Phosphorylation of the Respiratory Burst Oxidase Subunit p47\textsuperscript{phox} as Determined by Two-dimensional Phosphopeptide Mapping

PHOSPHORYLATION BY PROTEIN KINASE C, PROTEIN KINASE A, AND A MITOGEN-ACTIVATED PROTEIN KINASE*

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The respiratory burst oxidase is responsible for superoxide (O\textsubscript{2}\textsuperscript{-}) production by phagocytes and B lymphocytes. This multicomponent enzyme is dormant in resting cells but is activated on exposure of the cells to an appropriate stimulus. Upon activation, several serine residues on the cytosolic oxidase subunit p47\textsuperscript{phox} become phosphorylated. Using two-dimensional tryptic phosphopeptide mapping, we studied the phosphorylation of p47\textsuperscript{phox} in \textsuperscript{32}P\textsuperscript{-}loaded Epstein-Barr virus-transformed B lymphoblasts expressing wild type p47\textsuperscript{phox} or any of several p47\textsuperscript{phox} Ser → Ala mutants. We were able to identify the labeled peptides from wild type p47\textsuperscript{phox} as those containing Ser\textsuperscript{303/304}, Ser\textsuperscript{315}, Ser\textsuperscript{320}, Ser\textsuperscript{328} and/or Ser\textsuperscript{359/370}, and Ser\textsuperscript{345/348}, no \textsuperscript{32}P-labeled Ser\textsuperscript{310}-containing peptide was found. When purified p47\textsuperscript{phox} was phosphorylated in vitro by various protein kinases, varying phosphopeptide patterns were observed. Protein kinase C phosphorylated all the peptides except the one containing Ser\textsuperscript{345/348}; protein kinase A phosphorylated the peptide containing Ser\textsuperscript{320} and one or both of the peptides containing Ser\textsuperscript{328} and Ser\textsuperscript{359/370}, while mitogen-activated protein kinase phosphorylated only the peptide containing Ser\textsuperscript{345/348}. These findings suggest that these three kinases play distinct roles in the activation of the respiratory burst oxidase, each of them catalyzing the phosphorylation of a different group of serines in p47\textsuperscript{phox}.

The respiratory burst oxidase of phagocytes and B lymphocytes catalyzes the reduction of oxygen to superoxide (O\textsubscript{2}\textsuperscript{-}) at the expense of NADPH (1–6). In resting cells the enzyme is inactive, and its components are distributed between the cytosol and the membranes of secretory vesicles. When the cells are activated, the cytosolic components migrate to the membranes, where they associate with the membrane-bound components to assemble the catalytically active oxidase (1, 5, 7).

When the oxidase is activated, p47\textsuperscript{phox}, one of the cytosolic subunits, becomes phosphorylated on several serines (8, 9). We recently found that in human neutrophils serines Ser\textsuperscript{303}, Ser\textsuperscript{304}, Ser\textsuperscript{320}, Ser\textsuperscript{328}, Ser\textsuperscript{345}, Ser\textsuperscript{348}, and Ser\textsuperscript{359} and/or Ser\textsuperscript{370} are phosphorylated and that other serines lying between Ser\textsuperscript{303} and Ser\textsuperscript{379} could be phosphorylated (10). We further showed that at least one of these serines is absolutely required for oxidase activation in whole cells stimulated with PMA (11). In this study, we report the use of site-directed mutagenesis combined with two-dimensional phosphopeptide mapping to further characterize the phosphorylation of p47\textsuperscript{phox} in B lymphocytes and to compare the in vitro phosphorylation of purified p47\textsuperscript{phox} by various serine/threonine-specific protein kinases.

EXPERIMENTAL PROCEDURES

Reagents—Phorbol 12-myristate 13-acetate, leupeptin, pepstatin, aprotinin, phosphatidylserine, diacylglycerol, and protein kinase A (catalytic subunit) were purchased from Sigma. Protein kinase C was obtained from Calbiochem. MAP\textsuperscript{3} kinase was obtained from Santa Cruz Biotechnology, and DNase I and sequencing grade trypsin and Glu-C endopeptidase from Boehringer Mannheim. \textsuperscript{32}P (8500–9120 Ci/mmol) and [\textsuperscript{32}P\textsuperscript{3}]-ATP (3000 Ci/mmol) were purchased from DuPont NEN.

