM glutamine/0.1% FBS/1% PSF (GIBCO BRL, Gaithersburg, MD) (10^6 cell/ml), then transferred to fresh medium containing $^{32}P_i$ (0.2 mCi/ml; Dupont, NEN, Boston, MA) and incubated for an additional 4 h at 37°C. The cells were then activated for 20 min with phorbol myristate acetate (1 μg/ml), after which their p47^{phox} was isolated and purified by immunoaffinity chromatography as described earlier (11). For one-dimensional (1-D) peptide mapping, the purified ^{32}P-labeled p47^{phox} was eluted from the SDS-PAGE gel and cleaved with CNBr (12.5 mg/ml in 70% formic acid, 16 h, 25°C), Glu-C endopeptidase (100 μg/ml in 25 mM ammonium bicarbonate, pH 7.8, overnight, 25°C; Boehringer Mannheim Corp., Indianapolis, IN), or Lys-C endopeptidase (20 μg/ml in 50 mM Tris-HCl, pH 8.0, overnight, 37°C; Boehringer Mannheim) as indicated. The digests were lyophilized in a Speed-Vac and analyzed by Tris-Tricine SDS-PAGE (22) followed by autoradiography for 24 h at −70°C.

Respiratory burst oxidase activity. Respiratory burst oxidase activity

L. P. Faust and J. El Benna contributed equally to this work.

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Table I. Mutagenesis of p47^{phox}

Mutation	Oligonucleotide	Template plasmid	Restriction site change
S303A	AT GGA CGC GCG CCG GGG CGG CGC	WT	+ BssHII
S304A	CG GAT GGC CGA CCT GCG GGG	S303A	− BssHII
S310A	TG GAT GGC GTG CGC ATT CCG GAT GGA	WT	+ BsmI
S315A	TT CCG CGC GCG CTG ATG GAT	WT	+ BssHII
S320A	TC CTG GGC GAG CCG CTT CCG CGA	WT	− EcoNI
S328A	CG TAC GGC GTT GCG GCG ATA GGC	WT	+ BsiWI
S(345, 348)	ACC GGG GGC CTG CGG GCC CGG CCG CGC	S348A	+ ApaI
S348A	AG CGG GGC TCC GGG GCT CTG	WT	− SmaI
S359A	G TTT AGC GCG CTG CGT CTG CC	WT	+ BssHII
S370A	GTC GGC GGC AGG CCT CGG GGG CAC	WT	− SacII
S379A	G CTT GGT GCT CTC GGC GCA GC	WT	− PstI

Oligonucleotide sequences are those of the antisense strand of the p47^{phox} cDNA. Deviations from the wild-type sequence are shown in boldface. *WT*, wild-type.

was measured by chemiluminescence. Assays using whole cells were carried out as described elsewhere (23), except that 3×10^6 cells and 5 IU horseradish peroxidase were used in a final volume of 0.5 ml. The cells were warmed to 37°C, then activated for 5 min at the same temperature with phorbol myristate acetate (1 μg/ml). Chemiluminescence was then measured at 2-min intervals using a Monolight 2010 luminometer (Analytical Luminescence Laboratories, San Diego, CA). For measuring respiratory burst oxidase activity in a cell free system, the complete reaction mixtures contained 1.6×10^6 cell equivalents of neutrophil membranes, cytosol (0.2 mg protein) obtained from unstimulated lymphoblasts in the course of the translocation assays described below, 1 mM luminol, 5 IU horseradish peroxidase, 90 μM SDS, 160 μM NADPH, Hanks balanced salts solution containing 0.5 mM $CaCl_2$ and 1 mM $MgCl_2$, and 50 U of superoxide dismutase as indicated, in a final volume of 0.5 ml. The starting assay mixtures contained all the components except SDS and NADPH. The oxidase was then activated by adding SDS and incubating for 1 min at room temperature. After activation, the reaction was started with NADPH (final concentration 160 μM). Chemiluminescence was then measured at successive 10-s intervals.

Translocation of p47^{phox} from cytosol to plasma membrane. Transfected lymphoblasts were treated for 15 min at 4°C with diisopropylfluorophosphate as described elsewhere (24). After washing with PBS, the cells were resuspended in the same buffer at 10^8 cell/ml and incubated for 10 min at 37°C. Half the cells were then stimulated with phorbol myristate acetate (1 μg/ml), after which both stimulated and unstimulated cells were incubated for an additional 12 min. The cells were then resuspended in 2 ml of ice-cold relaxation buffer (10 mM Pipes, pH 7.4, 100 mM KCl, 3 mM NaCl, 3.5 mM $MgCl_2$, 1 mM ATP, and 1.5 mM EGTA) containing 1 mM PMSF, 50 μg/ml leupeptin, 25 μg/ml pepstatin, and 25 μg/ml aprotinin. Further procedures were carried out at 4°C. The cells were disrupted by nitrogen cavitation (400 psi for 15 min) and centrifuged (400 *g*, 10 min) to remove nuclei and unbroken cells. The supernatant was further centrifuged (100,000 *g*, 45 min) to separate membranes from cytosol. The membrane pellet was washed once in the antiproteinase-fortified relaxation buffer, then suspended in Laemmli sample buffer and heated in boiling water for 3 min. Finally, solid urea was added to the sample solution (final concentration 4 M) and dissolved by careful sonication, to improve the resolution of the gel.

