Phosphorylation of the NADPH Oxidase Component p67^{PHOX} by ERK2 and P38MAPK: Selectivity of Phosphorylated Sites and Existence of an Intramolecular Regulatory Domain in the Tetratricopeptide-Rich Region†

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ABSTRACT: p67^{PHOX}, a cytosolic component of the NADPH oxidase complex, is phosphorylated during neutrophil activation by several agonists. The intracellular signaling pathways leading to its phosphorylation in neutrophils may involve a PKC-dependent pathway and a PKC-independent pathway. Here, we analyzed p67^{PHOX} phosphorylation by ERK2 and p38MAPK. Both ERK2 and p38MAPK phosphorylated p67^{PHOX} in vitro, with similar K_m values (10 and 9 μM, respectively). Phosphopeptide mapping indicated that ERK2 and p38MAPK phosphorylate different subgroups of peptides. Using truncated forms of p67^{PHOX}, we found that the major phosphorylation target site of ERK2 was located in the N-terminal fragment (1−243), while the major phosphorylation target sites of p38MAPK were located in the C-terminal fragment (244−526). Furthermore, an additional peptide, which was not phosphorylated in the intact protein, appeared to be phosphorylated in the isolated C-terminal fragment (aa 244−526). This site may not thus be accessible in the intact protein. Indeed, incubation of the C-terminal fragment (244−526) with different N-terminal fragments (1−243, 1−210, or 1−199) containing the tetratricopeptide-rich region prevented phosphorylation of this C-terminal fragment. ERK1/2 and p38MAPK are also involved in p67^{PHOX} phosphorylation in intact neutrophils. Indeed, PD98059 and SB203580, two selective inhibitors of MEK1/2 and p38MAPK, respectively, inhibited p67^{PHOX} phosphorylation in fMLP- and PMA-stimulated neutrophils, with additive effects, thus suggesting that they also target different sites in vivo. Furthermore, the major peptides phosphorylated by ERK2 and p38MAPK in vitro were also phosphorylated in fMLP-stimulated neutrophils. Taken together, these results suggest not only that p67^{PHOX} is phosphorylated by ERK2 and p38MAPK in vitro and in intact neutrophils on several selective sites but also that a C-terminal phosphorylation site may become accessible after a conformational change of the protein.

The multicomponent phagocyte NADPH oxidase, which is responsible for superoxide anion production, plays a key role in host defenses against invading microorganisms (*1*−*4*). Upon neutrophil activation by several agonists, the cytosolic components p47^{PHOX}, p67^{PHOX}, and p40^{PHOX} become phosphorylated (*5*−*8*) and migrate with Rac2 to the outer membrane, where they associate with cytochrome b_{558} (which comprises gp91^{PHOX} and p22^{PHOX}) to assemble the active NADPH oxidase. The latter catalyzes NADPH-dependent reduction of oxygen to superoxide anion, a precursor of microbicidal oxidants.

p67^{PHOX} plays a central role in the formation of an active complex. For example, some patients with chronic granulomatous disease (CGD)[1] have an inherited mutation in the gene encoding for p67^{PHOX}, which impairs superoxide production via NADPH oxidase and leads to recurrent bacterial and fungal infections (*9*). p67^{PHOX} is an essential effector target for Rac (*10*, *11*), has affinity for p40^{PHOX} (*12*), binds directly to cytochrome b_{558} (*13*), possesses an activation domain which is probably involved in electron transfer from NADPH to the flavin center of cytochrome b_{558} (*14*), and is phosphorylated during NADPH oxidase activation (*6*, *7*, *15*). p67^{PHOX} phosphorylation occurs upon NADPH oxidase activation by several agonists such as PMA, fMLP, and serum-opsonized zymosan (*6*, *7*, *15*, *16*). The intracellular signaling pathways leading to p67^{PHOX} phosphorylation in neutrophils may involve a PKC-dependent pathway and a PKC-independent pathway (*7*). There is growing evidence that p47^{PHOX} phosphorylation is essential for the activation process, initiating assembly of the enzyme (*17*). However, the exact function of p67^{PHOX} phosphorylation is unclear. In contrast to p47^{PHOX}, which is phosphorylated on multiple sites in its C-terminal portion (*18*, *19*), p67^{PHOX} phosphorylation has been shown to occur on one major site (threonine 233) (*15*). Here, we sought other phosphorylation target sites on p67^{PHOX} and attempted to identify the kinases involved in this process.

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[1] Abbreviations: CGD, chronic granulomatous disease; DFP, diisopropyl fluorophosphate; DTT, dithiothreitol; IPTG, isopropyl β-D-thiogalactopyranoside; GST, glutathione *S*-transferase; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; NADPH, nicotinamide adenine dinucleotide phosphate, reduced form; phox, *ph*agocyte *ox*idase; MAPK, mitogen-activated protein kinase, ERK, extracellular-signal-regulated protein kinase; PKC, protein kinase C; FMLP, formylmethionylleucylphenylalanine; TPR, tetratricopeptide repeat.

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MATERIALS AND METHODS

Materials. Active recombinant ERK2 and p38MAPK were from New England Biolabs (Beverly, MA). [γ-^{32}P]ATP and [^{32}P]orthophosphoric acid were from NEN Life Science Products (Boston, MA). FMLP, PMA, ATP, MBP, proteases, and phosphatase inhibitors were from Sigma Chemical Co. (St. Louis, MO). Kinase inhibitors were from Calbiochem (La Jolla, CA). Endotoxin-free buffers and salt solutions were from Life Technologies (Cergy-Pontoise, France). Rabbit polyclonal antibodies against the C-terminal sequence of p67^{PHOX} were prepared as described in ref *7*. Reagents for SDS−PAGE were from Bio-Rad (Richmond, CA).