Site-directed Mutagenesis and Transfections—Most of the mutants used for these experiments have been previously reported (11). S303A, S304A was constructed by cloning the WT p47\textsuperscript{phox} GCGGGGCGGCGC (deviations from the WT sequence are shown in boldface) (11). The elimination of a BssHII site in the mutant was used for screening. S328A–S359A was constructed by the sequential introduction of the single mutations S348A, S345A, S359A, and S328A into the WT done using templates reported elsewhere (11). S370A, S379A was similarly created by the sequential introduction of the mutations S379A and S370A into the WT done. S328A–S359A was then constructed by replacing the Nad/NarI fragment of S370A, S379A with the Nad/NarI fragment of S328A–S359A. In every case, the mutations were confirmed by dideoxynucleotide-based sequencing (11). The wild type or mutant cDNAs were then excised from Bluescript, cloned into the XbaI/NotI sites of the mamma


dinated expression vector EBOpLP and transfected into p47\textsuperscript{phox}-deficient EBV-transformed B lymphocytes as described elsewhere (12). \textsuperscript{32}P Labeling of Transfected Cells and p47\textsuperscript{phox} Purification—Transfected B lymphoblasts were labeled with \textsuperscript{32}P as described previously (11). Briefly, the cells were incubated overnight in phosphate-free medium, then transferred to fresh medium containing \textsuperscript{32}P (0.2 mCi/ml) and incubated for 4 h at 37 °C. The cells were then activated for 12.5 min with PMA (1 μg/ml/10\textsuperscript{6} cells), after which their p47\textsuperscript{phox} was isolated and purified by immunoadfinity chromatography as described before (10).

In Vitro Phosphorylation of p47\textsuperscript{phox}—p47\textsuperscript{phox} was isolated by immunoprecipitation from resting neutrophils exactly as described elsewhere (10, 16). Labeling with protein kinase A was performed by incubating a reaction mixture containing 1 μl of p47\textsuperscript{phox}, 50 μl (1 μCi) [\textsuperscript{32}P\textsuperscript{3}]-ATP, and 0.5 μg of protein kinase A (catalytic subunit) in 50 μl of a buffer containing 40 mM HEPES (pH 7.3), 1 mM dithiothreitol, 20 mM MgCl\textsubscript{2}, 1 mM EGTA, 1 mM NaCl, and 1 mg/ml BSA.

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The abbreviations used are: MAP\textsuperscript{3} kinase, mitogen activated protein kinase; PMA, phorbol 12-myristate 13-acetate; WT, wild type; EBV, Epstein-Barr virus; PAGE, polyacrylamide gel electrophoresis; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.
RESULTS

Identification of Phosphopeptides on the Tryptic Peptide Map of Phosphorylated p47<sup>phox</sup>. Including a Phosphopeptide Containing Ser<sup>315</sup>—During the activation of the respiratory burst oxidase in phagocytes and B lymphocytes, p47<sup>phox</sup> becomes extensively phosphorylated. Using CNBr cleavage followed by proteolysis, Tricine gel electrophoresis, and Edman degradation, we showed that the targets of phosphorylation in p47<sup>phox</sup> are the serine residues lying between Ser<sup>303</sup> and Ser<sup>379</sup> inclusively, and that among these serines the following are phosphorylated: Ser<sup>303</sup>, Ser<sup>304</sup>, Ser<sup>320</sup>, Ser<sup>328</sup>, Ser<sup>345</sup>, Ser<sup>348</sup>, Ser<sup>359</sup> and/or Ser<sup>370</sup>, and Ser<sup>379</sup> (10, 11), the last phosphorylated peptide migrates much less extensively than the rest. The methods employed in that study were elaborate and time-consuming, however, and presented certain limitations: in qualitative analysis because manual Edman degradation is only reliable through the first 10–15 cycles (18) and in quantitative analysis because of unequal losses of phosphorylated peptides during the workup of the samples. A simpler and more quantifiable method would be the analysis of phosphorylation by two-dimensional tryptic peptide mapping (17). We therefore carried out experiments to identify the phosphopeptides on the tryptic peptide map of p47<sup>phox</sup>-labeled WT p47<sup>phox</sup>

