A Possible Role for Protein Phosphorylation in the Activation of the Respiratory Burst in Human Neutrophils

EVIDENCE FROM STUDIES WITH CELLS FROM PATIENTS WITH CHRONIC GRANULOMATOUS DISEASE*

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Two-dimensional gel electrophoresis was used to study protein phosphorylation in granules, membranes, and soluble fractions from human neutrophils that had been loaded with $^{32}$P. In resting cells, label was incorporated primarily into proteins of the membranes and the soluble supernatant; little appeared in the granules. Activation of $^{32}$P-loaded neutrophils resulted in an increase in the $^{32}$P content of a small number of membrane and soluble proteins without a change in the labeling of the granule fraction. The identity of the proteins affected by activation depended on the activating agent used; all of the activating agents, however, caused an increase in the labeling of a group of ~48-kDa proteins that appeared to be distributed between the membranes and the soluble supernatant.

To investigate the role of phosphorylation in the activation of the respiratory burst oxidase, the incorporation of $^{32}$P into phosphoproteins was studied in neutrophils from patients with chronic granulomatous disease. When these cells were exposed to phorbol myristate acetate, one of the agents used for the activation of normal neutrophils, the 48-kDa proteins in the membranes and supernatants failed to take up additional $^{32}$P. Phosphorylation patterns in normal neutrophils activated under nitrogen were similar to the patterns seen with cells activated in air, suggesting that the differences in phosphorylation between normal and chronic granulomatous disease neutrophils did not represent secondary effects of the oxidants produced by the normal cells, but reflected primary biochemical differences between the normal and the defective phagocytes. We postulate from these results that the uptake of phosphate by the 48-kDa protein group may be involved in the activation of the respiratory burst oxidase.

The enzyme responsible for the production of microbicidal oxidants during the respiratory burst is a membrane-bound oxidase that catalyzes the reduction of oxygen to $O_2^-$ at the expense of NADPH (1-3). This enzyme is dormant in resting cells, but is rapidly activated when the cells are exposed to appropriate stimuli such as opsonized bacteria or zymosan, phorbol myristate acetate (PMA), chemotactic peptides such as f-Met-Leu-Phe, or arachidonic acid (4-9). Despite extensive investigation, little is known about the molecular mechanism by which this oxidase is activated, although recent work suggests that activation requires elements from both the soluble and particulate fractions of the cell (10).

Other enzymes whose activities vary with the state of the cell are frequently regulated by phosphorylation (11). The idea that neutrophils contain such phosphorylation-controlled enzymes has received support from previous studies showing that neutrophils incubated with $^{32}$P incorporate phosphate into a large number of their proteins and that the extent of labeling of several of these proteins changes when the cells are exposed to activating agents (12-15). It has not yet been possible, however, to associate any of these phosphoproteins specifically with the activation of the $O_2^-$-forming oxidase, because consistent differences have not been observed between normal neutrophil phosphoprotein patterns and patterns in cases of chronic granulomatous disease, an inherited disorder affecting the activity of the $O_2^-$-forming oxidase (13). Inasmuch as studies to date employed only the relatively low resolution method of one-dimensional gel electrophoresis for the separation and analysis of phosphoproteins, it seems possible that subtle differences in the phosphoproteins of normal and chronic granulomatous disease neutrophils might have escaped detection. We have therefore reexamined neutrophil protein phosphorylation using two-dimensional gel electrophoresis as the analytical method. To further improve resolution, we separated the cells into subcellular fractions before carrying out the analysis. The results of these experiments are the subject of the present report.

MATERIALS AND METHODS

PMA, f-Met-Leu-Phe, superoxide dismutase, cytochrome c (Type VI), Nonidet P-40, p-nitrophenyl phosphate, N-ethylmaleimide, iodoacet acid, benzamidine, leupeptin, and pepstatin were purchased from Sigma Chemical Company, St. Louis, Missouri.

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1 The abbreviations used are: PMA, phorbol myristate acetate; CGD, chronic granulomatous disease; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PMA', f-Met-Leu-Phe, N-formyl-methionyl-leucyl-phenylalanine.
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from Sigma: Arachidonic acid was purchased from Sigma or from NeoClone, Elysin, MN. Diisopropyl fluorophosphate was obtained from Aldrich, carrier-free $^{32}$P; from Amersham, Macrodex and Ficollic-Paque from Pharmacia, tissue culture flasks (Falcon 3013) from Arthur H. Thomas, chemicals for gel electrophoresis (sodium dodecyl sulfate, acrylamide, N,N'-methylenebisacrylamide, molecular weight standards, and all basic reagents from Bio-Rad) from Bio-Rad, phosphorylase b-containing species with the following PI values: 4.1, 4.9, 6.4, 8.3, 9.7, and 10.6) from Calbiochem-Behring, and Ampholines from LKB, Paramus, NJ. Preparated gradient (10-20%) gel electrophoresis slabs were purchased from Separation Sciences, Attleboro, MA.