Immunoblotting. Transfected lymphoblasts were suspended at 2.5×10^7 cells/ml in detergent buffer (20 mM Tris-HCl, pH 7.4, 0.5% Triton X-100, 150 mM NaCl, 2.5 mM EGTA, 2.5 mM EDTA, 0.25 M sucrose, 1 mg/ml DNAse I, 50 μM leupeptin, 25 μM pepstatin, and 2 mM PMSF), sonicated for three 10-s intervals and centrifuged at 4°C for 15 min in an Eppendorf microfuge. An equal amount of 2× Laemmli sample buffer was then added to the sonicate, and proteins were separated by SDS-PAGE on a 10% polyacrylamide gel. The separated proteins were then transferred to nitrocellulose and the p47^{phox} bands were visualized with the ECL detection system (Amersham) or as described elsewhere (25), using an anti-peptide antibody at a dilution of 1:5,000.

Site-directed mutagenesis. For site-directed mutagenesis, the p47^{phox} cDNA was cloned into the XbaI/NotI fragment of pBluescript KSII. Mutagenesis was then performed by an oligonucleotide-directed technique, confirming each mutant by dideoxynucleotide-based sequencing. The primers and certain other details related to the mutagenesis procedures are shown in Table I. *Escherichia coli* strain BMH 71-18 (Clontech, Palo Alto, CA) was used for transformation to increase the recovery of the mutated plasmids. For ease of screening of the transformants, oligonucleotides were designed so as to contain an altered restriction site as well as the desired mutant codon. The mutant plasmids were cloned into the XbaI/NotI fragment of the mammalian expression vector EBOpLPP and transfected into p47^{phox}-deficient B lymphoblasts as described elsewhere (21).

Phosphorylation of S379. ^{32}P-labeled p47^{phox} was purified from $^{32}P_i$-loaded, phorbol-activated transfected lymphoblasts as described above and transferred to nitrocellulose by electroblotting. The portion of the blot containing the radioactive band was excised, placed in 0.25 ml ammonium bicarbonate buffer and cleaved with trypsin for 72 h at 37°C, adding 10 μg trypsin at the start of the incubation and every 12 h thereafter to ensure complete digestion (11). The digest was taken to dryness in a Speedvac, dissolved in 0.4 ml PBS containing 50 μM 2-mercaptoethanol and rotated for 2 h at room temperature with 50 μl 2-thiopyridine–activated thiopropyl agarose beads (Sigma Chem. Co., St. Louis, MO). The beads were then separated from the peptide solution by centrifugation, washed four times with 1-ml portions of PBS/0.5 M NaCl and twice more with 1-ml portions of PBS, then eluted by incubating for 20 min at room temperature with 100 mM dithiothreitol in PBS. The radioactivity in the peptide-containing supernatant, the washings and the final eluate was determined by Cerenkov counting in a liquid scintillation counter.

Results

p47^{phox} phosphorylation targets in B lymphoblasts. During activation, neutrophils phosphorylate p47^{phox} on serines lying between S303 and S379 inclusive (11). To determine whether these same serines are the phosphorylation targets in B lymphocytes, p47^{phox} from EBV-transformed normal B lymphoblasts that had been loaded with $^{32}P_i$ and then activated with phorbol myristate acetate was purified and analyzed by 1-D peptide

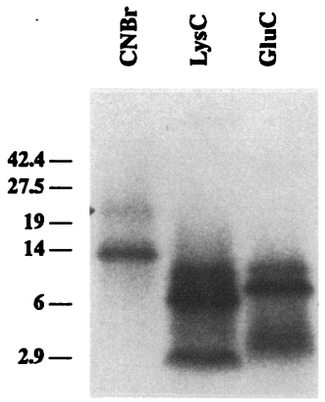

Figure 1. Phosphopeptide mapping of ^{32}P-labeled p47^{phox} purified from phorbol-activated B lymphoblasts. ^{32}P-labeled p47^{phox} was purified from 5 × 10^7 phorbol-activated lymphoblasts and cleaved with the indicated reagent as described in Methods. The products were separated by SDS-PAGE using a Tris-Tricine buffer system, and the labeled peptides were visualized by autoradiography. The serine residues present in the fragments were as follows: CNBr, S283-S388; LysC (9K peptide), S320-S381; LysC (3K peptide), S303-S315; GluC (10K peptide), S283-S348; GluC (4K peptide), S359-S379). The smallest labeled peptide in the GluC track probably arose through partial cleavage of the 4K peptide at D372, eliminating the 1K peptide L373-E380. The 9K peptide in the LysC digest appears because LysC is unable to cleave K-P bonds (30).