Our approach was to express p47<sup>phox</sup>-Ser → Ala mutants in EBV-transformed p47<sup>phox</sup>-deficient B lymphocytes; to load these lymphocytes with <sup>32</sup>P, and then activate them with PMA to label their p47<sup>phox</sup>, and finally to purify the labeled p47<sup>phox</sup> mutants, map them, and look for differences between those maps and the map of <sup>32</sup>P-labeled WT p47<sup>phox</sup>. In a tryptic digest of p47<sup>phox</sup>, the phosphorylation targets are distributed among several peptides (Table 1; trypsin is unable to split Lys-Pro and Arg-Pro bonds) (17). Of these, the peptide containing Ser<sup>379</sup> and Ser<sup>381</sup> would probably be difficult to see because it would contain very little <sup>32</sup>P relative to the other peptides (10, 11). The results (Fig. 1, left) showed that the map of WT p47<sup>phox</sup> contained six major phosphorylation targets (arrows), all of which were seen in each of 15 separate experiments, together with several minor phosphorylation sites in the maps. Inactivation of these sites was consistent. Taking into consideration the serines known to be phosphorylated during oxidase activation (10) and the peptides generated by tryptic digestion of p47<sup>phox</sup> (Table 1), we made a number of mutants in which two or more serines had been converted to alanines, and used these together with mutants containing a single Ser → Ala change to identify the labeled peptides on the two-dimensional map.

Restricting our analysis to the six constant phosphopeptides (Fig. 1), we found that maps of p47<sup>phox</sup> mutants containing single Ser → Ala mutations were the same as WT maps (Fig. 1, left) except for the map of S320A, which lacked a single spot, and the map of S315A, which lacked two spots (Fig. 1, left). The latter result suggests that at least two peptides were produced, probably because of partial cleavage at the sequence RKR (residues 316–318). Sequences containing basic residues in tandem are known to be susceptible to partial cleavage (17).

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Comparison of Peptide Maps of Phosphorylated p47<sup>phox</sup> from Activated Neutrophils and EBV-Transformed B Cells—Using tryptic phosphopeptide mapping, we compared the sites of phosphorylation of p47<sup>phox</sup> from activated human neutrophils and EBV-transformed normal B lymphocytes. The results (Fig. 3) showed that the same six major phosphopeptides appeared in both maps. These findings suggest that the p47<sup>phox</sup>-labeled phosphopeptide assignments made through the experiments described above are valid for neutrophils as well as B lymphocytes, and that this method can therefore be employed to study

2 There is too little <sup>32</sup>P in Ser<sup>379</sup> to produce a spot on the autoradiogram under the conditions used for these experiments (11).

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**Table 1**

| Peptide | Serine |
|---------|--------|
| SSIR    | 303, 304 |
| NAHSIHQR| 310    |
| SR/SRK/SKR| 315   |
| LSQDAYR | 320    |
| NSVR    | 328    |
| QARPQGSPGPLEEER | 345, 348 |
| SKQPQAPPRPADLILNR | 359, 370 |
| CSESTK  | 379, 381 |
the phosphorylation of p47phox during the activation of neutrophils and probably other phagocytes as well.

Phosphorylation of Respiratory Burst Oxidase Subunit p47phox by Various Protein Kinases—We employed tryptic phosphopeptide mapping to identify the sites phosphorylated by various kinases thought to be involved in the regulation of O2 production by the respiratory burst oxidase. For this purpose, p47phox immunopurified from resting neutrophils was incubated with [γ-32P]ATP together with the kinase of interest (protein kinase A, protein kinase C, or MAP kinase), then repurified and analyzed either by CNBr cleavage followed by Tris-Tricine SDS-PAGE (19) or by tryptic peptide mapping. As shown previously with p47phox that had been phosphorylated in intact cells (10), the sites phosphorylated in vitro by all three kinases were located in the C-terminal CNBr peptide of the labeled p47phox (Fig. 4). Peptide mapping showed that protein kinase C phosphorylated all the p47phox peptides except the peptide corresponding to Ser345/348 (Fig. 5, top), while protein kinase A, a more selective kinase, phosphorylated only the Ser320 peptide and the Ser328 and/or the Ser359/370 peptides (Fig. 5, middle). When p47phox phosphorylated by protein kinase C or protein kinase A was cleaved by Glu-C endopeptidase and then analyzed by SDS-PAGE, a labeled fragment appeared at 4 kDa (Fig. 6), indicating that the peptide containing Ser359/370 was phosphorylated. The status of Ser328 remained unresolved, although its phosphorylation in PMA-activated neutrophils suggests that it was probably phosphorylated at least by protein kinase C. MAP kinase, used here as an example of a proline-directed kinase, phosphorylated only the Ser345/348 peptide, as expected from the sequences around the target serines in p47phox (Fig. 5, bottom). Taken together, these results suggest that the three kinases could play different roles in regulating the activity of the respiratory burst oxidase.

On the phosphopeptide maps of purified p47phox that had been labeled with a known kinase, major spots were seen that...
were not present on the maps of p47\textsubscript{phox} labeled in whole cells. These spots were disregarded as irrelevant to the physiological labeling pattern of activated p47\textsubscript{phox}.