Preparation and Electrophoresis

Preparation and Electrophoresis of Plasma Membrane and Granule Fractions—The pooled sonicates from the 7 flask experiments were pooled. For each activation agent were carried out as described above. At the end of the incubations, the buffer in the gels was replaced with 1.5 ml of ice-cold deoxygenated phosphate-buffered saline containing 12.9 mCi of freshly prepared 8.5 m urea containing 2% Nonidet P-40, 1.6% $\beta$-mercaptoethanol.

The samples were then subjected to two-dimensional gel electrophoresis. For the first dimension, nonequilibrium isoelectric focusing (20) was performed in 4 x 120-mm cylindrical gels of 4% polyacrylamide containing 8.5 m urea, 2% Nonidet P-40, 1.6% $\beta$-mercaptoethanol, and 0.4% $\beta$-5-4.0 Ampholines, and 5% mercaptoethanol.

Under these conditions a linear pH 4-10 gradient was formed in the gels. After isoelectric focusing was complete, the gels were prepared for electrophoresis in the second dimension by equilibration at room temperature for two 1-h periods with sodium dodecyl sulfate-containing sample buffer (Laemmli: (21)). Electrophoresis in the second dimension was carried out by the Laemmli technique using 10-20% gradient or 12% straight polyacrylamide gels. Both the pelleted fractions and the cylindrical gels could be frozen for storage without affecting the final results.

 Autoradiography of the gel was carried out as previously described (13). For quantitation, the autoradiograms were scanned in one dimension using a Zeinin soft laser scanning densitometer with a 0.1-mm diameter beam. Each scan was carried out in the direction of the second electrophoresis, positioning the film in the instrument automatically. For each scan, the film was calibrated for the center of the gels or the particular experiment and whose density reflected the overall level of film exposure as judged by eye. The spots chosen for normalization differed from experiment to experiment. Quantitation was accomplished by drawing out and weighing peaks from Xerox copies of the scans. Normalized spot areas were calculated by dividing the weight of the peak corresponding to the spot to be quantified by the weight of the peak from the normalization scan.

Preparation and Electrophoresis of Particles and Soluble Supernatant—The 500 x g supernatant was centrifuged at 140,000 g for 15 min at 4 °C to remove whole cells and nuclei. Six ml portions of the supernatants were then fractionated by sucrose density gradient centrifugation as described elsewhere (16, 18, 19), except that the sucrose solutions from which the gradients were prepared contained inhibitors (except leupeptin and pepstatin) at the same final concentrations as were present in the sonicate. After centrifugation, pellets were suspended as described (33-36 seconds). Using myeloperoxidase determinations as a guide, the gruel containing fractions (in a typical experiment, fractions 1-12) and the plasma membrane-containing fractions (typically, fractions 24-34) were separately pooled. In a representative fractionation, the yield of plasma membrane was 18.8% of granules and 15.0 mg of plasma membrane. The particles from the pooled fractions were isolated by centrifugation at 140,000 g for 10 min at 4 °C, 12.9 mg of freshly prepared 8.5 m urea containing 2% Nonidet P-40, 1.6% $\beta$-mercaptoethanol.
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RESULTS

Fractionation of Adherent Cells—The sucrose gradient method used for fractionating neutrophils in this study had only been verified with homogenates from suspended cells. It was therefore necessary to establish that this method was also suitable for the fractionation of homogenates from adherent cells. For this purpose, neutrophil monolayers were treated with diisopropyl fluorophosphate, then disrupted in a small volume of inhibitor-containing buffer by means of a cup sonicator (13). The sonicates were then fractionated on sucrose density gradients as described under “Materials and Methods,” and the fractions were assayed for the following marker enzymes: myeloperoxidase (azurophil granules), cobalamin-binding protein (specific granules), acid phosphatase (plasma membrane), and lactate dehydrogenase (cytosol). The results (Fig. 1) show that for both resting and PMA-activated cells, the granules were well separated from the plasma membranes and cytosol, which were found in the top 7–8 fractions as indicated by the locations of the acid phosphatase and the lactate dehydrogenase (not shown). The top few fractions of the gradient from the activated cells also contained a substantial quantity of cobalamin-binding protein, presumably released from the specific granules during activation (23); cobalamin-binding protein was not present in the corresponding fractions from the resting cell gradient.

For fractionating cells that had been labeled with $^{32}$P$_o$, additional antiproteases and phosphatase and kinase inhibitors were included in the gradients. The presence of these additional inhibitors precluded the measurement of acid phosphatase, the marker used to locate the plasma membranes in the gradients shown in Fig. 1. By gel electrophoresis of the fractions, however, it was determined that the distribution of granules and plasma membrane in the gradients was not affected by the extra inhibitors.

