Assembly of the Neutrophil Respiratory Burst Oxidase

PROTEIN KINASE C PROMOTES CYTOSKELETAL AND MEMBRANE ASSOCIATION OF CYTOSOLIC OXIDASE COMPONENTS

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Activated human polymorphonuclear neutrophils (PMNs) convert molecular oxygen into superoxide anion, a process known as the respiratory burst, through the activity of a latent multicomponent NADPH-dependent oxidase. Components of this respiratory burst oxidase include the membrane-bound cytochrome b558 and the cytosolic factors p47-phox and p67-phox. We initiated these studies based on three observations: 1) that stimulation of PMN oxidase activity is associated with translocation of the cytosolic oxidase components to the plasma membrane; 2) that p47-phox is phosphorylated during PMN activation and that there is a sequential relationship between phosphorylation of p47-phox in the cytosol and appearance of the phosphoprotein in the membrane; and 3) that the predicted amino acid sequences of p47-phox and of p67-phox contain regions of homology to the SH3 or A domain of the src family of tyrosine kinases. We found in a variety of proteins which interact with the cytoskeleton or the subplasmalemmal cytoskeleton. Thus the purpose of our studies was to examine the role of protein kinase C (PKC)-dependent phosphorylation in the stimulus-induced association of p47-phox and p67-phox with the plasma membrane and the cytoskeleton. Using the PKC activator phorbol myristate acetate (PMA) as the agonist, we found that activation of the respiratory burst oxidase was associated with translocation of cytosolic p47-phox and p67-phox to the plasma membrane as well as redistribution of p47-phox to the Triton-insoluble cytoskeleton. Furthermore, the PKC inhibitor staurosporine inhibited phosphorylation of p47-phox, interrupted the redistribution of cytosolic oxidase factors, and blocked PMA-induced generation of superoxide anion. Taken together these results indicate that PKC-dependent phosphorylation of p47-phox correlates with association of p47-phox with the cytoskeleton and with translocation of p47-phox and p67-phox to the plasma membrane, with the ensuing assembly of an active superoxide-generating NADPH-dependent oxidase.

Stimulated human polymorphonuclear neutrophils (PMNs) undergo a surge in oxygen consumption known as the respiratory burst (reviewed in Ref. 1). This response results in the generation of a variety of reactive oxygen species including superoxide anion and hydrogen peroxide (2-4). The NADPH-dependent oxidase responsible for this activity is a multicomponent enzyme comprised of a membrane-bound b-type cytochrome (cytochrome b558), a flavoprotein, and at least two cytosolic factors, p47-phox and p67-phox. Genetically determined deficiencies of cytochrome b (5, 6), p47-phox (7, 8) or p67-phox (9) result in a failure of the respiratory burst and cause the clinical syndrome of chronic granulomatous disease (CGD). The specific functions of the cytosolic factors have not been established, although p47-phox is phosphorylated at multiple sites during PMN activation (10, 11) and, under some experimental conditions, this phosphorylation appears to be coupled to generation of superoxide (12-14). However neither the cellular site of phosphorylation of p47-phox nor the responsible kinase has been established.

We have recently demonstrated that during activation of the respiratory burst oxidase, p47-phox and p67-phox translocate from the cytosol to the plasma membrane (15). This translocation correlates with the generation of superoxide by stimulated PMNs and is directly related to the concentration of agonist as well as the temperature and duration of stimulation. Furthermore, we have recently examined translocation of these cytosolic factors in PMNs from subjects with various forms of CGD in order to determine the role of membrane-bound cytochrome b558 in this stimulus-coupled event (16). Taken together findings from these studies indicate that stimulus-induced assembly of the membrane-bound oxidase complex is the result of multiple interactions among cytochrome b558, p47-phox, and p67-phox. However the structural features of p47-phox which are important in the process of translocation remain undefined. The sequential relationship between the phosphorylation of p47-phox in the cytosol and the appearance of the phosphoprotein in membrane fractions points to a potential role for phosphorylation in this process (17). The CDNA sequences for p47-phox and p67-phox each encode two regions of ap-

