Protein Phosphorylation in Neutrophils From Patients With p67-phox–Deficient Chronic Granulomatous Disease

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Neutrophils are known to contain a major 67-kD protein that undergoes enhanced phosphorylation and translocation to the membrane during cell stimulation. Recent studies have assumed that this 67-kD phosphoprotein is the 67-kD subunit of the phagocyte oxidase (p67-phox). We compare here the protein phosphorylation patterns in lysates of normal neutrophils and neutrophils from patients with chronic granulomatous disease (CGD) that are completely deficient in p67-phox. The phosphoproteins were labeled by incubation of the cells with radioactive inorganic phosphate ($^{32}$P) or by the addition of [$\gamma$-$^{32}$P]ATP to electropermeabilized neutrophils. With either method, stimulation of the normal or CGD cells always resulted in an enhanced incorporation of $^{32}$P into two proteins in the 67-kD area. The extent of phosphorylation of these two proteins was very similar in the normal and CGD cells when permeabilized neutrophils loaded with [$\gamma$-$^{32}$P]ATP were compared. Moreover, no overall differences in the protein phosphorylation patterns were observed between the normal and CGD cells. Our data indicate that the major 67-kD phosphoproteins observed in stimulated neutrophils are clearly different from p67-phox.

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MATERIALS AND METHODS

Materials

$[\gamma$-$^{32}$P]ATP (3,000 Ci/mmol) was purchased from DuPont-New England Nuclear (Boston, MA). ATP (K+ salt), GTP (Na+ salt), and NADPH were purchased from Sigma Chemical Co (St Louis, MO). ImmunoPure immobilized protein A was obtained from Pierce (Rockford, IL). Antibodies to peptides that correspond to residues 340 to 355 of p47-phox (RPGHQPSGPLEEQK)20 and to residues 437 to 450 of p67-phox (DEPKISEKADANNQ)21 were raised in rabbits. Each peptide was conjugated to keyhole lympet hemocyanin before use as an immunogen. Sera were collected from these rabbits and tested by Western blotting against both the cytosolic fraction from normal neutrophils and from neutrophils of patients with CGD lacking either p47-phox or p67-phox to confirm the specificity for these two proteins.

Methods

Preparation of human neutrophils. Human neutrophils (purity, >99%) were prepared as described previously.22 The cells were suspended (2 × 10$^6$/mL) in phosphate-buffered saline (PBS; 138 mmol/L NaCl, 2.7 mmol/L KCl, 8.1 mmol/L Na$_2$HPO$_4$, and 1.47 mmol/L KH$_2$PO$_4$, pH 7.35) and maintained on wet ice before use.

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Labeling of neutrophils with $^{32}$P, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for studies on protein phosphorylation. These techniques were performed as described earlier, except that the amount of radioactivity was increased to 1.5 mCi $^{32}$P/mL and the labeling time was extended to 1 hour.

**Immunoblotting and immunoprecipitation experiments.** For SDS-PAGE, neutrophils (3 x 10^6/mL) were mixed with an equal volume of 2X concentrated loading buffer. The final composition of this buffer after mixing with cells was 50 mmol/L Tris (pH 6.8), 2.0% (wt/vol) SDS, 4.0% (vol/vol) glycerol, 1.0% (vol/vol) $\beta$-mercaptoethanol, and 0.002% (wt/vol) bromophenol blue. After immersion in a boiling water bath for 4 minutes, 20 $\mu$L samples were subjected to SDS-PAGE and Western blotting with antibodies to p47-phox and p67-phox exactly as described in Curtin et al. Alkaline-phosphatase-conjugated anti-IgG was used as the secondary antibody and the color was achieved with Promega's Wine Immobilion-PO4 kit (Promega, Madison, WI). For 2D-gel electrophoresis, a goat antirabbit IgG horse radish peroxidase conjugate was used as the secondary antibody (Bio-Rad, Hercules, CA), and antigen detection was performed with the enhanced chemiluminescence system (ECL) from Amersham (Arlington Heights, IL).