mapping using the approach employed in earlier studies with neutrophils (11). Results showed that, as with neutrophils, the p47^{phox} phosphorylation targets in B lymphoblasts were restricted to the carboxy-terminal CNBr fragment ($M_r \approx 12{,}000$), containing serines S283 through S388 (Fig. 1). Maps of products obtained by digestion with the endopeptidases Lys-C and Glu-C showed no radioactivity in peptides containing serines S283, S288, S381, and S388, leaving the serines between S303 and S379 (inclusive) as the targets of phosphorylation in B lymphoblasts, as they are in neutrophils.

p47^{phox}S(303-379)A. To ascertain whether phosphorylation of p47^{phox} was necessary for oxidase activity, we employed a plasmid (pEBOp47fx:*S[303–379]A*) that encoded a p47^{phox} mutant in which all the serines between S303 and S379 had been converted to alanines. The mutant protein, designated p47^{phox}S(303–379)A, was found not to become detectably phosphorylated during cell activation. Mutant protein purified from phorbol-activated p47^{phox}-deficient B lymphoblasts transfected with pEBOp47fx:*S(303–379)A* and loaded with $^{32}P_i$ contained negligible amounts of phosphate, whereas normal protein purified from activated $^{32}P_i$-loaded lymphoblasts transfected with the wild-type plasmid was heavily phosphorylated (Fig. 2, *right*). This difference between the mutant and wild-type p47^{phox} was not due to a deficiency of p47^{phox}S(303–379)A in the transfected lymphoblasts, because the mutant and

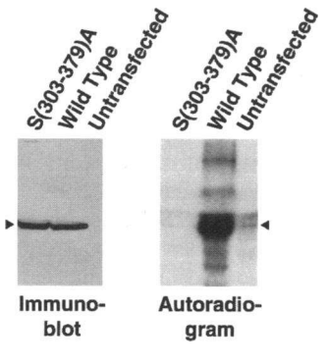

Figure 2. Phosphorylation of recombinant p47^{phox}S(303-379)A from transfected p47^{phox}-deficient B lymphoblasts activated with phorbol myristate acetate. The experiment was carried out as described in the text. Each track contains p47^{phox} from 3 × 10^7 cells. (*Left*) protein expression (immunoblot); (*right*) autoradiogram of the same immunoblot. The arrowheads show the location of p47^{phox}.

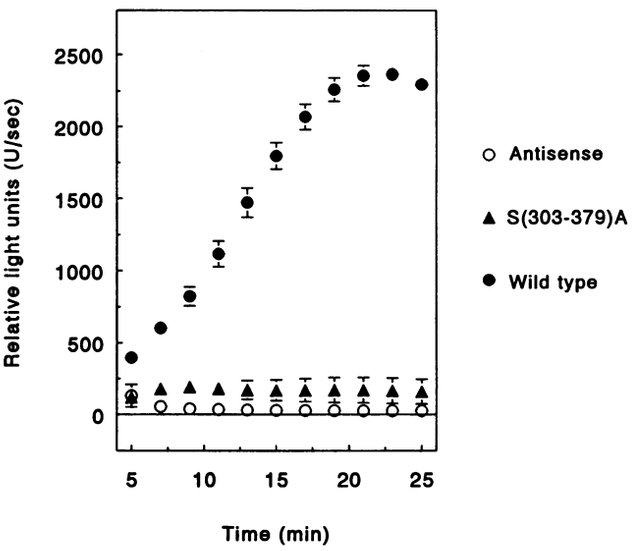

Figure 3. Oxidase activity of phorbol-activated p47^{phox}-deficient B lymphoblasts expressing recombinant p47^{phox}S(303–379)A. Oxidant production was measured as described in the text. Results represent the mean±SE of data from the following number of transfections: wild-type, 2; antisense, 3; S(303–379), 3. In this and subsequent figures, the absence of an error bar through a marker indicates that the error is smaller than the height of the marker.

wild-type proteins were expressed to about the same extent (Fig. 2, *left*).

We next asked whether p47^{phox}S(303–379)A could restore the respiratory burst to p47^{phox}-deficient lymphoblasts. Fig. 3 compares oxidase activity in phorbol-stimulated B lymphoblasts transfected with pEBOp47fx$^+$ (expressing wild-type p47^{phox}), pEBOp47fx$^-$ (antisense) and pEBOp47fx:*S(303–379)A.* Even though p47^{phox} expression was similar in the wild-type and mutant transfectants (see Fig. 2), oxidant production in cells expressing p47^{phox}S(303–379)A was greatly decreased compared with oxidant production in lymphoblasts expressing wild-type p47^{phox} (Fig. 3). These findings strongly suggest that the phosphorylation of p47^{phox} is essential for normal oxidase activation.

To rule out the trivial possibility that the very low oxidase activity observed in cells expressing the mutant p47^{phox} resulted from a failure of the mutant to fold into a functionally active conformation during its biosynthesis, we examined the ability of cytosols from lymphoblasts expressing wild-type and mutant p47^{phox} to support the function of the cell-free oxidase activating system, reasoning that if the mutant was able to support oxidase activity in the cell-free system, it had to have folded correctly during biosynthesis. The assays were carried out as usual (26), except that lymphoblast instead of neutrophil cytosol was used, and oxidase activity was measured by chemiluminescence. The results (Fig. 4) showed that p47^{phox}S(303–379)A supported oxidase activity in the cell-free system, indicating that the protein achieved an active conformation despite its content of S → A mutations.

