Assembly and Activation of the NADPH:O₂ Oxidoreductase in Human Neutrophils after Stimulation with Phorbol Myristate Acetate*

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*These studies were supported by Grants 900-503-091 from the Foundation for Medical Research (MEDIGON), which is subsidized by the Netherlands Organization for Scientific Research (NWO), by National Institutes of Health Grants AI-19423, and by the Fogarty International Fellowship Grant FO6-TWO 1357. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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Phagocytic leukocytes contain an activatable NADPH:O₂ oxidoreductase. Components of this enzyme system include cytochrome b₅₅₈, and three soluble oxidase components (SOC I, SOC II, and SOC III) found in the cytosol of resting cells. Previously, we found that SOC II copurifies with, and is probably identical to, a 47-kDa substrate of protein kinase C. In the present study we investigated the change in location of several of these oxidase components after activation of intact neutrophils with phorbol myristate acetate (PMA) and separation of subcellular fraction on sucrose density gradients.

On Western blots with fractions of resting cells, the α subunit of cytochrome b₅₅₈ was detected with a monoclonal antibody as a doublet of M₉ 22,000 and 24,000 in the specific granules and as a single band of M₉ 24,000 in the plasma membrane. PMA induced an increase of cytochrome b₅₅₈ in the plasma membrane, including the M₉ 22,000 band. PMA also induced translocation of the 47-kDa protein from the cytosol to the membrane fraction, as revealed by in vitro phosphorylation experiments. When NADPH oxidase activity was determined in a cell-free system in the presence of sodium dodecyl sulfate and GTP with plasma membranes from resting cells, cytosol from PMA-treated cells was deficient compared with cytosol from resting cells. This deficiency could be partially restored by the addition of SOC I. Concomitantly, SOC I activity appeared in the plasma membranes of PMA-treated cells. These studies support the hypothesis that PMA stimulation of neutrophils results in assembly of oxidase components from the cytosol and the specific granules in the plasma membrane with subsequent expression of NADPH oxidase activity.

Phagocytic cells are a major component of the body's defense against microbial invasion. Destruction of an invading organism occurs as a result of a complex sequence of events initiated by ingestion and sequestration of the microbe within the phagosome (1, 2). Concurrent with these processes, oxygen from the surrounding milieu is reduced to superoxide (O₂⁻), which subsequently leads to the formation of other toxic metabolites (3). Production of superoxide is catalyzed by an oxidoreductase which, upon appropriate stimulation, uses electrons from NADPH to reduce oxygen to superoxide (see Ref. 4 for a recent review). The importance of this first step in the oxidative antimicrobial system of the phagocyte is demonstrated by patients with chronic granulomatous disease (CGD),1 who are plagued by severe, recurrent bacterial infections. Phagocytes from these patients do not exhibit a respiratory burst upon addition of appropriate stimuli and as a result have defective microbicidal activity against bacteria and fungi (5–7).

The complexity of the NADPH oxidase has been illustrated by the identification of multiple components of this system and the demonstration that the heterogeneity of CGD can be attributed to defects in the various oxidase components (7, 8). One of these components is a h-type cytochrome, which is composed of an α subunit of M₉ 22,000 and a β subunit of M₉ 90,000 (9, 10). In the common form of X-linked, cytochrome b₅₅₈-negative, CGD the 90-kDa β subunit of this cytochrome b₅₅₈ is not properly synthesized in phagocytic cells (11), resulting in the absence of both the β and the α subunit. Patients lacking the cytochrome b₅₅₈ in their phagocytes due to an autosomal pattern of disease inheritance (8) probably suffer from a defect in the synthesis of the α subunit (12).