Immunoprecipitation of p67-phox from lysates of $^{32}$P-labeled neutrophils was performed by the procedure outlined in Springer. Before lysis, neutrophils were treated for 15 minutes at 4°C with 2.5 mmol/L diisopropyl fluorophosphate. The lysis buffer was supplemented with Complete Protease Inhibitor Cocktail (Boehringer Mannheim, Indianapolis, IN) at the recommended concentrations. An antibody raised to a recombinant p67-phox $\beta$-gal fusion protein was used in these studies at a 1:50 dilution. After the final wash, the protein A-coated beads containing bound p67-phox was resuspended in 0.2 mL of loading buffer. Samples were immersed in a boiling water bath for 4 minutes and the supernatants (0.05 mL/lane) were subjected to SDS-PAGE and analyzed for protein phosphorylation as described above.

**Cell permeabilization.** These studies were analyzed as described in Lu et al, except that 20 mmol/L [γ-$^{32}$P]ATP (10 $\mu$Ci) was used in place of nonradioactive ATP.

**Two-dimensional (2D) gel electrophoresis.** Neutrophils were treated with 2.5 mmol/L diisopropyl fluorophosphate for 15 minutes at 4°C and labeled with $^{32}$P as outlined above. Labeled cells were washed once; resuspended in PBS (1 x 10^6 cells/mL) containing 0.90 mmol/L CaCl$_2$, 0.50 mmol/L MgCl$_2$, and 7.5 mmol/L glucose; and equilibrated at 37°C for 3 minutes. After stimulation or treatment with reagents (see figure legends), the cells were rapidly pelleted in a microfuge and immediately lysed in 120 $\mu$L of lysis solution (9.0 mmol/L urea, 2% [vol/vol] Triton X-100, 2% [vol/vol] 2-mercaptoethanol, 2% [vol/vol] Pharmalyte 3-10 [Pharmacia Biotech, Uppsala, Sweden], and 8.0 mmol/L phenylmethylsulfonyl fluoride) at room temperature for 10 minutes. Samples were diluted with 80 $\mu$L of sample solution (composition the same as for the lysis solution except with 8.0 mmol/L urea and [0.5% vol/vol] Triton X-100) and centrifuged for 10 minutes at 19,000g. First-dimension isoelectric focusing was performed according to the manufacturer's instructions using a Pharmacia Multiphor II electrophoresis unit and immobilized pH gradients (110-mm Immobiline DryStrips, pH 3 to 10). Sample wells were placed at the cathode end of the strips and loaded with 60 $\mu$L of sample (equivalent to 3 x 10^6 cells). Electrophoresis was performed under a layer of mineral oil at 1,300 V for a total of 23,000 kVh, with cooling to 15°C. Isoelectric point markers (BioRad) were run in parallel under identical conditions.

Before the second-dimension run, Immobiline strips were equilibrated by agitation for two periods of 10 minutes each in a solution containing 6.0 mol/L urea, 1% (wt/vol) SDS, 30% (vol/vol) glycerol, and 50 mmol/L Tris/HCl, pH 6.8. For the first period of equilibration, the solution also contained 16.2 mmol/L diethiothreitol; for the second period of equilibration, it contained 240 mmol/L iodoacetamide and a few grains of bromophenol blue. The second-dimension was run by conventional SDS PAGE, as referenced above, using 1-mm thick 10% polyacrylamide gels and combs designed to take a single large sample (BioRad Protean II). Proteins were transferred to an Immobilon-P membrane and p67-phox was detected by Western blotting as described above. The chemiluminescent signal was allowed to decay for a minimum of 16 hours before autoradiography of the membrane was performed to detect the $^{32}$P-labeled proteins.

**Analysis of data.** All of the data presented in the autoradiograms for normal neutrophils were confirmed in at least three separate experiments performed on different preparations of cells. Data presented on CGD neutrophils deficient in p67-phox were observed in three patients studied at different times.