The individual target serines. The experiments with p47^{phox}S(303–379)A suggested that at least one of the serines between S303 and S379 was required for oxidase activity in whole cells. This may reflect an absolute requirement for a particular serine, or a requirement for one of a group of serines

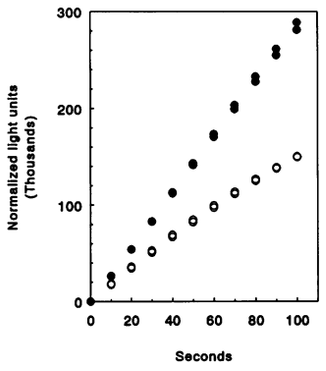

Figure 4. Oxidase activity in a cell-free oxidase activating system containing recombinant p47^{phox}S(303–379)A. Oxidant production was measured as described in the text. A different transfection was used for each assay. In each case, the addition of 50 U of superoxide dismutase to the assay mixture virtually abolished light production (not shown). The results shown represent the sums of the light units as measured over successive 10-s intervals, and are corrected for the fact that in the cytosols used for this experiment, the concentration of p47^{phox}S(303–379)A was 70% the concentration of wild-type p47^{phox}. •, wild-type; ○, p47^{phox}S(303–379)A.

any member of which could serve to support oxidase activation. To determine whether any of these 11 serines was essential as a single residue, we created a series of 11 constructs each containing a serine-to-alanine codon mutation involving a different target serine (see Table I), and measured oxidase activity in B lymphoblasts transfected with each of these constructs. The results (Fig. 5 and Table II) show that each of the p47^{phox} mutants was well expressed, and that of all the mutations, only S379A had a major effect on the ability of p47^{phox} to support oxidase activation. (The S345A mutation is represented in this study by the double mutant S(345,348)A. The control for this mutant is S348A.) Therefore, apart from S379, no individual target serine appears to be of major importance for the function of p47^{phox}.

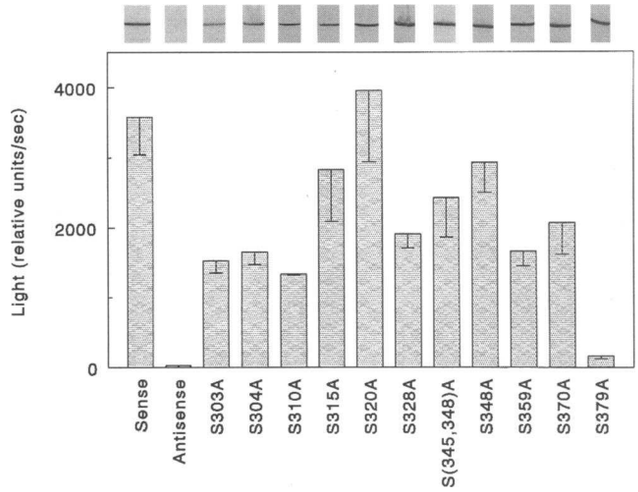

Figure 5. Oxidase activity of lymphoblasts expressing recombinant p47^{phox} containing single S → A substitutions involving the phosphorylation targets. Oxidant production by lymphoblasts expressing wild-type (*Sense*) p47^{phox}, mutant p47^{phox} as indicated, or no p47^{phox} (*Antisense*) was measured as described in the text. Except for the experiments with the antisense transfections, the results shown represent the peak chemiluminescence values, which were generally observed 10–20 min after adding phorbol. In the case of the antisense transfections, chemiluminescence declined steadily after activation with phorbol. For these transfections, the results used were the values observed 15 min after the addition of phorbol. Immunoblots above each bar indicate the levels of expression of the corresponding recombinant proteins, using 7.5×10^5 cells for mutants S310A, S315A, S320A, S359A, and S379A, and 5×10^5 cells for the rest of the transfectants. Chemiluminescence results represent the mean±SE of 3 experiments, except as follows: S310A and S(345,348)A, 2 each; S348A, 4; S379A, 5; S370A, 6; wild-type, 7. In calculating the statistics, outliers of 11071 (S310A) and 12882 (S328A) were disregarded.