The characterization of the molecular abnormality in the autosomal, cytochrome b₅₅₈-positive, form of CGD began with the discovery by Segal and co-workers (13) of a defect in the phosphorylation of a 47-kDa protein after stimulation of the patients' neutrophils with phorbol myristate acetate (PMA), an activator of protein kinase C. Further characterization of this defect has been made possible by the development of cell-free systems in which the respiratory burst can be activated with SDS or arachidonic acid in subcellular fractions derived from resting neutrophils (14–16). Oxidase activity in these cell-free systems requires a particulate fraction and a soluble, presumably cytosolic, fraction of the cells. Neutrophils of CGD patients lacking the membrane-bound cytochrome b₅₅₈...
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show a defective activity in the particulate fraction (16). Neutrophils of patients with the autosomal, cytochrome b$_{558}$-positive, form of CGD lack the activity of a cytosolic component (17, 18), which was tentatively identified as the 47-kDa substrate of protein kinase C (18). Subsequent studies by Volpp et al. (19) and Nuncio et al. (20) have indicated that in the neutrophil cytosol of one particular autosomal CGD patient the 47-kDa protein is present in normal amounts, but that in this case a 67-kDa protein is missing from the cytosol. This 67-kDa protein appears to be also required for generating requirement for yet another soluble component, which we have named soluble oxidase component (SOC) I. SOC I is a 67-kDa protein that may play a role in the translocation of these components to the plasma membrane.

EXPERIMENTAL PROCEDURES

Materials—Phosphatidylcholine, phosphatidylserine, phenylmethylsulfonyl fluoride (PMSF), acetyl-L-leucyl-L-leucyl-L-arginine methyl ester (substrate of protein kinase C (18), which was tentatively identified as the 47-kDa substrate of protein kinase C (18). Subsequent studies by Volpp et al. (19) and Nuncio et al. (20) have indicated that in the neutrophil cytosol of one particular autosomal CGD patient the 47-kDa protein is present in normal amounts, but that in this case a 67-kDa protein is missing from the cytosol. This 67-kDa protein appears to be also required for generating requirement for yet another soluble component, which we have named soluble oxidase component (SOC) I. SOC I is a 67-kDa protein that may play a role in the translocation of these components to the plasma membrane.

Preparation of Neutrophils—Neutrophils were prepared from the blood as previously described (24). After dilution of the buffy coat with an equal volume of cold Dulbecco's phosphate-buffered saline (PBS, 140 mM NaCl, 5.5 mM glucose, and 0.5% (w/v) human albumin), the mixture was vortexed vigorously. For in vitro phosphorylation, the following assay mixture, pH 7.4, was used: 7.5 mM MgCl$_2$, 2 mM Ca$^{2+}$/EGTA buffer with a free Ca$^{2+}$ concentration of 1 nM (as determined by indo-1 fluorescence), 30 mM Tris-HCl, and 25 µl of the phospholipid mixture (Phosphatidyserine, Phosphatidylethanolamine, and Phosphatidylcholine, Phosphorus Chemicals, Utrecht, the Netherlands) in a total volume of 50 µl. After equilibration for 5 min, SDS (final concentration 100 µM) was added, the reaction vessel was closed, and the rate of oxygen consumption was recorded. NADPH (final concentration 200 µM) was added 3 min later in some experiments, oxygen consumption by plasma membrane fractions was determined at 37°C, and the rate of oxygen consumption was recorded.

In Vitro Phosphorylation of Proteins—Protein kinase C was isolated from rat brain and contained a mixture of protein kinase C isoforms. The phospholipid mixture for the stimulation of protein kinase C was made as follows: 10 µg of phosphatidylserine and 3 µg of phosphatidylcholine were dispersed in 250 µl of water by sonication at 4°C for 3 min at 21 kHz frequency and 8-µm peak-to-peak amplitude. Then 0.5 µg of PMA in Me$_2$SO was added, and the mixture was vortexed vigorously. For in vitro phosphorylation, the following assay mixture, pH 7.4, was used: 7.5 mM MgCl$_2$, 2 mM Ca$^{2+}$/EGTA buffer with a free Ca$^{2+}$ concentration of 1 nM (as determined by indo-1 fluorescence), 30 mM Tris-HCl, and 25 µl of the phospholipid mixture in a total volume of 50 µl. After equilibration for 5 min, SDS (final concentration 100 µM) was added, the reaction vessel was closed, and the rate of oxygen consumption was recorded. NADPH (final concentration 200 µM) was added 3 min later in some experiments, oxygen consumption by plasma membrane fractions was determined at 37°C, and the rate of oxygen consumption was recorded.
dried and exposed to either X-Omat AR or X-Omat S films for 1–3 days at -70 °C with an intensifying screen.