**RESULTS AND DISCUSSION**

**Protein Phosphorylation Examined by SDS-PAGE/Autoradiography.**

Figure 1A presents an autoradiogram of the $^{32}$P-labeled proteins in unstimulated neutrophils and neutrophils treated with 320 nmol/L PMA for 3 minutes. The positions of p47-phox and p67-phox are indicated in the corresponding Western blot (Fig 1B). These cells were labeled by a preincubation with radioactive inorganic phosphate ($^{32}$P; see Methods). Although considerable variability is observed in labeling experiments of this type, stimulation of neutrophils with PMA always resulted in a marked incorporation of $^{32}$P into a 47-kD protein (solid arrow) along with two proteins in the 67-kD region (bracketed area designated "S"). The 47-kD phosphoprotein was positively identified as p47-phox by the observation that this entity was not present in neutrophils from patients with CGD deficient in this protein (data not shown, Okamura et al and Segal et al). Several other proteins also exhibited an enhanced incorporation of $^{32}$P upon cell stimulation, including a pair of proteins in the 80-kD range (area designated "E"). Changes in this area were difficult to quantitate due to the large increase in the background radioactivity that occurred during cell stimulation. Figure 2 compares the time-course for incorporation of $^{32}$P into p47-phox and the 67-kD proteins. The lower phosphoprotein band in the 67-kD area (broken arrow) and p47-phox exhibited significant labeling 1 minute after stimulation, whereas the upper band in the 67-kD region (arrowhead) did not display appreciable incorporation of $^{32}$P until 2 minutes after stimulation. The delayed labeling of the upper 67-kD band is similar to that reported by Korchak et al for a 66-kD protein in neutrophils that is a substrate for the Ca$^{2+}$-independent, phosphatidyserine/diglyceride-dependent protein kinase (nPKC). The kinetics of phosphorylation of the lower 67-kD band and p47-phox are similar to the time-course for O$_2$ release from neutrophils stimulated with PMA.

To determine if one or both of the phosphoproteins in the 67-kD region was or were p67-phox, neutrophils from patients with CGD that lacked p67-phox were examined. The phosphoproteins in $^{32}$P-labeled normal neutrophils and neutrophils from two sisters with CGD resulting from a complete deficiency in p67-phox (CGD1 and CGD2) are compared in Fig 3B. Although all three cell preparations exhibited considerable differences in the overall degree of labeling...
Because several other phosphoproteins in this sample also exhibited reduced incorporation of $^{32}$P (e.g., p47-phox and the 80-kD proteins designated “E”). Neutrophils from neither of the CGD patients used in Fig 3 exhibited any p67-phox when their proteins were subjected to Western blotting with a peptide antibody generated against residues 437 to 450 of this protein (Figs 3A or 4A). Similarly, p67-phox was not detected in these same patients during immunoblotting with an antibody raised against the 10 C-terminal residues of p67-phox, a polyclonal antiserum (B-L) that recognized the native p67-phox protein, or with a polyclonal antibody generated against a recombinant p67-phox β-gal fusion protein (data not shown).