Table II. Expression of p47^{phox} Mutants in Transfected p47^{phox}-deficient B Lymphoblasts Relative to the Expression of the Wild-type Protein

p47^{phox}	Expression	
	% wild-type	*n*
Wild-type	100	(7)
S303A	66±12	(3)
S304A	69±15	(3)
S310A	100	(1)
S315A	57	(1)
S320A	118±6	(2)
S328A	107±4	(3)
S345/348A	140±38	(2)
S348A	101±9	(3)
S359A	108±17	(2)
S370A	102±8	(3)
S379A	132±15	(6)
S(303-379A)	83±7	(4)

Immunoblots of p47^{phox}-deficient B lymphoblasts expressing wild-type or mutant p47^{phox} were probed with anti-p47^{phox} antibody (see Methods), visualizing with a peroxidase-labeled secondary antibody as described elsewhere (25). All tracks on each blot represented samples from cells that had been transfected on the same day and collected at the same time. The identities of the mutants varied from blot to blot, but for use as standard, one track on each blot was run with lymphoblasts expressing wild-type p47^{phox}. The blots were scanned with a Zeinieh laser scanner, and quantities of p47^{phox} were determined from the peak heights (in earlier studies we had shown that the heights of the p47^{phox} peaks on immunoblots visualized with a peroxidase-labeled secondary antibody were directly proportional to the amounts of p47^{phox} loaded onto the original gel). Amounts of mutant protein relative to the amount of protein in the lymphoblasts expressing wild-type p47^{phox} were calculated for each blot by dividing the height of the mutant p47^{phox} peak by the height of the standard peak, correcting if necessary for differences in loading of the gel. The results are expressed as the mean±1 SE. The figures in parentheses indicate the number of samples used for each calculation.

Fig. 6 shows in more detail the activity of the oxidase in phorbol-treated p47^{phox}-deficient B cells expressing p47^{phox}-S379A, as compared with the same cells transfected with pEBOp47fx$^+$ (sense) or pEBOp47fx$^-$ (antisense). The mutant protein was adequately expressed in the transfected B lymphoblasts (Fig. 5, Table II), but the conversion of S379 to alanine greatly hampered the ability of the protein to support oxidase activation. Oxidase activity in a cell-free system containing p47^{phox}S379A, however, was nearly normal (Fig. 7), showing that p47^{phox}S379A, like p47^{phox}S(303-379)A, acquired a native conformation during biosynthesis.

Activation of the respiratory burst oxidase is accompanied by the transfer of p47^{phox} (and p67^{phox}) from the cytosol to the plasma membrane. To determine whether S379 was important

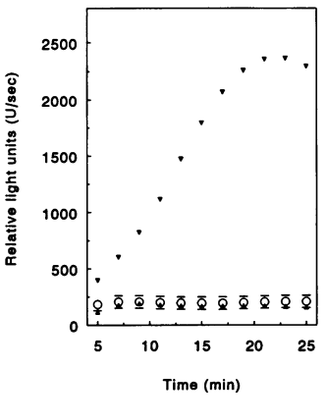

Figure 6. Oxidase activity of phorbol-activated p47^{phox}-deficient B lymphoblasts expressing recombinant p47^{phox}S379A. Results (○) represent the mean±SE of data from three transfections. Mean values for the wild-type (▼) and S(303–379)A (▲) experiments, presented in Fig. 3, are presented again here for purposes of comparison.

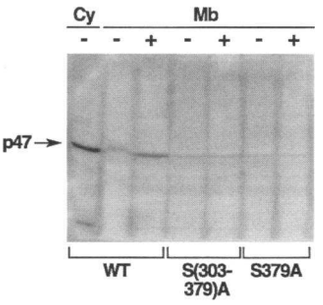

Figure 8. Translocation of p47^{phox} in phorbol-activated p47^{phox}-deficient B lymphoblasts expressing wild-type and mutant recombinant p47^{phox}. The experiment was carried out as described in the text, visualizing the p47^{phox} bands by chemiluminescence. The track labeled *Cy* contained 1.5×10^6 cell equivalents of cytosol; each of the *Mb* tracks contained 1.25×10^7 cell equivalents of membrane. +, activated cells; −, resting cells. This experiment is representative of six experiments with p47^{phox}S379A and two experiments with p47^{phox}S(303–379)A.

for this transfer, we performed an experiment in which plasma membranes from p47^{phox}-deficient B lymphoblasts expressing various forms of recombinant p47^{phox} were assayed for p47^{phox} before and after activation with phorbol. The results (Fig. 8) showed that wild-type p47^{phox} was transferred to the membrane after activation, but little transfer of p47^{phox}S(303–379)A or p47^{phox}S379A was detected. These findings are consistent with earlier work showing that the phosphorylation inhibitor staurosporine prevented translocation of p47^{phox} in whole neutrophils (27), and indicate that S379 participates in the translocation of p47^{phox} that takes place during oxidase activation, suggesting that the defect in O_2^- production in B lymphoblasts expressing p47^{phox}S379A is due at least in part to a failure of translocation.

To determine whether S379 becomes phosphorylated when the respiratory burst oxidase is activated, the phosphate content of the tryptic peptide containing this serine was measured. For this purpose, p47^{phox}-deficient lymphoblasts expressing either wild-type p47^{phox} or p47^{phox}S379A were loaded with $^{32}P_i$ and activated with phorbol. The ^{32}P-labeled p47^{phox} species from these two lymphoblast preparations were purified and digested with trypsin, and the ^{32}P in the combined cysteine-containing peptides from this digest was determined after isolation of the peptides by transthiolation of activated thiopropyl agarose. Among the 4 cysteine-containing tryptic peptides from p47^{phox}, only one (CSESTK, corresponding to amino acids 378–383) contained a target serine, and that serine was S379, the serine of interest. The cysteine peptides obtained from wild-type p47^{phox} contained twice as much ^{32}P as the peptides from p47^{phox}S379A (Table III), indicating that S379 was phosphorylated. Although the amount of phosphate in S379 was small, amounting to only ~ 0.12% of the total, it was in the range expected for a single phosphorylated serine in the small fraction of p47^{phox} that had been incorporated into the active oxidase (see Discussion).