Preparation of Soluble Oxidase Components—Pooled cytosol fractions were dialyzed against elution buffer containing 1 mm EGTA, 1 mm PMPS, and 10 mm MFS at pH 6.8, and the resultant dialysate was applied to a carboxymethyl-Sepharose column that had been equilibrated with elution buffer (16). A packed volume of 0.5 ml/10° cell equivalents was used. After the absorbance of the flow through at 280 nm had dropped to zero, a linear gradient from 0 to 0.2 M NaCl in elution buffer was applied to the column. Fractions of 2 ml were collected. The fraction that did not bind to the carboxymethyl-Sepharose column and eluted before the gradient was designated SOC I (16). This fraction and another fraction eluting at 0.125 M NaCl (containing both SOC II and III) were sufficient to elicit activity of the oxidase enzyme system in plasma membranes from resting cells. The carboxymethyl-Sepharose fractions were kept at 4 °C and used within 2 days after their separation.

Western Blot with a Monoclonal Antibody against the a Subunit of Cytochrome b558—For Western blotting, SDS-PAGE was carried out on 10% (w/v) slab gels containing 0.1% (w/v) SDS. Preparation of samples and electrophoresis were performed as described above. After electrophoresis, the gels were blotted onto nitrocellulose using 1 A for 1 h in a buffer containing 200 mm glycine, 2.4% (v/v) methanol, 0.1% (w/v) SDS, and 25 mm Tris-HCl, pH 8.3. The blots were then washed for 15 min in 1% TCA and stored overnight at 4 °C in PBS with 0.2% (v/v) Tween 20 and 1.0% (w/v) casein at 4 °C. The blots were subsequently washed three times with PBS-Tween and incubated for 1.5 h at room temperature with a monoclonal antibody to the a subunit of cytochrome b558 (23) that was raised in our laboratory (mAb 449, 5 ml of a 1:200 dilution of ascites fluid in PBS-Tween 20). After five washes with PBS-Tween, the blots were incubated with goat-anti-mouse IgG conjugated to horseradish peroxidase (5 ml of a 1/200 dilution in PBS-Tween 20) for 1.5 h. The blots were then washed three times with PBS-Tween 20 and PBS and staining was completed by incubation with the peroxidase substrate solution (0.012% (v/v) H2O2 and 0.1 mg/ml 4-chloro-1-napthol in 100 ml of PBS). Typically, the maximal intensity of reactivity of mAb 449 with blotted samples took 3–10 min to appear. The reaction was stopped by washing the blots with distilled water.

RESULTS
Separation of Subcellular Fractions—Subcellular fractions were prepared from neutrophils disrupted by nitrogen cavitation and were separated with sucrose density gradients that combined the characteristics of both continuous and discontinuous sucrose density gradients. Analysis of the fractions removed from the density gradients resulted in the pattern of vitamin Bi2-binding protein, and /3-glucuronidase, and represent the specific granules of the neutrophil, respectively. There were two peaks of lysozyme activity, which coincided with the peaks of vitamin-B12-binding protein and /3-glucuronidase activity (data not shown). Lactate dehydrogenase, the marker for cytosol, along with most of the protein, remained on top of the gradient. Using the peaks of the marker proteins, we pooled fractions for cytosol, plasma membrane, specific granule, and azurophilic granule components. The marker protein/enzyme profile of these pools is summarized in Table I. As can be seen, each subcellular fraction contained the highest amount of its specific marker protein, with only small amounts of marker proteins from other subcellular fractions. Subcellular fractions of cells stimulated with PMA were separated on the sucrose gradients in a manner that was qualitatively identical to resting neutrophils. However, absolute quantities of alkaline phosphatase in membrane fractions, and vitamin-B12-binding protein and lysozyme in specific granules, were decreased (data not shown).