The variability in labeling neutrophils with $^{32}$P is probably the result of individual differences in the rates of $^{32}$P transport into cells and the metabolism of this anion into ATP. These problems can be circumvented by the direct addition of [$\gamma$-$^{32}$P]ATP to permeabilized cells. A previous study has shown that permeabilized neutrophils stimulated with PMA release normal amounts of $O_2$ when ATP, GTP, and NADPH are added to the medium. Figure 4B presents the autoradiograms for permeabilized neutrophils that were incubated with 20 mmol/L [$\gamma$-$^{32}$P]ATP (10 μCi), 100 mmol/L GTP, and 1.0 mmol/L NADPH for 5 minutes and then stimulated with 320 nmol/L PMA for 3 minutes. Although the same cell preparations were used in these studies as in Fig 3, all of the samples now exhibited comparable amounts of labeling. Stimulation of the normal and CGD cells again resulted in the incorporation of $^{32}$P into two proteins in the 67-kD area. Under these conditions, the amounts of $^{32}$P in these two bands were very similar in all cases and substantially greater than that observed in stimulated cells labeled (see below), increased incorporation of $^{32}$P into the two proteins in the 67-kD area was clearly evident in the stimulated CGD cells. Similar results were observed with neutrophils from a third patient with CGD who was deficient in p67-phox (data not shown). Less labeling of these two proteins occurred in the CGD2 neutrophils than in the other samples (N and CGD1). However, this diminution probably reflects a lower specific activity of the [[$\gamma$-$^{32}$P]ATP in these cells,
Fig 3. Protein phosphorylation in neutrophils from patients with CGD deficient in p67-phox. Proteins from normal neutrophils (N) and neutrophils from 2 CGD patients deficient in p67-phox (CGD1 and CGD2) were subjected to both Western blotting (A) and autoradiography (B). Unstimulated (−) and stimulated neutrophils (+) were prepared by treating the cells for 3 minutes with 0.25% (vol/vol) Me2SO or 320 nmol/L PMA, respectively. Neutrophils were labeled with [32P]PI, and the proteins were subjected to SDS-PAGE as described in the Methods. The gel was cut in half and the proteins in one portion (A) were transferred to an Immobilon-P membrane and immunoblotted with antibodies to p47-phox and p67-phox. The remaining portion of the gel was subjected to autoradiography (B). The position of p47-phox is designated by an arrow. Pairs of phosphoproteins with molecular masses of approximately 67 and 80 kD are bracketed in (B) and designated by “S” and “E,” respectively. The arrowhead (upper band) and dashed arrow (lower band; B, right margin) denote the positions of two proteins with molecular masses of approximately 67 kD that undergo enhanced phosphorylation during cell stimulation.

Fig 4. Protein phosphorylation in permeabilized neutrophils loaded with [γ-32P]ATP. Comparison of the phosphoproteins in normal neutrophils and cells deficient in p67-phox. Proteins from permeabilized normal neutrophils (N) and neutrophils from 2 patients deficient in p67-phox (CGD1 and CGD2) were subjected to both Western blotting (A) and autoradiography (B). Unstimulated (−) and stimulated (+) permeabilized neutrophils were prepared by treating the cells for 3 minutes with 0.25% (vol/vol) Me2SO and 320 nmol/L PMA, respectively. Cells were permeabilized and loaded with [γ-32P]ATP and the proteins were subjected to SDS-PAGE as described in the Methods. The gel was cut in half and the proteins in one section (A) were transferred to an Immobilon-P membrane and immunoblotted with antibodies to p47-phox and p67-phox. The remaining portion of the gel was subjected to autoradiography (B). The position of p47-phox is designated by an arrow. Pairs of phosphoproteins with molecular masses of approximately 67 and 80 kD are bracketed in (B; right margin) and designated by “S” and “E,” respectively. The arrowhead (upper band) and dashed arrow (lower band; B, right margin) denote the positions of the two proteins with molecular masses of approximately 67 kD that undergo enhanced phosphorylation during cell stimulation.
with $^{32}$P. This probably results from a higher specific activity of [$\gamma$-$^{32}$P]ATP in the permeabilized cells and a lower background compared with cells labeled with $^{32}$P. In addition, the samples in Fig 4 clearly exhibited the pair of proteins in the 80-kD area along with several other proteins that underwent marked phosphorylation during cell stimulation. In contrast, p47-phox consistently displayed less phosphorylation in stimulated permeabilized neutrophils than in intact cells (compare Figs 3 and 4). The basis for this decrease in the labeling of p47-phox in permeabilized neutrophils is not known. However, the extent of phosphorylation of p47-phox in stimulated cells is known to be dependent on its interaction with cytochrome b$_{55}$ in the cytoskeleton/plasmalemma.$^5$ Recent studies have provided evidence that