A small amount of ^{32}P was also found in the cysteine-containing peptides from p47^{phox}S379A. This could be the result of nonspecific binding of peptides to the column, but it might also represent the phosphorylation of S381 during oxidase activation. The failure to detect a radioactive Glu-C peptide containing S381 (STKRKLASAV, the COOH-terminal decapeptide of p47^{phox}) suggests that nonspecific binding is the most likely explanation, but a clear decision between these two possibilities will require additional experiments.

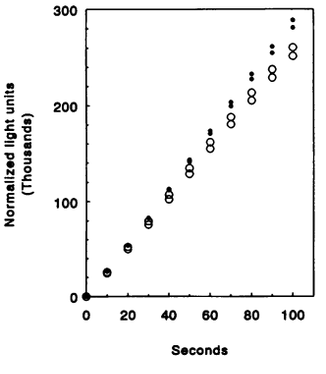

Figure 7. Oxidase activity in a cell-free oxidase activating system containing recombinant p47^{phox}S379A. Oxidant production was measured as described in the text. In each case, the addition of 50 U of superoxide dismutase to the assay mixture virtually abolished light production (not shown). A different transfection was used for each assay. The results shown represent the sums of the light units as measured over successive 10-s intervals. The average concentrations of wild-type and mutant p47^{phox} in the lymphoblast cytosols were essentially equal, so it was not necessary to correct for differences between cytosols in the concentrations of p47^{phox}S379A. The wild-type results are the same as those shown in Fig. 4. ●, wild-type; ○, p47^{phox}S379A.

Discussion

The relationship between respiratory burst oxidase activation and protein phosphorylation—in particular, the phosphorylation of p47^{phox}—has been the subject of speculation ever since the discovery of a 44 kD (Named p47^{phox} because on SDS-PAGE, this protein migrated at M_r 47-48 × 10^3.) protein that became phosphorylated when neutrophils were activated and was missing in certain patients with chronic granulomatous disease, an inherited deficiency of respiratory burst oxidase activity. Correlative studies, inhibitor studies and demonstrations of an ATP requirement for oxidase activation have all been consis-

Table III. Phosphorylation of S379

	^{32}P			
	Experiment 1		Experiment 2	
	WT	S379A	WT	S379A
	cpm		*cpm*	
Total counts	24749	29145	64528	73014
Final wash	31	25	20	33
Eluate	84	51	179	129
^{32}P in CSESTK (%)*	0.26	0.11	0.24	0.14

* Corrected for background (20 cpm).
The experiments were carried out as described in the text.

tent with the idea that the phosphorylation of $p47^{phox}$ is an essential element of oxidase activation, but they all left open the possibility that the true oxidase-activating phosphorylation might involve some other protein, and that the phosphorylation of $p47^{phox}$ was merely an epiphenomenon. The results presented here furnish further evidence for the proposition that the phosphorylation of $p47^{phox}$ is causally related to the activation of the respiratory burst oxidase.

Of the 11 phosphorylation targets in $p47^{phox}$, only the elimination of S379 led to a major defect in the translocation of $p47^{phox}$ and the activity of the oxidase. The conversion of any one of the other 10 target serines to alanine had relatively little effect on oxidase activity (and presumably on translocation as well). The occurrence, however, of low levels of oxidant production by cells expressing $p47^{phox}$S379A, as opposed to the absence of oxidant production by cells transfected with the antisense plasmid, suggests that the story of $p47^{phox}$ phosphorylation is not yet complete. Additional chapters may involve such subjects as combinations of target serines, activating agents besides phorbol myristate acetate, or other topics yet to be investigated.

Only a very small percentage of the phosphate in phosphorylated $p47^{phox}$ was located on S379. Although this result suggests that the phosphorylation of S379 may not be related to oxidase activation, the following line of reasoning indicates that this quantity might be enough to account for the activation of the oxidase. First, only about 2% of the total $p47^{phox}$ in activated cells becomes associated with the active oxidase, even though most of the $p47^{phox}$ molecules in the cell become phosphorylated to one degree or another (Fig. 7 in reference 28). Second, S379 represents $< 10\%$ of the target serines in the molecule. Therefore the level of phosphorylation of S379 observed in these experiments could be accounted for by the complete phosphorylation of S379 in all the $p47^{phox}$ molecules associated with the active oxidase, and would accordingly be compatible with a requirement for S379 phosphorylation in oxidase activation.