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The abbreviations used are: PMN, polymorphonuclear leukocytes; CGD, chronic granulomatous disease; EGTA, ethylenebis(oxyethylene)tetraacetic acid; p47-phox, 47-kDa cationic cytosolic phagocyte oxidase factor; p67-phox, 67-kDa cytosolic phagocyte oxidase factor; PKC protein kinase C; PMA, phorbol myristate acetate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide electrophoresis; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PIPESS, 1,4-piperazineethanesulfonic acid.
proximately 50 amino acids which are homologous to a non-catalytic domain of the src family of tyrosine kinases (18-20). This region, known as either the SH3 or A domain (21-24), is also present in a variety of proteins that have associations with the subplasmalemmal cytoskeleton, implying that the SH3 regions may represent functional domains governing interactions with the cytoskeleton and the plasma membrane (25).

The purpose of the studies described here was to examine the role of protein kinase C-dependent phosphorylation in the stimulus-induced association of the cytosolic oxidase components p47-phox and p67-phox with the plasma membrane and the cytoskeleton.

MATERIALS AND METHODS

Neutrophils—Human PMNs were isolated from venous blood obtained from normal subjects after informed consent and in accordance with guidelines established by the Human Subjects Committee of the Iowa City Department of Veterans Affairs and the University of Iowa. PMNs (approximately 95% purity) were isolated by sequential dextran sedimentation and Ficoll-Hypaque differential density centrifugation and hypotonic lysis of contaminating erythrocytes, as described previously (26). PMNs for all studies were pretreated with 2 mM diisopropylfluorophosphate (Sigma) at 4 °C for 20 min, washed, and resuspended in phosphate-buffered saline with glucose (PBS-G): 10 mM NaCl, 15 mM NaH2PO4, 2.8 mM KCl, 10 mM d-glucose at 5 x 10^-25/ml and kept on ice until use. Diisopropylfluorophosphate had no effect on the activity of the respiratory burst oxidase (27, 28). Staurosporine (Calbiochem) was prepared at 2.5 mM aqueous stock solution. In all experiments, PMNs were incubated for 10 min at room temperature in varied concentrations of staurosporine, as detailed in text and figure legends, prior to stimulation.

Neutrophil Activation and Fractionation—For studies of membrane-associated cytosolic oxidase factors, PMNs on PBS-G were stimulated with PMA (or MeSO4, as solvent control), resuspended in relaxation buffer (100 mM KCl, 3 mM NaCl, 3.5 mM MgCl2, 1.25 mM EGTA, 10 mM PIPES, pH 7.3), and disrupted by sonication as previously described (15). The high speed supernatant obtained from disrupted cells represented the soluble cytosolic fraction, whereas the pellet from disrupted PMNs after ultracentrifugation represented the membrane fraction after washing with relaxation buffer as described previously (15).

Solubilization in Triton X-100—Cytoskeleton was operationally defined as the Triton-insoluble material remaining after PMNs were sonicated in lysis buffer. PMNs stimulated as described above, pelleted, and resuspended at 5-10 x 10^6/100 μl of lysis buffer were suspended in lysis buffer (1.0% Triton X-100 in 100 mM Tris, pH 7.4) containing a mixture of protease inhibitors (100 μg/ml leupeptin, 100 μg/ml antipain, 25 mM benzamidine, 0.05 mM trypsin inhibitor, 1 mM phenylmethylsulfonyl fluoride). The cells were then sonicated for 7 s on ice and placed on ice for 10 min. Sonicates were loaded at 300 μl of 6% sucrose containing 1.0% Triton X-100 and 100 mM Tris, pH 7.4, and centrifuged 72,000 rpm (Beckman TLA 100.2 rotor) at 4 °C for 38 min. The top 100 μl represented the Triton-soluble fraction, and the pellet represented the Triton-insoluble material (29).