Studies Using 2D-Gel Electrophoresis

Phosphorylation of p67-phox may be masked by one of the major phosphoproteins in the 67-kD area. This possibility was investigated by 2D-gel electrophoresis (Fig 5). To determine the exact position of p67-phox in the resulting autoradiographs, the separated $^{32}$P-labeled proteins were transferred to Immobilon-P membranes and each membrane was analyzed by both Western blotting and autoradiography. p67-phox was well separated from the major phosphoproteins in the 67-kD region by this technique and did not exhibit an enhanced phosphorylation upon stimulation of the
cells with PMA (Fig 5, right-hand panels). The measured isoelectric point (pI) for p67-phox in both unstimulated and stimulated neutrophils was approximately 5.7 to 5.9 (Fig 5, left-hand panels), which is close to the theoretical value of 5.88. Thus, the majority of p67-phox in stimulated neutrophils did not exhibit the shift in pI of approximately 0.5 to 1.0 pH units that is expected for the incorporation of a phosphate group into a protein.

Previous studies that reported phosphorylation of p67-phox used a relaxation buffer to disrupt the cells that contained 500 μmol/L okadaic acid. This compound is a potent and selective antagonist of type 1 and 2A protein phosphatases. Okadaic acid is known to inhibit O2 release from PMA-stimulated human neutrophils at a concentration of 5.0 μmol/L. Moreover, antagonists of type 1 and 2A protein phosphatases promote a dramatic hyperphosphorylation of numerous proteins in neutrophils, particularly those in the greater than 50-kD area. Treatment of neutrophils with 5.0 μmol/L okadaic acid alone also results in a small population of p67-phox undergoing an increase in mass that is consistent with phosphorylation. Treatment of neutrophils with 5.0 μmol/L okadaic acid alone also results in a small population of p67-phox undergoing an increase in mass that is consistent with phosphorylation. Effects of okadaic acid on the phosphorylation of neutrophil proteins were therefore investigated with 2D-gel electrophoresis (Fig 6). As reported previously, treatment of neutrophils with okadaic acid (5.0 μmol/L) alone for 10 minutes resulted in a remarkable hyperphosphorylation of a large number of proteins. A slight increase in the incorporation of 32P was observed in the area of p67-phox in 3 of 3 experiments (Fig 6B), but no further increase was observed upon subsequent stimulation of the cells with PMA (Fig 6C). Thus, the combination of okadaic acid and PMA did not result in the appearance of a major phosphoprotein band in the area of the autoradiogram occupied by p67-phox. Finally, immunoprecipitation experiments with an antibody raised to a recombinant p67-phox β-gal fusion protein also did not show any phosphorylation of p67-phox during cell stimulation. In these experiments, p67-phox was immunoprecipitated from lysates of 32P-labeled neutrophils after treatment of the cells at 37°C with 0.25% (vol/vol) Me2SO for 3 minutes or 320 nmol/L PMA for 3 minutes. No incorporation of 32P into p67-phox was observed under these circumstances (data not shown; n = 2). A parallel experiment was also run and subjected to Western blotting to confirm that precipitation of p67-phox had occurred under the conditions used.

As noted above, recent studies have presumed that a major 67-kD phosphoprotein observed in cell lysates or fractions of stimulated neutrophils was p67-phox. Models for the assembly and activation of the NADPH-oxidase complex were based on this identification. We show here that this assumption is unfounded. In particular, we report that the two proteins in the 67-kD range that undergo a major incorporation of 32P during cell stimulation are present in both stimulated normal neutrophils and in cells deficient in p67-phox. Identifying and characterizing these phosphoproteins will be important to a complete understanding of stimulus-response phenomena in neutrophils. Although it is possible that a small population of p67-phox may undergo phosphorylation in stimulated neutrophils that cannot be detected by the procedures used herein, p67-phox is clearly shown to be different from the major 67-kD phosphoproteins. Subsequent studies on the possible phosphorylation of p67-phox during the assembly of the NADPH-oxidase system are likely to require reconstitution methods with cell-free systems that require protein phosphorylation.

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