By what mechanism might the phosphorylation of $p47^{phox}$ result in the activation of the oxidase? We showed earlier that activation of the oxidase in the cell-free system is accompanied by the appearance of a membrane-binding site on a cytosolic oxidase component (25). This binding site is likely to be at least partially on $p47^{phox}$, since $p47^{phox}$ can migrate on its own from the cytosol to the membrane during oxidase activation, but $p67^{phox}$ and Rac2, the other cytosolic oxidase components, can only migrate to the membrane in the presence of $p47^{phox}$ (2). Further support for this idea comes from recent studies indicating that an interaction between an SH3 domain in $p47^{phox}$ and a proline-rich region in the protein is eliminated during oxidase activation, exposing a region that is concealed in the resting protein (29). It may be that in the resting cell, the membrane binding site of $p47^{phox}$ is concealed, not only by the SH3 domain-mediated interaction, but also by an interaction involving the protein's highly cationic carboxy-terminal tail, whose positive charges could associate with a concentration of negative charge elsewhere on the surface of the molecule. Phosphorylation could reverse this positive charge, releasing the tail and exposing the membrane binding site to allow assembly of the active oxidase.

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References

1. Chanock, S. J., J. El Benna, R. M. Smith, and B. M. Babior. 1994. The respiratory burst oxidase. *J. Biol. Chem.* 269:24519–24522.

2. Heyworth, P. G., J. T. Curnutte, W. M. Nauseef, B. D. Volpp, D. W. Pearson, H. Rosen, and R. A. Clark. 1991. Neutrophil nicotinamide adenine dinucleotide phosphate oxidase assembly. Translocation of p47-phox and p67-phox requires interaction between p47-phox and cytochrome b558. *J. Clin. Invest.* 87:352–356.

3. Volpp, B. D., W. M. Nauseef, and R. A. Clark. 1988. Two cytosolic neutrophil oxidase components absent in autosomal chronic granulomatous disease. *Science (Wash. DC).* 242:1295–1297.

4. Nunoi, H., D. Rotrosen, J. I. Gallin, and H. L. Malech. 1988. Two forms of autosomal chronic granulomatous disease lack distinct neutrophil cytosol factors. *Science (Wash. DC).* 242:1298–1301.

5. Lomax, K. J., T. L. Leto, H. Nunoi, J. I. Gallin, and H. L. Malech. 1989. Recombinant 47kD cytosol factor restores NADPH oxidase in chronic granulomatous disease [published erratum appears in Science 246:987 (1989)]. *Science (Wash. DC).* 245:409–412.

6. Volpp, B. D., W. M. Nauseef, J. E. Donelson, D. R. Moser, and R. A. Clark. 1989. Cloning of the cDNA and functional expression of the 47-kilodalton cytosolic component of human neutrophil respiratory burst oxidase [published erratum appears in Proc Natl Acad Sci U S A 1989 Dec;86(23):9563]. *Proc. Natl. Acad. Sci. USA.* 86:7195–7199.

7. Babior, B. M., T. Hayakawa, K. Suzuki, and S. Suzuki. 1984. A deficiency in membrane protein phosphorylation in X-linked chronic granulomatous disease. *Clin. Res.* 32:550a.

8. Segal, A. W., P. G. Heyworth, S. Cockcroft, and M. M. Barrowman. 1985. Stimulated neutrophils from patients with autosomal recessive chronic granulomatous disease fail to phosphorylate a Mr-44,000 protein. *Nature (Lond.).* 316:547–549.

9. Hayakawa, T., K. Suzuki, S. Suzuki, P. C. Andrews, and B. M. Babior. 1986. A possible role for protein phosphorylation in the activation of the respiratory burst in human neutrophils. *J. Biol. Chem.* 261:9109–9115.

10. Okamura, N., J. T. Curnutte, R. L. Roberts, and B. M. Babior. 1988. Relationship of protein phosphorylation to the activation of the respiratory burst in human neutrophils. Defects in the phosphorylation of a group of closely related 48K proteins in two forms of chronic granulomatous disease. *J. Biol. Chem.* 263:6777–6782.

11. El Benna, J., L. P. Faust, and B. M. Babior. 1994. The phosphorylation of the respiratory burst oxidase component $p47^{phox}$ during neutrophil activation. Phosphorylation of sites recognized by protein kinase C and by proline-directed kinases. *J. Biol. Chem.* 269:23431–23436.

12. Combadiere, C., J. El Benna, E. Pedruzzi, J. Hakim, and A. Perianin. 1993. Stimulation of the human neutrophil respiratory burst by formyl peptides is primed by a protein kinase inhibitor, staurosporine. *Blood.* 82:2890–2898.

13. Kessels, G. C. R., K.-H. Krause, and A. J. Verhoeven. 1993. Protein kinase C activity is not involved in *N*-formylmethionyl-leucyl-phenylalanine-induced phospholipase D activation in human neutrophils, but is essential for concomitant NADPH oxidase activation: Studies with a staurosporine analogue with improved selectivity for protein kinase C. *Biochem. J.* 292:781–785.