Recombinant Proteins. The following truncated forms of p67^{PHOX}, N-terminal (1−243), N-terminal (1−210), N-terminal (1−199), and C-terminal (244−526), were constructed as described by Dang et al. (*20*), except that the C-terminal fragment (244−526) was subcloned into PGEX-5X-3 in order to eliminate the potential phosphorylation site represented by the polylinker region of PGEX-6P3. p67^{PHOX} and its truncated forms were expressed in *Escherichia coli* as GST fusion proteins and purified with glutathione−Sepharose beads. The proteins were then separated from GST while on the beads by cleavage with PreScission protease or factor Xa (Amersham Pharmacia Biotech, Piscataway, NJ). p47^{PHOX}, p40^{PHOX}, and Rac2 were expressed in *E. coli* as described in ref *13*. Protein concentrations were determined with the Bio-Rad assay kit using bovine serum albumin as standard.

In Vitro Phosphorylation of p67^{PHOX}. p67^{PHOX}, p67^{PHOX} N-terminal fragment (1−243), and p67^{PHOX} C-terminal fragment (244−526) were phosphorylated by ERK2 and p38-MAPK (New England Biolabs) by incubating 5 μg of protein in a reaction mixture containing 40 mM Hepes, pH 7.5, 10 mM $MgCl_2$, 10 mM DTT, and 100 μM ATP (3 μCi per assay) in a total volume of 40 μL at 30 °C for 30 min. The reaction was stopped by adding hot 2× Laemmli sample buffer. For K_m determination, the reaction was stopped after 10 min, and 20 μL of the reaction mixture was spotted onto Whatman p81 paper (Tewksbury, MA), followed by three washes with 0.85% H_3PO_4, before radioactivity was measured. The remaining fraction (20 μL) was mixed with hot 2× Laemmli sample buffer. In some assays, the p67^{PHOX} C-terminal fragment (244−526) was phosphorylated in the presence of increasing concentrations of p67^{PHOX} N-terminal fragments (1−199, 1−210, and 1−243). Proteins were separated by SDS−PAGE on 12% Tris−glycine gel using standard techniques (*21*), then transferred to nitrocellulose, and revealed by autoradiography and Ponceau red staining.

Coprecipitation of the p67^{PHOX} N-Terminal Fragment by the p67^{PHOX} C-Terminal Fragment. p67^{PHOX} N-terminal fragment (1−210) and p67^{PHOX} C-terminal fragment (244−526), 0.216 nmol each, expressed as fusion proteins with GST, were incubated together in the phosphorylation reaction mixture and then immunoprecipitated overnight with an antibody directed against the C-terminal end of p67^{PHOX} (residues 511−526) in the presence of Gamma-bind G-Sepharose beads (Amersham-Pharmacia, Orsay, France). Bound proteins were eluted in 2× Laemmli buffer and separated by SDS−PAGE on 12% Tris−glycine gel, then transferred to nitrocellulose, and revealed with an antibody directed against GST (Santa Cruz Biotechnology, Santa Cruz, CA).

Neutrophil Preparation. Human neutrophils were obtained in LPS-free conditions by dextran sedimentation and Ficoll-Hypaque centrifugation (*7*). To avoid protein degradation, neutrophils were treated with the serine protease inhibitor diisopropyl fluorophosphate (DFP) (2.7 mM) for 20 min at 4 °C before use.

^{32}P-Labeling of Neutrophils: Stimulation and Fractionation. Cells were incubated in phosphate-free loading buffer (10 mM Hepes, pH 7.4, 137 mM NaCl, 5.4 mM KCl, 5.6 mM D-glucose, 0.8 mM $MgCl_2$, and 0.025% bovine serum albumin) containing 0.5 mCi of [^{32}P]orthophosphoric acid per 10^8 cells per milliliter for 60 min at 30 °C, as previously reported (*7*). Neutrophils were stimulated with 10^{-6} M fMLP for 1 min or with 0.5 μg/mL PMA for 6 min. Where necessary, neutrophils were pretreated with 14 nM calyculin and 5 μM cytochalasin B for 5 min before stimulation. In some cases, cells were pretreated with PD98059 (50 μM) or SB203580 (10 μM), two selective inhibitors of MEK1/2 and p38MAPK, respectively, for 30 min at 37 °C before stimulation with fMLP or PMA. The reaction was stopped by addition of ice-cold buffer and centrifugation at 400*g* for 6 min at 4 °C. The cells were lysed by resuspending them in lysis buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% Triton X-100, 0.25 M sucrose, 2.5 mM EGTA, 2.5 mM EDTA, 1 mg/mL DNase I, protease, and phosphatase inhibitors) as previously described (*7*). The suspension was sonicated for 3 × 15 s and allowed to solubilize for 1 h on ice. Unsolubilized materials were removed by centrifugation at 100000*g* for 30 min at 4 °C in a TL100 ultracentrifuge (Beckman Inc.).

Immunoprecipitation of p67^{PHOX}. The cleared supernatant was incubated overnight with anti-p67^{PHOX} antibody (1/200) in the presence of Gamma-bind G-Sepharose beads (Amersham-Pharmacia, Orsay, France) for immunoprecipitation. Beads were then washed as previously described (*7*).