NADPH-Oxidoreductase Activity in Membrane Fractions of Resting and PMA-treated Cells—To ascertain that the plasma membrane fraction acquired NADPH oxidase activity upon PMA stimulation of neutrophils, oxygen consumption by plasma membranes was assayed at 37 °C in the presence of NADPH, but without cytosolic components or SDS. The membrane fraction from PMA-stimulated neutrophils expressed an increased rate of oxygen consumption (115.0 ± 25.0 nmoI oxygen consumed/min/mg protein, mean ± S.E., n = 4) compared with membranes from resting cells (7.8 ± 2.7, n = 4; p < 0.05, unpaired t test). These results indicate that after stimulation of the neutrophils with PMA, the isolated plasma membranes contain an active NADPH oxidase.

Location of Cytochrome b558—One of the best characterized components of the oxidase enzyme system is cytochrome b558, most of which is found in subcellular fractions that contain specific granules (22, 23). Only small amounts of this protein are found in the plasma membrane from resting neutrophils, but after stimulation of the cells with PMA, most of the cytochrome b558 becomes associated with the plasma membrane (21, 22). mAb 449 was found to react with the a subunit of cytochrome b558 (23), which is missing in the common X-linked and the rare autosomal form of CGD (10, 12). Fig. 2 is representative of four experiments evaluating the reactivity of this antibody in subcellular fractions of resting and PMA-treated neutrophils by Western blot. No reactivity was seen in the cytosol and azurophilic granules of either resting or PMA-treated cells (data not shown). However, the a subunit of cytochrome b558 was demonstrated in membranes and specific granules from resting neutrophils. Of note was the appearance of mAb 449 reactivity as a doublet (M, 22,000 and 24,000) in the specific granules, whereas in the plasma membranes only the 24-kDa band was identified. Upon PMA stimulation, the reactivity of mAb 449 became almost exclusively associated with the plasma membrane, including the 22-kDa band.

Since these results provide only qualitative evaluation of cytochrome b558, quantitative data were obtained by absorbance spectroscopy. In resting cells, plasma membranes contained 24 ± 4% (mean ± S.E., n = 5) of the total cell content of cytochrome b558, while the specific granules contained 70 ± 6% In PMA-treated cells, cytochrome b558 in the membrane accounted for 63 ± 7% (n = 4) of the total and that in the specific granules for 31 ± 2%. Taken together, the data from both techniques confirm the translocation of cytochrome b558 to the membrane upon stimulation, but suggest additional qualitative differences in this protein that are associated with assembly and activation of the NADPH:0 oxidoreductase.

Localization of the 47-kDa Phosphoprotein by in Vitro Phosphorylation—In vitro phosphorylation by protein kinase C was used to determine the presence of the 47-kDa protein in the various subcellular fractions of human neutrophils (Fig. 3) To eliminate possible influences of protein kinase C present in the fractions, phosphorylation was also carried out by adding protein kinase C (purified from rat brain) to samples, that had been heated for 10 min at 60 °C. After this treatment, both phosphorylation of the 47-kDa protein by endogenous protein kinase C, and dephosphorylation by phosphatases could no longer be detected in any of these samples (data not shown).

In resting neutrophils, the 47-kDa protein could only be detected in the cytosol and not in the plasma membrane, specific granules, or azurophilic granules (Fig. 3A, data for azurophilic and specific granules not shown). The exclusive cytosolic location was also observed when phosphorylation...
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1.200 – 1.100

1.000 – 0.900

0.800 – 0.700

0.600 – 0.500

0.400 – 0.300

0.200 – 0.100

0.000 – 0.000

fraction number

FIG. 1. Pattern of marker enzymes/proteins from a typical sucrose density gradient. Unstimulated neutrophils (4 x 10⁶) were disrupted by nitrogen cavitation, the postnuclear supernatant was layered over the discontinuous/continuous sucrose gradient and centrifuged for 2 h at 100,000 x g, and the gradient was unloaded as described under “Experimental Procedures.”