14. Naccache, P. H., C. Gilbert, A. C. Caon, M. Gaudry, C.-K. Huang, V. A. Bonak, K. Umezawa, and S. R. McColl. 1990. Selective inhibition of human neutrophil functional responsiveness by erbstatin, an inhibitor of tyrosine protein kinase. *Blood.* 76:2098–2104.

15. Zhou, M., and E. J. Brown. 1994. CR3 (Mac-1, $\alpha_M\beta_2$, CD11b/CD18) and FcgammaRIII cooperate in generation of a neutrophil respiratory burst: requirement for FcgammaRII and tyrosine phosphorylation. *J. Cell Biol.* 125:1407–1416.

16. Ding, J., and J. A. Badwey. 1992. Effects of antagonists of protein phosphatases on superoxide release by neutrophils. *J. Biol. Chem.* 267:6442–6448.

17. Ohtsuka, T., N. Okamura, and S. Ishibashi. 1986. Involvement of protein kinase C in the phosphorylation of 46 kDa proteins which are phosphorylated in parallel with activation of NADPH oxidase in intact guinea-pig polymorphonuclear leukocytes. *Biochim. Biophys. Acta.* 888:332–337.

18. Gaut, J. R., and R. A. Carchman. 1987. A correlation between phorbol diester-induced protein phosphorylation and superoxide anion generation in HL-60 cells during granulocytic maturation. *J. Biol. Chem.* 262:826–834.

19. Cohen, H. J., and M. E. Chovaniec. 1978. Superoxide production by digitonin-stimulated guinea-pig granulocytes. The effect of NEM, divalent cations and glycolytic and mitochondrial inhibitors on the activation of the superoxide generating system. *J. Clin. Invest.* 61:1088–1096.

20. Lu, D. J., and S. Grinstein. 1990. ATP and guanine nucleotide dependence of neutrophil activation: evidence for the involvement of two distinct GTP-binding proteins. *J. Biol. Chem.* 265:13721–13729.

21. Chanock, S. J., L. P. Faust, D. Barrett, C. Bizal, F. E. Maly, P. E. Newburger, J. M. Ruedi, R. M. Smith, and B. M. Babior. 1992. O_2^- production by B-lymphocytes lacking the respiratory burst oxidase subunit p47-phox after transfection with an expression vector containing a p47-phox cDNA. *Proc. Natl. Acad. Sci. USA.* 89:10174–10177.

22. Schagger, H., and G. von Jagow. 1990. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal. Biochem.* 166:368–379.

23. Thrasher, A., M. Chetty, C. Casimir, and A. W. Segal. 1992. Restoration of superoxide generation to a chronic granulomatous disease-derived B-cell line by retrovirus mediated gene transfer. *Blood.* 80:1125–1129.

24. Crowley, C. A., J. T. Curnutte, R. E. Rosin, J. Andre-Schwartz, J. I. Gallin, M. Klempner, R. Snyderman, F. S. Southwick, T. P. Stossel, and B. M. Babior. 1980. An inherited abnormality of neutrophil adhesion: its genetic transmission and its association with a missing protein. *N. Engl. J. Med.* 302:1163–1168.

25. Park, J-W., M. Ma, J. M. Ruedi, R. M. Smith, and B. M. Babior. 1992. The cytosolic components of the respiratory burst oxidase exist as a Mr 240,000 complex that acquires a membrane-binding site during activation of the oxidase in a cell-free system. *J. Biol. Chem.* 267:17327–17332.

26. Smith, R. M., J. T. Curnutte, and B. M. Babior. 1989. Affinity labeling of the cytosolic and membrane components of respiratory burst oxidase by the 2′,3′-dialdehyde derivative of NADPH. Evidence for a cytosolic location of the nucleotide-binding site in the resting cell. *J. Biol. Chem.* 264:1958–1962.

27. Nauseef, W. M., B. D. Volpp, S. McCormick, K. G. Leidal, and R. A. Clark. 1991. Assembly of the neutrophil respiratory burst oxidase. Protein kinase C promotes cytoskeletal and membrane association of cytosolic oxidase components. *J. Biol. Chem.* 266:5911–5917.

28. El Benna, J., J. M. Ruedi, and B. M. Babior. 1994. Cytosolic guanine nucleotide-binding protein Rac2 operates *in vivo* as a component of the neutrophil respiratory burst oxidase. Transfer of Rac2 and the cytosolic oxidase components p47*phox* and p67*phox* to the submembranous actin cytoskeleton during oxidase activation. *J. Biol. Chem.* 269:6729–6734.

29. Sumimoto, H., Y. Kage, H. Nunoi, H. Sasaki, T. Nose, Y. Fukumaki, M. Ohno, S. Minakami, and K. Takeshige. 1994. Role of Src homology 3 domains in assembly and activation of the phagocyte NADPH oxidase. *Proc. Natl. Acad. Sci. USA.* 91:5345–5349.

30. Boyle, W. J., P. van der Geer, and T. Hunter. 1991. Phosphopeptide mapping and phosphoamino acid analysis by two-dimensional separation on thin-layer cellulose plates. *Methods Enzymol.* 201:110–149.