Two-Dimensional Tryptic Phosphopeptide Mapping. Tryptic digestion of p67^{PHOX} on nitrocellulose, thin-layer electrophoresis, and thin-layer chromatography were performed as previously described (*7*). The nitrocellulose area containing ^{32}P-labeled p67^{PHOX} was incubated for 30 min at 37 °C with poly(vinylpyrrolidone), then washed, and incubated overnight with trypsin (50 μg/mL) in carbonate buffer. Released peptides were washed three times in a Speed-Vac, redissolved in electrophoresis buffer (17 volumes of water/3 volumes of 88% formic acid), and applied to one corner of a thin-layer cellulose plate (Merck). After electrophoresis (1000 V for 20 min), chromatography was performed as previously described (*7*). The plates were autoradiographed for 1 week at −75 °C.

RESULTS

In Vitro Phosphorylation of p67^{PHOX} by ERK2 and p38MAPK. We have previously shown that a PKC-dependent pathway and a PKC-independent pathway are involved in p67^{PHOX} phosphorylation (*7*). In an initial attempt to identify the kinases involved in the PKC-independent pathway, in vitro phosphorylation was carried out with two kinases belonging to the MAPK family, namely, ERK2 and p38-MAPK. As shown in Figure 1, ERK2 and p38MAPK phosphorylated increasing concentrations of p67^{PHOX}. The K_m values of ERK2 and p38MAPK for p67^{PHOX}, calculated from

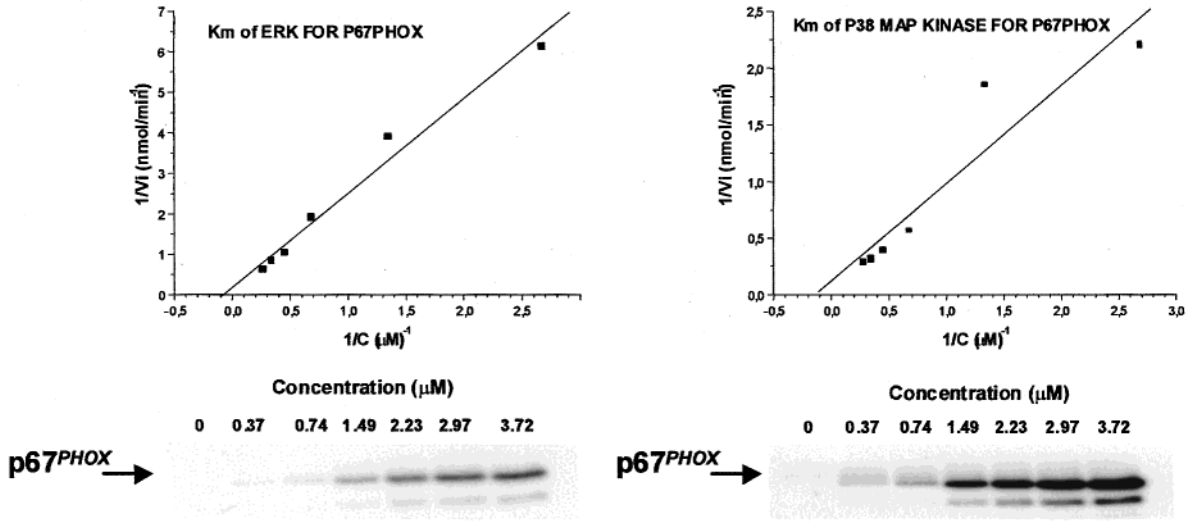

FIGURE 1: ERK2 and p38MAPK phosphorylate recombinant p67^{PHOX} in vitro. p67^{PHOX} at various concentrations was phosphorylated, as described in Materials and Methods, with active ERK2 and p38MAPK. Reactions were stopped in hot Laemmli sample buffer or on Whatman p81 paper. Samples were electrophoresed for autoradiography (lower) or analyzed by Cerenkov counting (upper). Data are representative of three experiments.

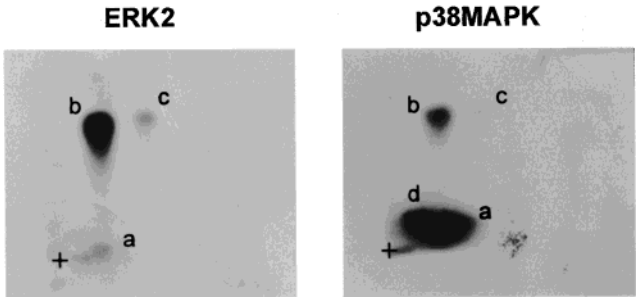

FIGURE 2: Phosphopeptide mapping of in vitro phosphorylated p67^{PHOX}. p67^{PHOX} was phosphorylated in vitro by ERK2 and p38MAPK and then cleaved with trypsin and analyzed by thin-layer electrophoresis and chromatography as described in Materials and Methods. The phosphopeptides were detected by autoradiography. Data are representative of three experiments.