Phosphorylation—Phosphorylation of neutrophil proteins was examined in three subcellular fractions: plasma membranes, combined granules (azurophil plus specific), and cytosol. Results were represented graphically in the form of densitometric scans of autoradiograms, with the percentages of total protein on each autoradiogram being normalized to 100.

FIG. 1. Fractionation of neutrophil homogenates by sucrose gradient centrifugation. Fractionation and assays were carried out as described in the text. Cbl, cobalamin.

FIG. 2. Uptake of $^{32}$P by membrane proteins in resting neutrophils and neutrophils activated with phorbol myristate acetate. Experiments were carried out as described in the text. Cells were activated for 2 min with PMA (0.1 μg/ml). A gradient gel was used for electrophoresis in the second dimension. Proteins that took up added label in response to the activating agent are indicated by the arrows. The arrow with the asterisk indicates the 48-kDa group. In this and subsequent figures, the gels are oriented with their basic ends to the left. Top, the complete autoradiograms; bottom, detail of areas showing proteins that took up added label when the cells were activated.
As seen in Fig. 2, plasma membranes from resting cells contained a relatively large number of phosphorylated proteins, some resolved, but an indeterminate number represented by a vaguely defined haze. Labeling of some of the resolved proteins intensified when the cells were activated with PMA (arrows). In particular, PMA treatment caused a clear increase in the labeling of a group of basic proteins of mass ~48 kDa (arrow with asterisk). Labeling of the 48-kDa group was well under way by 20 s (data not shown). Activation of the neutrophils with arachidonate (80 μM) or f-Met-Leu-Phe (0.6 μM) also resulted in an increase in the incorporation of label into various plasma membrane phosphoproteins, each activator affecting a slightly different set of proteins. Labeling of the 48-kDa group, however, was stimulated by all 3 activators.

The labeling of proteins in the soluble supernatants from resting and PMA-activated neutrophils is shown in Fig. 3. Those phosphoproteins that showed consistent differences in labeling between the resting and activated preparations are indicated by the arrows. Among them is a group at ~48 kDa that is very similar in location to the group of 48-kDa membrane proteins that took up label on activation of the neutrophils (arrow with asterisk; see Fig. 2). It is possible that these represent a single set of proteins that are distributed between the plasma membrane and the cytosol.

Labeling was also examined in supernatants from cells activated with arachidonate. For this experiment, advantage was taken of the reversibility of arachidonate-mediated neutrophil activation to fully label proteins that are phosphorylated in the resting state but lose their phosphate during activation. The results (Fig. 4) showed that, like the membrane phosphoproteins, the cytosolic phosphoproteins showed distinct differences in labeling patterns depending on whether arachidonate or PMA was the activating agent. For example,
in arachidonate-treated cells, a 21-kDa protein (arrowhead) that was unaffected by PMA (cf. Fig. 3) was found to take up label during the transition from the activated to the resting state and to lose it again upon reactivation. The 48-kDa group, however, responded to arachidonate just as it did to PMA.

Proteins in the neutrophil granules took up little 32P in either the resting or activated state.

Protein Phosphorylation in Chronic Granulomatous Disease—CGD is an inherited condition in which neutrophils fail to express a respiratory burst (24). In most cases, including the most common type (X-linked inheritance), the disease appears to be due to a defect in activation of the oxidase rather than to an abnormality in the enzyme itself (25, 26). To ascertain whether any of the changes in phosphorylation seen in these experiments might be related to the activation of the oxidase, we examined the protein phosphorylation patterns in a group of male patients with CGD.

Membrane protein phosphorylation was studied in 4 of these patients. The results of a representative experiment are shown in Fig. 5. A clear difference between the phosphorylation patterns of normal and CGD membranes was seen: In normal neutrophils, all of the proteins in the 48-kDa group took up label when the cells were activated with PMA, while in CGD neutrophils only the leftmost protein took up added label. Identical results were obtained in 2 of the other 3 experiments; in one, however, there was a minor increase in labeling of the other 48-kDa proteins, although the extent of the increase was much smaller than normal.

Phosphorylation of cytosolic proteins in response to PMA activation was also studied in CGD neutrophils. The results (Fig. 6) showed that the increase in labeling of the soluble 48-kDa protein group normally elicited by PMA did not take place. Moreover, the abnormality affected all of the soluble 48-kDa group proteins, in contrast to the situation in CGD.