Labeling of PMNs with "32P—PMNs were suspended at 2.5 x 10^6/ml in labeling buffer (150 mM NaCl, 20 mM HEPES, pH 7.4, 1.85 mM CaCl2, 100 μM MnCl2, 100 μM MgCl2) and incubated at room temperature with "32Porthophosphate (37 MBq/ml, carrier-free, Du Pont) for 60 min with gentle mixing every 10-15 min. Cells were washed and resuspended in PGM-Na (Na2Na phosphate buffer, pH 7.4, 138 mM NaCl, 2.7 mM KCl, 0.6 mM CaCl2, 10 mM MgCl2, 5 mM d-glucose) with or without staurosporine, as described in the text and figure legends. After 10 min preincubation at room temperature, PMA (or appropriate amounts of MeSO4) was added and the cells (2.5 x 10^6/ml) agitated at 37 °C for 5 min. The reaction was stopped by adding the contents of the tube to 250 μl of cold 50% trichloroacetic acid (final concentration 10%). The precipitate was pelleted by centrifugation, washed with cold water and, dissolved in SDS-sample buffer for analysis by SDS-PAGE and autoradiography.

Electrophoresis, Immunoblotting, and Autoradiography—Samples were analyzed by SDS-PAGE using methods described previously (7). Immunoblots were processed with B-1, a rabbit polyclonal antiserum to cytosolic oxidase factors p47- and p67-phox, and iodinated protein A, as described previously (7). Fractions from phosphorylated cells were separated by SDS-PAGE and the resulting gel dried for autoradiography. Processed nitrocellulose filters or dried gels were exposed to x-ray film at ~80 °C in cassettes containing intensifying screens. In some cases relative signal intensity on autoradiographs or positive photographic prints was quantitated by densitometry using a Shimadzu CS-930 scanning densitometer (Shimadzu, Kyoto, Japan) kindly provided by Dr. T. A. Koerner (Department of Pathology, University of Iowa).

Superoxide Generation—Production of superoxide anion was quantitated using the superoxide-dismutase inhibitable reduction of ferricytochrome c at 37 °C, as described previously. Two assays were used to quantitate the superoxide production by PMA-stimulated PMNs, one which determined the maximum rate by continuously monitoring the reduction of ferricytochrome c (continuous assay (28)) and the other which determined the total amount of ferricytochrome c reduced in 10 min (static assay (31)).

RESULTS

Previously we have demonstrated that both p47-phox and p67-phox are distributed exclusively in the cytosol of resting PMNs but become associated with the plasma membrane during stimulation (15). Therefore we examined the interaction of p47-phox and p67-phox with the cytoskeleton, operationally defined as the detergent-insoluble fraction obtained after solubilizing intact PMNs in 1% Triton X-100. Using immunoblots processed with B-1 antiserum, we compared the distribution of p47-phox and p67-phox in Triton-soluble and Triton-insoluble fractions after PMNs were exposed to the protein kinase C (PKC) activating agent PMA (Fig. 1). In the absence of any stimulation, p67-phox was detected exclusively in the Triton-insoluble fraction, whereas p47-phox remained with the Triton-soluble material. The presence of p67-phox in the Triton-insoluble fraction of unstimulated PMNs was not due to a nonspecific precipitation of the protein by the

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**Fig. 1.** Effect of PMA on distribution of p47-phox and p67-phox with the Triton-soluble and Triton-insoluble fractions. PMNs were incubated with 100 ng/ml PMA or with 0.004% MeSO4 (solvent control) at 37 °C for the indicated times. Triton-soluble and Triton-insoluble fractions were isolated (see "Materials and Methods"), mixed with SDS-sample buffer, and separated in a 9% polyacrylamide gel. Proteins were electrophoresed to nitrocellulose paper and the blots processed with B-1 antiserum as described previously (7). The locations of p47-phox and p67-phox and molecular mass standards are indicated.
PKC-dependent Redistribution of PMN Cytosolic Oxidase Factors