**DISCUSSION**

Tryptic peptide mapping of p47\textsubscript{phox} labeled with \textsuperscript{32}P either in intact cells or in a cell-free system provides an efficient way of identifying which of the target peptides are phosphorylated, and in combination with image analysis of radioactivity could yield important information on the relative quantities of phosphate on various of the serines of the protein. The results obtained by tryptic peptide mapping retain a certain amount of ambiguity, however, because they provide no information as to which of the two serines on a two-serine peptide is (are) phosphorylated. Whether it is important to answer that question will depend on studies correlating structure and function in Ser\textsuperscript{3}Ala mutants of p47\textsubscript{phox}, although a partial answer is provided by our recent report showing that Ser\textsuperscript{3}Ala mutations of individual serines from position 303 to 370 have little effect on oxidase activity (11).

The present results provide some information as to the order of phosphorylation of the target serines on p47\textsubscript{phox}. Except for the mutant S315A, whose anomalous properties were discussed above, mutations affecting the serines on a single tryptic peptide caused the loss of at most one spot on the phosphopeptide map. This finding suggests that there is no target serine whose phosphorylation is absolutely dependent on the phosphorylation of a serine on a different peptide, or the phosphorylation of a group of such serines. Rather, it appears that these serines can be phosphorylated in any order.

We previously showed that when the respiratory burst oxidase is activated, serines Ser\textsuperscript{303}, Ser\textsuperscript{304}, Ser\textsuperscript{320}, Ser\textsuperscript{328}, Ser\textsuperscript{345}, Ser\textsuperscript{348}, Ser\textsuperscript{359} and/or Ser\textsuperscript{370} of p47\textsubscript{phox} are phosphorylated (10, 11). The present studies confirm the earlier results by another method, and in addition have shown that Ser\textsuperscript{315} is also phosphorylated, bringing the total number of phosphorylated serines in the C-terminal region of activated p47\textsubscript{phox} to 9 or 10. Ser\textsuperscript{379} has already been found to play an important role in oxidase activation and p47\textsubscript{phox} translocation, and it is likely that protein kinase-mediated phosphorylation of other target serines is equally important. In fact, several lines of evidence already support a role for protein kinase C in oxidase activation. For example, PMA, an activator of several forms of protein kinase C, is a powerful stimulator of O\textsubscript{2}\textsuperscript{-} production in whole cells (20). Purified p47\textsubscript{phox} is a good substrate for protein kinase C in vitro (21), while staurosporine, a potent inhibitor of protein kinase C (and other kinases), blocks PMA-induced O\textsubscript{2}\textsuperscript{-} generation as well as the phosphorylation of p47\textsubscript{phox} (22, 23). Finally, we show in this study that the phosphopeptide map of p47\textsubscript{phox} isolated from PMA-activated neutrophils and EBV-transformed B lymphocytes is identical to the phosphopeptide map of p47\textsubscript{phox} phosphorylated in vitro by protein kinase C, except for the absence of the Ser\textsuperscript{345/348} peptide from the latter map. These findings suggest that one or more of the PMA-responsive forms of protein kinase C could be a critical mediator of oxidase activation. We showed recently that Ser\textsuperscript{345} and Ser\textsuperscript{348} are not required for oxidase activation (11), since the S345A, S348A mutant of p47\textsubscript{phox} is fully active in EBV-transformed B cells. This finding suggests that, in contrast to pro-
tein kinase C, phosphorylation of p47phox by proline-directed kinases such as MAP kinase may have little to do with oxidase activation. The role of target serines in the regulation of oxidase activity is currently under investigation in our laboratory.

Phosphorylation of p47phox was also shown to occur upon addition of dibutyryl cAMP to neutrophil cytoplasts or cytosol, suggesting that p47phox is also a substrate for protein kinase A (24). Dibutyryl cAMP did not induce O2\(^\text{=}\) production, however, indicating that phosphorylation by protein kinase A alone is not sufficient to activate the oxidase. It is possible, in fact, that the phosphorylation of p47phox by protein kinase A prevents the assembly of the oxidase, since the elevation of neutrophil cAMP inhibits O2\(^\text{=}\) production (25). Our results show that protein kinase A phosphorylates fewer target serines than protein kinase C, phosphorylating only peptides Ser320, Ser359/370, and possibly Ser328 (a maximum of four target serines), in contrast to the five peptides (up to seven target serines) phosphorylated by protein kinase C. The protein kinase A targets could be responsible for the negative regulation of p47phox phosphorylation and oxidase activation by protein kinase A.

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