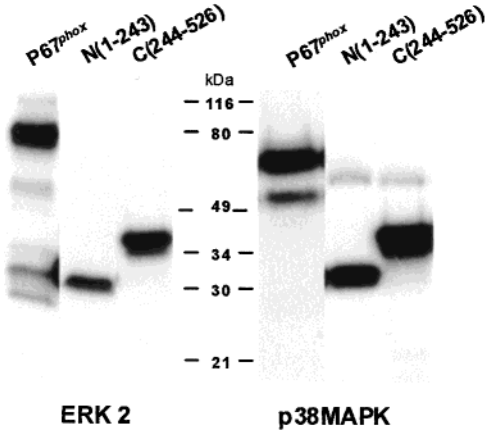

FIGURE 3: p67^{PHOX} is phosphorylated by ERK2 and p38MAPK in its N-terminal region (1−243) and C-terminal region (244−526). The p67^{PHOX} N-terminal fragment (1−243) and C-terminal fragment (244−526) were phosphorylated with active ERK2 and p38MAPK as described in Materials and Methods. Reactions were stopped in hot Laemmli sample buffer. Samples were electrophoresed for autoradiography. Data are representative of three experiments.

a Lineweaver−Burk representation, were found to be close (10 and 9 μM, respectively), indicating that p67^{PHOX} was a good substrate for these two types of kinases (Figure 1).

To determine if the same peptides were phosphorylated by ERK2 and p38MAPK, we performed phosphopeptide mapping of in vitro phosphorylated p67^{PHOX}. Figure 2 (left) shows that three peptides (designated **a**, **b**, and **c**) were phosphorylated by ERK2; **b** was the major phosphorylated peptide, while **a** and **c** were only weakly phosphorylated. The same peptides were phosphorylated by p38MAPK, together with an additional peptide, peptide **d** (Figure 2, right). However, the degree of peptide **a**, **b**, and **c** phosphorylation by ERK2 and p38MAPK differed greatly, indicating that ERK2 and p38MAPK preferentially catalyze the phosphorylation of different subgroups of peptides: peptide **b** was strongly phosphorylated by ERK2, while its phosphorylation by p38MAPK was weaker (Figure 2). Likewise, peptides **a** and **d** were strongly phosphorylated by p38MAPK but only weakly phosphorylated (peptide **a**) or not phosphorylated (peptide **d**) by ERK2 (Figure 2). These results indicate that the subgroups of p67^{PHOX} peptides preferentially phosphorylated by ERK2 and p38MAPK are different.

Localization of the Phosphorylated Peptides on p67^{PHOX}. We then located the regions of p67^{PHOX} targeted by ERK2 and p38MAPK by using the p67^{PHOX} N-terminal fragment (1−243) and the p67^{PHOX} C-terminal fragment (244−526). Fragment 1−243 was phosphorylated by both ERK2 and p38MAPK, as was fragment 244−526 (Figure 3). This suggested that, in addition to the previously identified phosphorylated residue Thr^{233} (*15*), other sites between residues 244 and 526 are phosphorylated by ERK2 and p38MAPK. Phosphopeptide mapping of the phosphorylated N-terminal fragment and the phosphorylated C-terminal fragment showed that peptides **b** and **c** were recovered in the N-terminal fragment (Figure 4A), while peptides **a** (phosphorylated by ERK2 and p38MAPK) and **d** (phosphorylated only by p38MAPK) were recovered in the C-terminal fragment (Figure 4B). Interestingly, the different degrees of phosphorylation of peptides **b** and **a** observed between ERK2 and p38MAPK when the intact protein was used were no longer found when the N-terminal part and the C-terminal part were examined separately (Figure 4). Furthermore, an additional peptide, peptide **e**, which was not phosphorylated

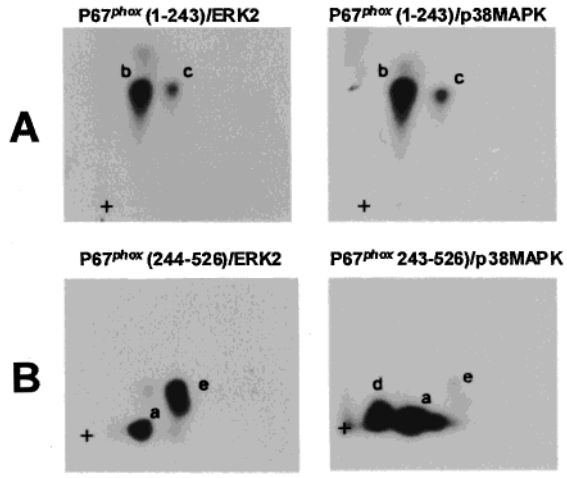

FIGURE 4: Phosphopeptide mapping of the p67^{PHOX} N-terminal fragment (1−243) and p67^{PHOX} C-terminal fragment (244−526). The N-terminal fragment (1−243) (A) and C-terminal fragment (244−526) (B) were cleaved with trypsin and analyzed by thin-layer electrophoresis and chromatography as described in Materials and Methods. The phosphopeptides were detected by autoradiography. Data are representative of three experiments.

by ERK2 or by p38MAPK on the entire protein appeared to be phosphorylated by both kinases in the isolated C-terminal fragment (strongly by ERK2 and weakly by p38MAPK).