Stimulation of PMNs with PMA results in phosphorylation of p47-phox (32). Work by a variety of investigators has implicated PKC as an important participant in stimulus-coupled phosphorylation during activation of the NADPH-dependent oxidase (32–38). Since PMA acts primarily through PKC (39), we examined the effects of staurosporine, a potent inhibitor of PKC (40), on the PMA-induced redistribution of the cytosolic oxidase factors. In the presence of staurosporine alone, the distribution of factors in the membrane as well as the cytoskeletal preparations resembled that seen in unstimulated cells under control conditions; i.e. both factors were present exclusively in the cytosol fraction, whereas in the detergent fractionation of p47-phox and p67-phox were detected in the Triton-soluble and Triton-insoluble fractions, respectively (Fig. 4). Treatment with PMA, which by itself caused both factors to translocate to the plasma membrane and p47-phox to become associated with the Triton-insoluble fraction, did not alter the distribution of either protein when added to PMNs in the presence of 200 nM staurosporine. The immunoblot analysis of fractions from PMNs stimulated by PMA in the presence of staurosporine resembled the pattern of distribution seen with PMNs in the unstimulated state. The inhibition of translocation by staurosporine was dose-dependent (Fig. 5). At 50 nM staurosporine, partial PMA-mediated translocation of cytosolic factors to membrane and of p47-phox to the Triton-insoluble fraction was detected, whereas 150–200 nM of the inhibitor nearly completely blocked redistribution of cytosolic oxidase factors (Table I).

To determine whether staurosporine altered the phosphorylation of p47-phox, we labeled PMNS with 32P and examined the intensity and cellular distribution of radiolabelled p47-phox. Phosphorylation of p47-phox was dependent on stimulation of PMNs, as there was very little phosphorylation of proteins in the 47-kDa molecular mass range in the absence of PMA (Fig. 6). In the presence of staurosporine (100 nM), phosphorylation of p47-phox was reduced in comparison to that detected in the presence of PMA alone. Inhibition of phosphorylation was seen to a similar extent in samples obtained using the membrane translocation protocol (12.2% of control) as in those from detergent fractionation studies of the cytoskeletal association of cytosolic factors (16.3% of control). To determine the functional correlates of the inhibition of cytosolic oxidase factor redistribution, we examined the effects of staurosporine on superoxide produc-
concentrations of staurosporine (0-200 nM) prior to treatment with
pelleted and resuspended in the buffer appropriate for membrane
fractionation or Triton-insolubilization (see "Materials and Meth-ods"). Fractions were collected and analyzed by SDS-PAGE and
immunoblotting with B-1 antiserum as described previously. Because
the cytosolic oxidase factors redistributed to membrane fraction and
to the Triton-insoluble fraction, the results of immunoblots of those
fractions are shown. The locations of p47-phox, p67-phox, and the
molecular mass standards are indicated.

FIG. 5. Dose response of concentration of staurosporiniae (ST)
with the redistribution of p47-phox and p67-phox to the
membrane and of p47-phox to the Triton-insoluble fraction.
PMNs were incubated at room temperature for 10 min with varied
concentrations of staurosporine (0-200 nM) prior to treatment with
100 ng/ml PMA or 0.004% Me2S0, for 5 min at 37 °C. Cells were
pelleted and resuspended in the buffer appropriate for membrane
fractionation or Triton-insolubilization (see "Materials and Meth-
ods"). Fractions were collected and analyzed by SDS-PAGE and
immunoblotting with B-1 antiserum as described previously. Because
the cytosolic oxidase factors redistributed to membrane fraction and
to the Triton-insoluble fraction, the results of immunoblots of those
fractions are shown. The locations of p47-phox, p67-phox, and the
molecular mass standards are indicated.