TABLE I

Amount of marker enzymes or proteins in subcellular fractions of resting neutrophils separated on discontinuous/continuous sucrose density gradients

Assays for marker enzymes and proteins were performed as described under “Experimental Procedures.” Fractions containing peak activities of different marker enzymes were pooled, and the percentage of activity present in each pool was calculated by comparison with the postnuclear supernatant. Consequently, not all of the activity present in the fractions of the gradient was saved in the various pools.

<table>
<thead>
<tr>
<th>Subcellular fraction</th>
<th>Marker enzymes/proteins</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Protein</td>
</tr>
<tr>
<td>Cytosol</td>
<td>61 ± 8</td>
</tr>
<tr>
<td>Membrane</td>
<td>5 ± 1</td>
</tr>
<tr>
<td>Specific granules</td>
<td>5 ± 1</td>
</tr>
<tr>
<td>Azurophilic granules</td>
<td>7 ± 1</td>
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* Numbers represent mean ± S.E. of results from three different preparations.

was catalyzed by exogenous protein kinase C. In contrast, the 47-kDa protein had a dual location in PMA-treated neutrophils (Fig. 3B). The presence of this protein in the cytosol of PMA-treated cells could only be demonstrated after addition of exogenous protein kinase C, confirming the disappearance of protein kinase C from the cytosol after PMA stimulation (28). Together, these results show the cytosolic location of the 47-kDa protein in resting neutrophils and its partial translocation to the plasma membrane after stimulation with PMA.

Location of Soluble Oxidase Components—Previous studies from our laboratory (18) have shown that the soluble fraction obtained by sonication of resting neutrophils can be separated by ion-exchange chromatography into two fractions, both of which are necessary with resting plasma membranes for full oxidase activity. These fractions were termed soluble oxidase component (SOC) I and SOC II. Subsequent studies showed that the SOC II fraction actually contains two soluble oxidase components, the 47- and the 67-kDa protein, which were renamed SOC II and III, respectively (33).

For the present study, we first investigated possible influences of DFP treatment and choice of disruption method (sonication versus cavitation under nitrogen) on the recovery of soluble oxidase components. No differences in the separation characteristics or activities were found with either method of cell disruption, nor after pretreatment of the neutrophils with DFP. These results support the cytosolic location generally assumed for these components.

Second, we explored the possibility that appearance of NADPH oxidase activity in the plasma membrane after PMA stimulation is accompanied by the loss of soluble components from the cytosol. For this purpose, plasma membranes from resting cells were added to cytosol fractions from resting or PMA-treated cells, and oxygen consumption was measured in the cell-free system in the presence of SDS. Resting membranes alone exhibited a very low level of oxygen consumption. When these membranes were reconstituted with cytosol...
from unstimulated neutrophils, a pronounced increase in the rate of NADPH-dependent oxygen consumption was observed, depending on the amount of cytosol added (Fig. 4, open bars). However, the efficacy of cytosol of PMA-stimulated cells to support NADPH oxidase activity was much less (Fig. 4, closed bars). These data suggest that the cytosol from PMA-treated neutrophils is deficient in one or more cytosolic oxidase components.

For the characterization of the cytosol deficiency induced by PMA, we chose to explore the possibility that PMA induces the depletion of SOC I activity. Addition of SOC I to cytosol of unstimulated cells slightly decreased the ability of this cytosol to support NADPH oxidase activity in the cell-free system (Fig. 5, left). In contrast, addition of SOC I did increase the activity of cytosol isolated from PMA-treated cells, albeit not to the control level (Fig. 5, right). Therefore, part of the diminished activity expressed by cytosol from PMA-treated neutrophils could be due to disappearance of SOC I activity.

Finally, we attempted to demonstrate SOC I activity in the membrane fraction of PMA-stimulated neutrophils. For this purpose, plasma membrane fractions from resting or PMA-stimulated cells were incubated with a mixture of the 47- and 67-kDa protein (SOC II/III), and NADPH-dependent oxygen consumption was measured in the presence of SDS (Fig. 6). To elicit significant NADPH oxidase activity in membranes from resting cells, addition of SOC I is also required (18), as illustrated in Fig. 6 (left). When the 47- and 67-kDa proteins were added to the membrane fraction of PMA-stimulated cells, the rate of oxygen uptake was 40% higher than the rate observed in the absence of these soluble components (Fig. 6, right). This increase was not observed when SDS was omitted from the reaction mixture (data not shown). These results suggest that in the membranes from PMA-treated cells SOC I is already present, capable of interacting with the other soluble components in the presence of SDS. Taken together with the analysis of the cytosol of PMA-treated cells (Fig. 5), they support the idea that SOC I is translocated from the cytosol to the plasma membrane upon PMA stimulation of neutrophils.