The N-Terminal Fragments (1−243, 1−210, and 1−199) Prevent ERK2 Phosphorylation of the C-Terminal Fragment (244−526). Peptides **e** and **a** are not phosphorylated or only weakly phosphorylated by ERK2 in the intact protein. However, they appeared to be clearly phosphorylated by ERK2 in the C-terminal (244−526) fragment. To determine whether phosphorylation of the C-terminal fragment was masked by the N-terminal portion in the intact protein, we phosphorylated the C-terminal fragment (244−526) with ERK2 in the presence of increasing concentrations of the N-terminal (1−243) portion. Phosphorylation of the C-terminal fragment was inhibited in a concentration-dependent manner (from 1 to 8 equimolar) by the N-terminal (1−243) portion (Figure 5). Ponceau red staining showed that the amount of the C-terminal protein incubated with increasing concentrations of the N-terminal fragment was the same in each lane (Figure 5). To rule out the possibility that the reduction in C-terminal fragment phosphorylation by the N-terminal fragment was due to substrate competition, the same experiments were performed with N-terminal fragments that are not phosphorylated by ERK2. The N-terminal fragment (1−210) also inhibited, in concentration-dependent manner, phosphorylation of the C-terminal fragment (244−526) (Figure 6A), as did a shorter N-terminal fragment (1−199) mainly composed of four TPR domains (Figure 6B). Finally, the observed inhibition was not due to an effect of the N-terminal fragment on ERK2 activity, as phosphorylation of MBP, a well-known ERK2 substrate, was not inhibited by the N-terminal fragment (1−210) (Figure 7). To determine which peptide was affected when N-terminal fragments are added back, we recovered the phosphorylated C-terminal fragment (244−526) and performed a peptide mapping. Figure 8 shows that both peptides **e** and **a** are inhibited by the N-terminal region of p67^{PHOX}. Furthermore, N-terminal and C-terminal fragments associate with each other in these conditions of the phosphorylation mixture. Indeed, using an antibody directed against the C-terminal end of p67^{PHOX} (residues 511−526), we showed that the C-terminal fragment could coprecipitate the N-terminal fragment (Figure 9, lane 3). These results show that the N-terminal fragment of p67^{PHOX} inhibits phosphorylation of the C-terminal fragment by ERK2 and suggest that peptides **e** and **a** are not phosphorylated or only weakly phosphorylated in the intact protein because they are masked by an N-terminal domain. The region responsible for this inhibition appears to be located between residues 1 and 199, which is mainly composed of four TPR domains.

The above results suggest that a conformational change of p67^{PHOX} is necessary for peptide **e** to be phosphorylated and peptide **a** to be fully phosphorylated by ERK2. Rac2-GTPγS, p47^{PHOX}, and p40^{PHOX}, which are known to interact with p67^{PHOX} (*10*, *11*, *22*), are candidate cellular factors permitting this conformational change. We thus phosphorylated intact p67^{PHOX} with ERK2 in the presence of Rac2-GTPγS and p47^{PHOX} plus p40^{PHOX} and performed a phosphopeptide map. Neither Rac2-GTPγS nor p47^{PHOX} plus p40^{PHOX} permitted peptide **e** phosphorylation (data not shown) or increased peptide **a** phosphorylation, indicating that these factors are not responsible for the relevant conformational change.

ERK1/2 and p38MAPK Are Involved in p67^{PHOX} Phosphorylation in Intact Neutrophils. We then examined whether ERK1/2 and p38MAPK were involved in p67^{PHOX} phosphorylation in intact neutrophils. For this purpose, ^{32}P-labeled neutrophils were pretreated with PD98059 and SB203580, two selective inhibitors of MEK1/2 and p38MAPK, respectively, and then stimulated with fMLP and PMA. As shown in Figure 10 (top), PD98059 and SB203580 both inhibited p67^{PHOX} phosphorylation in fMLP-stimulated neutrophils

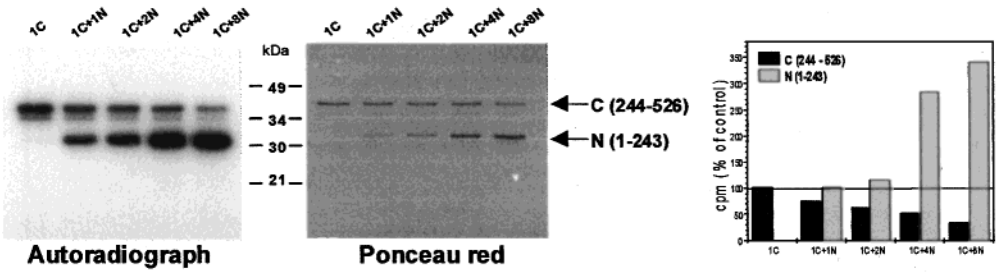

FIGURE 5: The N-terminal fragment (1−243) prevents C-terminal fragment (244−526) phosphorylation. Phosphorylation of the p67^{PHOX} C-terminal fragment (244−526) (0.216 nmol) by ERK2 was carried out in the presence of increasing concentrations of the p67^{PHOX} N-terminal fragment (1−243) (from 1 to 8 equimolar of the C-terminal fragment). Proteins were separated by SDS−PAGE on 12% Tris−glycine gel using standard techniques (*21*) and then transferred to nitrocellulose and revealed by autoradiography and Ponceau red staining. Phosphorylation was quantified by Cerenkov counting of the nitrocellulose bands. Data are representative of two experiments.