**FIG. 5.** Uptake of 32P by membrane proteins from normal and CGD neutrophils in response to PMA. For experimental conditions, see legend to Fig. 2. The 48-kDa protein group is indicated by the arrow. Uptake of label by the 48-kDa proteins in response to PMA is seen to occur in normal but not CGD cells. Top, the complete autoradiograms; bottom, detail of areas showing the 48-kDa protein group.
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Fig. 6. Uptake of $^{32}$P by soluble proteins from resting and PMA-activated neutrophils; comparison of normal and CGD cells. Each row shows the results of a single experiment in which labeling of soluble proteins from preparations of normal and CGD cells was measured on the same day. The 4 experiments were carried out with cells from 4 different normal donors and 4 different patients with chronic granulomatous disease. Only the region around the 48-kDa protein group (arrow) is shown (see Fig. 3 for the appearance of the entire autoradiogram). The experimental conditions are given in the legend to Fig. 3.

TABLE I
Stimulation of $^{32}$P uptake into the 48-kDa protein group by PMA treatment of normal and CGD neutrophils

<table>
<thead>
<tr>
<th>Cells</th>
<th>$^{32}$P ratio (activated/resting)*</th>
<th>48-kDa group (60 kDa)</th>
<th>Controls (53 kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>Membrane</td>
<td>5.2 ± 2.2</td>
<td>1.4 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>Soluble</td>
<td>4.1 ± 1.5</td>
<td>0.77 ± 0.05</td>
</tr>
<tr>
<td>CGD</td>
<td>Membrane</td>
<td>1.4 ± 0.4</td>
<td>1.4 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>Soluble</td>
<td>0.92 ± 0.13</td>
<td>0.96 ± 0.12</td>
</tr>
</tbody>
</table>

* $^{32}$P (normalized value) in the proteins from activated cells divided by the corresponding value from resting cells.

membranes, in which one of the 48-kDa proteins still appeared to take up label in response to PMA.

The changes in the labeling of the 48-kDa proteins in response to neutrophil activation were quantified by scanning as described under "Materials and Methods." The results (Table I) showed a 4- to 5-fold increase in the amount of $^{32}$P incorporated into the 48-kDa proteins upon exposure of the cells to PMA, and confirmed that this PMA-induced increase in labeling was greatly diminished or absent in CGD neutrophils.

To determine whether the inability of activated CGD neutrophils to phosphorylate the proteins in the 48-kDa group was secondary to their failure to generate reactive oxidants, an examination was made of phosphorylation by resting and activated normal neutrophils incubated in an oxygen-free atmosphere, under which conditions the normal cells would be unable to manufacture those oxidants. For these experiments, plasma membrane phosphorylation patterns were determined by electrophoresis of unfractonated particles from the labeled sonicates; these particles are made up of plasma membrane and granules, but since the granules take up little phosphate (see above), the phosphorylation pattern of the unfractionated particles was essentially that of the plasma membranes alone. The results showed that the phosphorylation patterns of particles and soluble fractions from resting and activated neutrophils incubated under anaerobic conditions could not be distinguished from the patterns obtained with the corresponding fractions from neutrophils incubated in air (not shown). In particular, the increase in labeling of the 48-kDa proteins in response to activation was found to take place under both room air and nitrogen.

DISCUSSION

Previous studies have shown that neutrophils contain a large number of proteins that become labeled when the cells are incubated with $^{32}$P, and that in several of these proteins the extent of labeling is altered when the cells are exposed to activating agents (12-15). The alteration in labeling is not due to a stimulus-induced change in the specific activity of the intracellular $[^{32}]$P$\text{ATP}$ (15), but rather appears to reflect a true change in the phosphorylation of the protein. The present findings confirm the earlier studies on protein phosphorylation in neutrophils, although both the number of labeled proteins and the number of proteins affected by activating agents are smaller than previously observed, differences that are probably methodological in origin.

Studies on subcellular distribution showed that labeled proteins were abundant in both the plasma membrane and the soluble fractions, but not in the granules. In the case of the 48-kDa group, the proteins appeared to be distributed in the cytosol and plasma membrane include red cell glyceraldehyde-3-phosphate dehydrogenase (27) and protein kinase C (28).

To examine the relationship between protein phosphorylation and the respiratory burst, studies were carried out with neutrophils from patients with chronic granulomatous disease. These studies revealed that the increase in labeling of the 48-kDa group proteins that normally took place during activation did not occur in CGD. This phosphorylation defect was not a secondary effect of oxidants generated by normal but not CGD cells, because the normal phosphorylation pattern, not the CGD pattern, was seen in normal neutrophils incubated under anaerobic conditions. The defect appears instead to reflect primary biochemical differences between normal and CGD cells. These results thus suggest that phosphate uptake (or at least phosphate turnover) by this group of 48-kDa proteins is involved in the activation of the respiratory burst oxidase.

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Addendum—While this manuscript was under review a paper appeared demonstrating a defect in protein phosphorylation in autosomal recessive chronic granulomatous disease (29).

REFERENCES