**FIG. 2.** Western blot analysis of subcellular fractions from resting and PMA-stimulated neutrophils with mAb 449. Fractions containing plasma membranes and specific granules (each containing the equivalent of 4 $\times 10^6$ neutrophils) from resting and PMA-stimulated neutrophils were subjected to SDS/PAGE and Western blotting as described under "Experimental Procedures." The experiment shown is representative for four experiments with similar results.

**FIG. 3.** Localization of the 47-kDa phosphoprotein in subcellular fractions of resting and PMA-stimulated neutrophils by in vitro phosphorylation. Fractions containing cytosol (equivalent to 1 $\times 10^6$ neutrophils) and plasma membranes (PM; 2 $\times 10^6$ cell equivalents) from resting and PMA-stimulated neutrophils were phosphorylated in vitro in the presence of activators of protein kinase C in the absence (–) or presence (+) of rat brain protein kinase C, as described under "Experimental Procedures." When exogenous protein kinase C was added, the samples were heated at 60 °C for 10 min prior to the addition of [γ-32P]ATP. The position of the 47-kDa phosphoprotein is indicated by the arrow. No phosphorylation at the 47-kDa level was observed in specific or azurophilic granules from resting or PMA-stimulated cells. The experiment shown is one out of three with identical results. A, subcellular fractions from control neutrophils; B, subcellular fractions from PMA-stimulated neutrophils.

**DISCUSSION**

It has been known for many years that the respiratory burst of phagocytes is catalyzed by an NADPH:O2 oxidoreductase that is associated with the plasma membrane after stimulation of the cells with a variety of agonists. More recently, it has been reported that membranes from resting cells are able to express a similar activity provided cytosol and arachidonic acid or SDS are also present (14–16). The direct relationship between the activity present in membranes from activated cells and the activity elicited in membranes from resting cells in the presence of cytosol is strongly suggested by experiments with membranes and cytosol from CGD neutrophils, which have been shown to be defective in both assay systems (15–
Fig. 4. Oxygen consumption by membranes from resting cells in the presence of SDS and cytosol from resting or PMA-stimulated neutrophils. Plasma membranes from resting neutrophils (equivalent to 2 x 10^6 cells) were incubated with different amounts of cytosol (2 and 5 x 10^6 cell equivalents), and NADPH-dependent oxygen consumption was determined in the presence of SDS as described under "Experimental Procedures." Cytosol from control neutrophils (hatched bars) or from PMA-stimulated neutrophils (closed bars) was used to support NADPH oxidase activity. Results are expressed as nmol oxygen consumed/min. The bars represent mean and S.E. of three experiments.

Fig. 5. Effect of SOC I addition on the cytosol activity of control and PMA-stimulated neutrophils. Experimental conditions were the same as described in the legend to Fig. 4, in the presence of 2 x 10^6 cell equivalents of cytosol from control (hatched bars) or PMA-stimulated neutrophils (closed bars). As indicated, incubations were carried out in the absence or presence of 50 μl of SOC I (derived from about 5 x 10^6 cell equivalents). Results are expressed as percentage of the activity obtained in the presence of a mixture of carboxymethyl-Sepharose fractions containing all soluble oxidase components (amounting to 0.9 ± 0.1 nmol of oxygen/min for both cytosol fractions, mean ± S.E. of three experiments). Bars represent mean and S.E. of measurements with three different cytosol preparations.