TABLE I
Relative amounts of p47-phox and p67-phox associated with
membrane and triton-insoluble fractions in the presence of
staurosporine as measured by densitometry

| Staurosporine (nM) | Membrane p47-phox | Membrane p67-phox | Triton-insoluble p47-phox |
|-------------------|-------------------|-------------------|--------------------------|
| 0                 | 100               | 100               | 100                      |
| 50                | 6.7               | 26.6              | 35.2                     |
| 100               | 6.6               | 13.6              | 32.7                     |
| 150               | 5.3               | 3.9               | 13.2                     |
| 200               | 1.4               | 0.2               | 2.8                      |

FIG. 6. The effect of staurosporine (ST) on the PMA-stimu-
lated phosphorylation of p47-phox and its redistribution to
the membrane and the Triton-insoluble fractions. PMNs were
labeled with 32P as described under "Materials and Methods" and
stimulated with 100 ng/ml PMA for 5 min at 37 °C in the presence
or absence of 100 nM staurosporine. Cells were pelleted and resus-
pended in the buffer appropriate for preparation of membrane frac-
tions or for Triton-solubilization, as described previously. Fractions
were mixed with SDS-sample buffer and the proteins separated on a
9% polyacrylamide gel which was dried prior to autoradiography.

DISCUSSION

FIG. 7. Effect of staurosporine on PMA-stimulated super-
oxide production by PMNs. PMNs were stimulated with 100 ng/
ml of PMA at 37 °C described under "Materials and Methods" in the
presence of varied concentrations of staurosporine. Superoxide pro-
duction was measured spectrophotometrically as the superoxide-dis-
mutable reduces of reduced ferricytochrome c in both a con-
tinuous (dotted line) and a static (solid line) assay. Data are the mean of
four separate experiments, each done in triplicate, and are depicted
as the percent of daily control. The average control value for the
static assay was 36.08 ± 1.63 nmol/2.5 x 10^5 PMNs in 10 min (n = 4),
and the average control value for the continuous assay was 6.93 ±
0.28 nmol/l2 x 10^5 PMN/min (n = 4).

These studies demonstrate that exposure of human PMNs
to PMA, an agent that activates PKC and the respiratory
burst oxidase, induced the translocation of cytosolic oxidase
factors p47-phox and p67-phox from the cytosol to the plasma
membrane as well as the redistribution of p47-phox to the
Triton-insoluble cytoskeleton. Moreover these events were
blocked by the PKC inhibitor staurosporine. Concentrations of
staurosporine that interrupted the translocation and redis-
tribution of the cytosolic oxidase components also blocked
PKC-dependent Redistribution of PMN Cytosolic Oxidase Factors

PMA-induced phosphorylation of p47-phox and generation of superoxide. In all cases, the observed inhibition occurred in a direct dose-response relationship to the concentration of staurosporine.

Protein kinase C has been implicated as a major participant in stimulus-coupled signal transduction in a wide variety of cells including PMNs (41). Nanomolar concentrations of PMA stimulate PKC, activate the NADPH-dependent superoxide-generating system of PMNs, and result in phosphorylation of numerous proteins, including the cytosolic oxidase factor p47-phox (13, 17, 32-38). The kinases responsible for phosphorylation of specific PMN proteins during activation have not been identified, although considerable evidence suggests that PMN responses to most stimuli are mediated through a PKC-dependent signal transduction pathway (13, 34, 42). With PMN stimulation, cytosolic PKC translocates to the plasma membrane (43). In the case of the agonist formylmethionyleucylphenylalanine, a 47-kDa phosphoprotein appears only transiently during stimulation (12), and there is generation of a proteolytically activated form of PKC, called PKM, which is present for a short period of time in PMNs and is believed to be important in exocytosis of PMN granule contents (44).

Staurosporine is a potent inhibitor of PKC and acts primarily on the catalytic domain of the enzyme (45). Findings obtained from experiments which employ inhibitors must be interpreted with caution, since rarely are they exclusively selective in their targets. In the case of PKC inhibitors, their effect on enzymatic activity is often a function of the substrates being examined and the nature of the kinase (e.g., PKC versus PKM). Staurosporine at nanomolar concentrations inhibits the PKC-activated phosphorylation of either histone or protamine but has no effect on the activity of PKM (46).