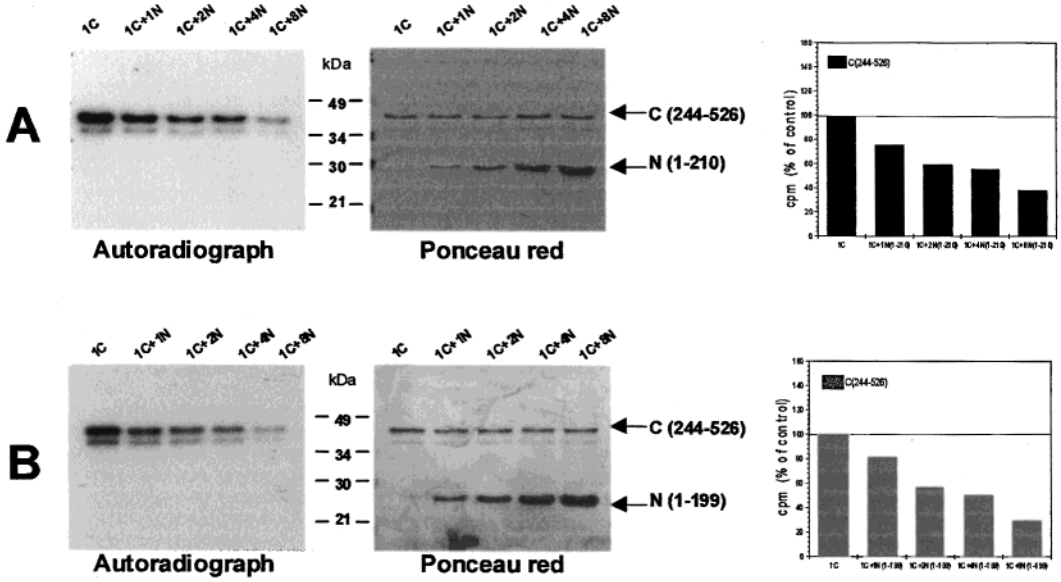

FIGURE 6: The N-terminal fragment (1−210) and N-terminal fragment (1−199) prevent C-terminal fragment (244−526) phosphorylation. Phosphorylation of the p67^{PHOX} C-terminal fragment (244−526) (0.216 nmol) by ERK2 was carried out in the presence of increasing concentrations of the p67^{PHOX} N-terminal fragment (1−210) (A) or N-terminal fragment (1−199) (B) (from 1 to 8 equimolar of the C-terminal fragment). Proteins were separated by SDS−PAGE on 12% Tris−glycine gel using standard techniques (*21*) and then transferred to nitrocellulose and revealed by autoradiography and Ponceau red staining. Phosphorylation was quantified by Cerenkov counting of the nitrocellulose bands. Data are representative of two experiments.

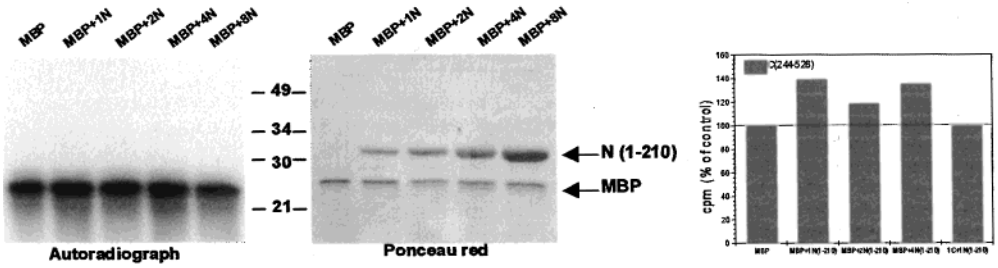

FIGURE 7: MBP phosphorylation is not inhibited by the N-terminal region of p67^{PHOX}. MBP phosphorylation was conducted as described in Materials and Methods in the presence of increasing concentrations of the p67^{PHOX} N-terminal fragment (1−210). Proteins were separated by SDS−PAGE on 12% Tris−glycine gel using standard techniques (*21*) and then transferred to nitrocellulose and revealed by autoradiography and Ponceau red staining. Phosphorylation was quantified by Cerenkov counting of the nitrocellulose bands. Data are representative of two experiments.

relative to the control. Furthermore, PD98059 and SB203580 had additive effects (Figure 10, top), suggesting that ERK1/2 and p38MAPK preferentially target different sites, confirming in vitro findings. PD98059 and SB203580 also inhibited p67^{PHOX} phosphorylation in PMA-stimulated neutrophils, and their effects were again additive (Figure 10, bottom).

To determine if peptides that are phosphorylated in vitro by ERK2 and p38MAPK are also phosphorylated in intact cells, we performed a phosphopeptide map of immunoprecipitated p67^{PHOX} from fMLP-stimulated neutrophils. To prevent the action of phosphatases, calyculin A (14 nM), an inhibitor of PP1/2, was added to each assay. Furthermore, to increase the response to fMLP, cytochalasin B, a non-G protein receptor mediated priming agonist, was preincubated with neutrophils 5 min before stimulation at a concentration of 5 μM. In these conditions, the major peptides phosphorylated by ERK2 and p38MAPK in vitro, peptides **a**, **b**, **d**, and **e**, were found to be phosphorylated in intact cells (Figure 11). Phosphorylation of these peptides occurs in resting cells (Figure 11, left) and was increased by fMLP (Figure 11, right). The basal phosphorylation seen in resting cells may be due to the presence of calyculin and cytochalasin B.

DISCUSSION

p67^{PHOX} phosphorylation is known to occur during neutrophil activation by agonists such as PMA, fMLP, and serum-opsonized zymosan (*6*, *7*, *15*), but the underlying mechanisms remain to be determined. p67^{PHOX} phosphorylation is a more subtle event than p47^{PHOX} phosphorylation. Indeed, p67^{PHOX} phosphorylation in activated cells is less intense than p47^{PHOX} phosphorylation (*7*, *16*) and is thought to involve a single major peptide (*15*), whereas the C-terminus of p47^{PHOX} contains multiple phosphorylation sites (*18*, *19*). For these reasons, p67^{PHOX} phosphorylation is more difficult to analyze. In the present study, we found that ERK2 and p38MAPK were both involved in p67^{PHOX} phosphorylation both in vitro and in intact neutrophils. In vitro p67^{PHOX} phosphorylation models are useful for identifying both major and minor phosphorylation sites and also the kinases involved. Using such a model, we found that ERK2 and p38MAPK phosphorylated p67^{PHOX} on selective sites: the major phosphorylation site targeted by ERK2 (peptide **b**) was located in the N-terminal fragment (1−243), while the major phosphorylation sites targeted by p38MAPK (peptides **a** and **d**) were located in the C-terminal fragment (244−526). This