Western blot analysis with monoclonal mAb 449, directed against the α subunit of cytochrome b_{558}. Analysis of the subcellular fractions for their content of cytochrome b_{558} confirmed earlier studies (21, 22), demonstrating transfer of the cytochrome from the specific granule fraction to the plasma membrane upon stimulation. Furthermore, with Western blots heterogeneity in the α subunit of cytochrome b_{558} was found, depending on its location inside the cell (Fig. 2). It seems unlikely that this is caused by proteolytic breakdown during preparation of the subcellular fractions, because several protease inhibitors were present during the experiments. Heterogeneity in the α subunit of cytochrome b_{558} has also been reported by Teahan et al. (29). Possibly, the difference in the two forms of cytochrome b_{558} is of importance for the expression of oxidase activity in the plasma membrane.

Previously, we have shown that one of the soluble components of the NADPH oxidase, the 47-kDa protein deficient in most autosomal CGD neutrophils (19), is a substrate for protein kinase C (30). Therefore, phosphorylation in vitro followed by SDS-PAGE and autoradiography was used to detect the 47-kDa protein in subcellular fractions. Our studies clearly show that the 47-kDa protein is a cytosolic protein in resting neutrophils and becomes associated with the plasma membrane after stimulation of neutrophils with PMA. The association with the plasma membrane does not result in a complete disappearance from the cytosol, although density scanning of the autoradiographs indicated a 30% decrease in 47-kDa protein phosphorylation in the presence of exogenous protein kinase C (results not shown). The PMA-induced translocation of the 47-kDa protein was not apparent after only Coomassie Blue staining of the gels, indicating the relative low abundance of this protein. It is of interest, however, that in the membrane fraction of PMA-stimulated cells the 47-kDa protein is the most prominent substrate when endogenous protein kinase C activity is used, but not when exogenous protein kinase C (purified from rat brain) is added (Fig. 3 B). We cannot rule out the possibility that PMA treatment of neutrophils alters the susceptibility to phosphorylation of 47-kDa protein already present in the membranes of resting cells. It is not clear, however, by what mechanism this effect of PMA would be mediated. The translocation of the 47-kDa protein revealed in these experiments might even be underestimated because PMA-induced phosphorylation of this protein in the intact neutrophils may have influenced the results.
Previous work, however, has shown that upon disruption of cells the 47-kDa protein becomes rapidly dephosphorylated in the absence of phosphatase inhibitors (30).

The cell-free system for measuring NADPH oxidase activity has been proven an important tool in elucidating the role of cytosolic and membrane-bound components in the assembly of the oxidase. In addition, the antibodies against various components which recently have been described, can be of great help in this analysis. These include antibodies against both subunits of cytochrome b$_{558}$ (11, 24, 31), and the anti-serum B-1, recognizing the 47- and the 67-kDa protein in the cytosol of resting neutrophils (19). As yet, studies on SOC I have to rely on activity measurements only. To determine changes in the location of SOC I after stimulation with PMA, we first measured the activity of whole cytosol in the cell-free system with plasma membranes from resting neutrophils. Cytosol from PMA-treated cells was found to have strongly diminished activity. This was also observed by Tanaka et al. (32). Addition of SOC I partially reconstituted the cytosol of PMA-treated cells, and replenishment with all soluble components restored the activity to the level of resting cytosol. We tried to further evaluate the fate of SOC I by determining the effect of a mixture of both the 47- and 67-kDa protein on the activity of the membranes of PMA-treated neutrophils. The activity of these membranes increased 40% in the presence of these proteins, consistent with the presence of SOC I in the membranes of neutrophils treated with PMA. Another explanation would be that PMA stimulation induces a stable change in the membrane by the activation and subsequent inactivation of SOC I without actual translocation of this component. Therefore, further characterization of this oxidase component is required to substantiate the translocation proposed here.

In conclusion, we have demonstrated the dual location of cytochrome b$_{558}$ in the plasma membrane and in the specific granules of the soluble oxidase components in the cytosol of resting neutrophils. Upon stimulation with PMA, there is a loss of soluble components from the cytosol. Concomitantly, cytochrome b$_{558}$, the 47-kDa protein and SOC-1 activity appear in the plasma membrane. These results suggest that, upon stimulation of phagocytes, the NADPH oxidase becomes assembled from components that have different locations inside the resting cell.

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