Thus it seems likely that the effects of staurosporine on PMN responses are the result of inhibition of either PKC or a novel PMN kinase (other than PKM) which is staurosporine-sensitive.

Previous studies have observed inhibition by staurosporine of superoxide production (47, 48), although the mechanism of inhibition of the respiratory burst has not been described. The data presented in this paper demonstrate that staurosporine inhibited the translocation of the cytosolic oxidase factors p47-phox and p67-phox to the plasma membrane and the association of p47-phox with the cytoskeleton and that features of this inhibition (i.e., the kinetics and dose dependence) were paralleled in studies of superoxide generation. Based on these correlations we suggest that staurosporine inhibits the NADPH oxidase of PMN-stimulated cells by blocking the intracellular redistribution of cytosolic factors and thereby preventing the assembly of the membrane oxidase complex.

In addition these data indicate that the stimulus-induced assembly of the oxidase complex is in part related to phosphorylation of some site(s) on p47-phox, since partial inhibition of its phosphorylation by staurosporine correlated with inhibition of p47-phox redistribution and superoxide generation. The kinase(s) responsible for phosphorylation of p47-phox and the specific sites in p47-phox which are substrates for each kinase are not known. The primary amino acid sequence of p47-phox predicted from the cDNA contains 6 serines in the arginine-rich carboxyl-terminal domain that are likely targets for serine/threonine kinases (19). Electrophoretic analyses of ³²P-labeled p47-phox demonstrate up to six charge-variant species (37, 38). It has been suggested that the phosphorylation of p47-phox occurs in a stepwise fashion, with the addition of phosphate groups in series to different domains within the protein. Some of these sites are phosphorylated when p47-phox is still cytosolic and some may be phosphorylated after translocation of p47-phox to the plasma membrane. Thus the responsible kinase and the phosphorylated domain may vary with the subcellular compartment in which phosphorylation occurs. For example, cytosolic kinases, PKC or a novel PMN kinase, may be responsible for the phosphorylation of p47-phox in cytosol, before translocation to the plasma membrane; other kinases, perhaps PKM, may be responsible for terminal phosphorylation after translocation.

Based on studies presented here we suggest that a staurosporine-susceptible kinase, either PKC or a novel PMN kinase, phosphorylates p47-phox in a domain which is critical for association of the molecule with the cytoskeleton and to the plasma membrane. Alternatively, it is possible that PKC mediates some currently unidentified event which is critical for activation and that the phosphorylation of p47-phox is a nonessential reaction. However, we believe that the correlation between staurosporine inhibition of PKC activation, of p47-phox translocation, and of assembly of a functional oxidase in the plasma membrane is compelling evidence that phosphorylation of p47-phox is a necessary event in NADPH-oxidase activation. We have recently examined membrane translocation of p47-phox and p67-phox in PMNs from patients with various forms of CGD (16). Neither cytosolic oxidase component translocated to the plasma membrane in PMNs from subjects with the cytochrome b₅₆₇-deficient form of CGD. Furthermore, there appeared to be an interaction between the two cytosolic factors, as judged from studies in patients whose CGD was the result of a deficiency of one of these factors. In PMNs from patients with a deficiency of p47-phox as the cause of their CGD, p67-phox failed to translocate to the plasma membrane with stimulation. In contrast, p47-phox translocated to the PMN plasma membrane in patients with the p67-deficient form of CGD. Based on these findings, one would predict that p67-phox would fail to become membrane-associated if p47-phox translocation were inhibited. Thus we suggest that the primary effect of staurosporine is inhibition of p47-phox phosphorylation, that this results in a failure of the protein to associate with cytoskeletal and membrane elements, and that secondary failure of p67-phox translocation occurs due to a lack of membrane-associated p47-phox.