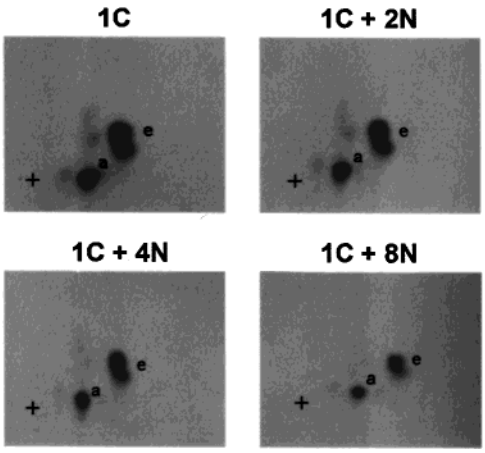

FIGURE 8: Phosphorylation of both peptide **e** and peptide **a** is inhibited by the N-terminal region of p67^{PHOX}. Phosphorylation of the p67^{PHOX} C-terminal fragment (244−526) (0.216 nmol) by ERK2 was carried out in the presence of increasing concentrations of the p67^{PHOX} N-terminal fragment (1−199) (from 2 to 8 equimolar of the C-terminal fragment). Proteins were separated by SDS−PAGE on 12% Tris−glycine gel and then transferred to nitrocellulose and revealed by autoradiography and Ponceau red staining. The nitrocellulose area containing the ^{32}P-labeled p67^{PHOX} C-terminal fragment (244−526) was then recovered, and phosphopeptide mapping was performed as described in Materials and Methods.

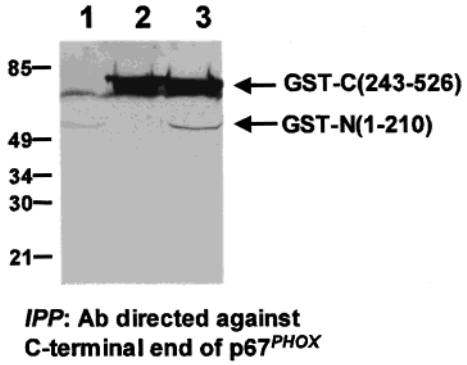

FIGURE 9: N-Terminal and C-terminal fragments associate with each other in the conditions of the phosphorylation mixture. The p67^{PHOX} N-terminal fragment (1−210) and p67^{PHOX} C-terminal fragment (244−526), 0.216 nmol each, expressed as fusion proteins with GST were incubated together in the phosphorylation reaction mixture and then immunoprecipitated overnight with an antibody directed against the C-terminal end of p67^{PHOX} (residues 511−526) in the presence of Gamma-bind G-Sepharose beads (Amersham-Pharmacia, Orsay, France). Bound proteins were revealed with an antibody directed against GST (Santa Cruz Biotechnology, Santa Cruz, CA) as described in Materials and Methods. Lanes: 1, GST−N-terminal (1−210) alone; 2, GST−C-terminal (244−526) alone; 3, GST−C-terminal (244−526) plus GST−N-terminal (1−210). Data are representative of two experiments.

selective phosphorylation suggests that these two kinases may have different roles in regulating p67^{PHOX} function.

We also obtained evidence that an additional peptide (peptide **e**), which was not phosphorylated in the intact protein, was phosphorylated in the isolated C-terminal fragment (244−526) in vitro by ERK2. One cannot exclude that phosphorylation of peptide **e** is an artifact of the use of the truncated protein; however, this site was also found to be phosphorylated in intact neutrophils, suggesting that its weak phosphorylation may actually happen under physiological conditions. Furthermore, phosphorylation of peptide **a** by ERK2 was also increased in the isolated C-terminal

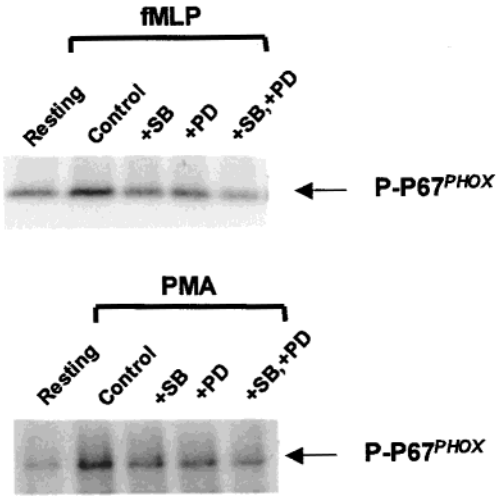

FIGURE 10: p67^{PHOX} phosphorylation is inhibited by SB203580 and PD980059 in fMLP- and PMA-stimulated neutrophils. ^{32}P-Labeled neutrophils (50×10^6/mL) were incubated with SB203580 (SB; 10 μM) and PD98059 (PD; 50 μM) at 37 °C for 30 min before stimulation with 10^{-6} M fMLP for 1 min or 0.5 μg/mL PMA for 6 min. p67^{PHOX} was immunoprecipitated and analyzed by SDS−PAGE transfer and revealed by autoradiography (p-p67^{PHOX}) and anti-p67^{PHOX} antibody labeling as described in Materials and Methods. Data are representative of three experiments.