Cytochrome b₅₆₇-deficient CGD patients form all but the two most acidic species of the six phosphoproteins seen at 47-kDa in two-dimensional gel analysis (37, 38). In light of the results from translocation studies in CGD patients discussed above, it appears that the final phosphates are added to p47-phox after translocation to the membrane. Consistent with this suggestion are the studies of Okamura et al. (38) which demonstrate phosphorylation of all six phosphoproteins in the 47-kDa range in PMNs from a patient with a rare variant form of CGD which has a missense mutation in the cytochrome b sequence, resulting in a nonconservative amino acid substitution (Pro to His (49)). We have found normal translocation of cytosolic oxidase components to the plasma membrane in PMNs from one of these patients (16), consistent with the notion that some sites in p47-phox are phosphorylated after translocation to the plasma membrane. Additional studies using deletional and site-directed mutants of p47-phox should elucidate the structure-function relationships of its specific phosphorylation sites.

Cytoskeletal proteins exhibit ordered structural and functional associations with certain integral membrane proteins. Although this has been most thoroughly characterized in erythrocytes (reviewed in Ref. 50), it appears to apply to a
variety of cells (reviewed in Ref. 51), including PMNs (29). It may be that the association of p47-phox and p67-phox with cytoskeletal elements promotes the translocation of these oxidase components to the plasma membrane where the final assembly of the fully active oxidase occurs. Using PMA-stimulated PMNs, Quinn et al. (52) have localized NADPH-dependent superoxide activity in a population of plasma membrane vesicles which is also enriched for the cytoskeletal elements actin and fodrin. The superoxide-generating activity remains associated with the insoluble pellet after detergent extraction of these vesicles. These findings are consistent with the existence of active PMNs of plasma membrane-associated complex which includes the assembled and fully activated NADPH-dependent oxidase and elements of the subplasmalemmal cytoskeleton. It is noteworthy that our studies indicate quantitative differences in cytoskeletal association (greater than 50%) versus membrane association (approximately 10%) of the cytosolic oxidase factors. The studies presented here do not address the basis for this difference but at least two possible explanations can be offered. First, it is conceivable that only a portion of the cytoskeleton-associated p47-phox and p67-phox becomes redistributed with the membrane fraction. Second, it is possible that the assay used to quantitate the membrane-associated cytosolic oxidase factors underestimates the amount which has translocated, whereas the cytoskeletal preparations indicate the redistribution with greater fidelity.

The significance of the constitutive cytoskeletal association of p67-phox, in contrast to the stimulus dependence of the p47-phox association, it not clear. The data clearly indicate that Triton X-100 does not nonspecifically cause p67-phox to pellet into the detergent-insoluble fraction. The apparent constitutive association of the p67-phox with the cytoskeleton should be viewed with some caution, since we used an operational definition of cytoskeletal which is relatively unrefined. Additional studies using more sophisticated definitions of cytoskeleton, such as those of Quinn et al. (52) and some of our previous work (29), will explore this association further and may elucidate its basis. Obviously, the relationship of p67-phox to cytoskeletal elements, unlike its membrane translocation, is independent of p47-phox. Apart from the phosphorylation of p47-phox, the structural determinants of cytoskeletal association of the cytosolic oxidase components are unknown. However, the presence in both p47-phox and p67-phox of two copies of a domain homologous to a noncatalytic region (SH3 or A) of the src family of tyrosine kinases is of considerable interest in this regard. Among the proteins containing this region of homology are several that are firmly associated with the cytoskeleton and/or cytoplasmic face of the plasma membrane including pp60"src" (53), PLC-γ (22, 23), GAP (54, 55), myosin Ib of Acanthamoeba (56), α-spectrin (57), cdc25 (58), and fus1 (59, 60), and actin binding protein 1p of Saccharomycyes (61). In addition, Matsuda et al. (62) have suggested that the SH2 and SH3 regions of p47"src"modulate interactions with pp60"src" by a mechanism which is phosphorysine-dependent. The role of the SH3 domains of p47-phox and p67-phox in cytoskeletal and membrane association and NADPH oxidase assembly, although speculative at present, should be explored with mutant recombintant proteins and ecaryotic expression systems.

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