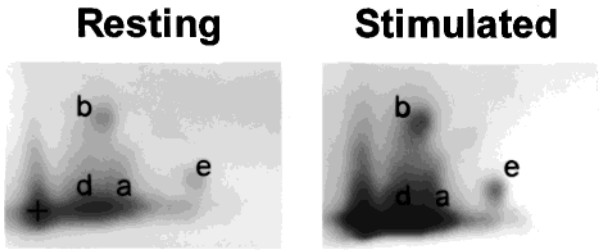

FIGURE 11: Phosphorylation of p67^{PHOX} in fMLP-stimulated neutrophils. ^{32}P-Labeled neutrophils (25×10^6 cells/mL, 450×10^6 cells total for each assay) were pretreated with 14 nM calyculin and 5 μM cytochalasin B for 5 min and then stimulated with 10^{-6} M fMLP for 1 min (right) or not (left). p67^{PHOX} was then immunoprecipitated and cleaved by trypsin and analyzed by thin-layer electrophoresis and chromatography as described in Materials and Methods. The phosphopeptides were detected by autoradiography. Data are representative of three experiments.

fragment (244−526). This suggests that both sites, **e** and **a**, are masked by the N-terminal domain in the intact protein. Indeed, we found that the N-terminal fragments (1−243, 1−210, and 1−199) all inhibited, in a concentration-dependent manner, phosphorylation of C-terminal fragment (244−526) by ERK2. Peptide mapping indicated that both peptides **e** and **a** were inhibited. Thus, an intramolecular domain in the TPR-rich region may regulate peptide **e** and **a** phosphorylation by ERK2. The significance of this regulation is currently under investigation.

Previous studies have shown that fMLP and PMA activate the ERK and p38MAPK pathways (*23*−*26*) and that SB 203580 and PD98059, two selective inhibitors of p38MAPK and MEK, respectively, inhibit superoxide anion production by human neutrophils (*23*−*26*). With regard to NADPH oxidase activation, p47^{PHOX} has been identified as a target of ERK (*27*), while the target(s) of p38MAPK is/are unknown. Our results indicate that p67^{PHOX} may be targeted by both p38MAPK and ERK in stimulated neutrophils. Indeed, PD98059 and SB203580 both inhibited p67^{PHOX}

phosphorylation in fMLP- and PMA-stimulated neutrophils. Furthermore, their effects were additive, suggesting that ERK and p38MAPK target different sites in vivo, as observed here in vitro. The fact that PD98059 and SB203580 inhibit $p67^{PHOX}$ phosphorylation in PMA-stimulated neutrophils suggests that $p67^{PHOX}$ phosphorylation by ERK1/2 and p38MAPK in intact neutrophils could be in part regulated by an upstream PKC-dependent pathway. Phosphorylation of $p47^{PHOX}$ by ERK1/2 and $p67^{PHOX}$ by both ERK1/2 and p38MAPK could explain the inhibitory effect of MEK and p38MAPK inhibitors on NADPH oxidase activation (*23*−*26*). The involvement of ERK and p38MAPK in vivo is also supported by the fact that major sites phosphorylated in vitro by ERK and p38MAPK (sites **a**, **b**, and **d)** are also phosphorylated in fMLP-stimulated neutrophils that had been pretreated with calyculin A and cytochalasin B. In addition, phosphorylation of peptide **e** that is unmasked in the truncated $p67^{PHOX}$ may be relevant to the in vivo situation, since this site is also phosphorylated in intact cells. Phosphorylation of these peptides occurs also weakly in resting state and may be due to pretreatment of neutrophils by calyculin, a phosphatase inhibitor, and cytochalasin B, a non-G protein receptor mediated priming agonist.

Phosphorylation of the C-terminal part of intact $p67^{PHOX}$ by p38MAPK (peptides **a** and **d**) and also, to a lesser extent, by ERK2 (peptide **a**) could trigger a conformational change to an active configuration. Indeed, in a recent study, Hata et al. (*28*) showed that even without the amphiphiles which, in cell-free systems, mimic phosphorylation by conferring negative charges, the oxidase is activated in vitro by C-terminally truncated $p47^{PHOX}$, retaining the N-terminal and two SH3 domains, and the N-terminus (1−242) of $p67^{PHOX}$. Furthermore, when either truncated $p47^{PHOX}$ or $p67^{PHOX}$ is replaced by the corresponding intact protein, the amphiphiles are crucial for activation. This indicates that the C-terminal part of $p67^{PHOX}$ is also targeted by the amphiphiles and that it plays a negative regulatory role. In intact cells, phosphorylation of the C-terminal portion of $p67^{PHOX}$ could abrogate this negative regulation.

Finally, $p67^{PHOX}$ phosphorylation by ERK2 and p38MAPK on more than one site is in keeping with previous studies showing that $p67^{PHOX}$ can be phosphorylated on both threonine and serine residues (*7*, *15*) and the fact that two other serines, located at positions 312 and 332, are potential targets for proline-directed kinases. Site **b** may correspond to threonine 233, since it is located in the N-terminal fragment comprising residues 1−243, and that shorter N-terminal fragment (comprising residues 1−210 and 1−199) could not be phosphorylated (Figure 6). The identification of the other sites is currently under investigation.

In conclusion, our results suggest that $p67^{PHOX}$ can be phosphorylated by ERK2 and p38MAPK on several selective sites both in vitro and in vivo. One of the C-terminal sites appears to be cryptic, becoming accessible after a conformational change of the protein.

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