Cytosolic Guanine Nucleotide-binding Protein Rac2 Operates in Vivo as a Component of the Neutrophil Respiratory Burst Oxidase

TRANSFER OF Rac2 AND THE CYTOSOLIC OXIDASE COMPONENTS p47phox AND p67phox TO THE SUBMEMBRANOUS ACTIN CYTOSKELETON DURING OXIDASE ACTIVATION

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The respiratory burst oxidase is responsible for O2 production in stimulated neutrophils and B lymphocytes. Components of this oxidase include cytochrome b558, a membrane-bound flavohemoprotein; the cytosolic polypeptides p47phox and p67phox; and one or more small G proteins including Rac1, Rac2, and/or Rap1A. We found that when normal neutrophils were activated, small percentages of each of the cytosolic proteins p47phox, p67phox, and Rac2 were transferred to the membrane cytoskeleton. However, Rac2 was not transferred to the membrane during activation of p47phox-deficient neutrophils. In normal cells, some p47phox also became associated with the non-cytoskeletal portion of the plasma membrane, but p67phox, Rac2, and O2--forming activity were restricted to the cytoskeleton. Neutrophil activation also causes the phosphorylation of multiple serines in p47phox. The most heavily phosphorylated forms of p47phox were found solely in the membrane cytoskeleton. These results suggest that 1) the membrane cytoskeleton participates in respiratory burst oxidase activation, 2) the fully phosphorylated p47phox is located in the active oxidase, which resides in the membrane cytoskeleton, and 3) Rac2 acts like a dedicated component of the respiratory burst oxidase.

The respiratory burst oxidase of neutrophils catalyzes the NADPH-dependent reduction of oxygen to O2 (1, 2). It is dormant in resting cells but acquires catalytic activity when the cells are stimulated. In resting cells, oxidase components are distributed between the cytosol (p47phox, p67phox, and a small guanine nucleotide-binding protein (either Rac1 or Rac2 (3-7)) and the plasma membrane (or the membrane of a newly recognized organelle (8)) (cytochrome b558 and the small guanine nucleotide-binding protein Rap1A (9-13)). During activation, p47phox becomes phosphorylated on multiple serines (14-16), and the cytosolic components transfer to the plasma membrane (17-19).

Earlier work suggested a close association between the respiratory burst oxidase and the neutrophil cytoskeleton (20-23). It is of interest that both p47phox and p67phox contain SH3 domains, a motif typically found in proteins known to associate with the cytoskeleton (24). In this communication, we describe further studies on the relationship between the membrane cytoskeleton of the neutrophil and cytosolic components that have been associated with the respiratory burst oxidase: p47phox, p67phox, and Rac2.

EXPERIMENTAL PROCEDURES

Materials—Cytochrome c (type IV), bovine superoxide dismutase, NADPH, rabbit muscle actin, sucrose, and phorbol myristate acetate (PMA), 1 were obtained from Sigma. 32P-Labeled phosphoric acid (carrier free, 9000 Ci/mmol) was obtained from DuPont NEN. DNsase I was from Boehringer Mannheim, amphotolines were from Pharmacia LKB Biotechnology Inc., p1 markers were from Calbiochem, and all of the SDS-polyacrylamide gel electrophoresis reagents were from Bio-Rad. Rabbit polyclonal antibodies against Rac2 were the generous gift of Dr. Alan Hall or were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). A rabbit polyclonal antibody against Rac1 was purchased from Santa Cruz Biotechnology. The commercial anti-Rac1 and anti-Rac2 antibodies were each raised against unique peptides near the C termini of Rac1 and Rac2, respectively.

Preparation of Neutrophil Cytoskeletons—Neutrophils were obtained from normal adult human subjects and from a patient with p47phox-deficient chronic granulomatous disease as described previously (25). The neutrophils (10⁶ cells/ml) were suspended in Ca2+- and Mg2+-free Dulbecco’s phosphate-buffered saline, treated with 2.4 mM diisopropyl fluorophosphate (DFP) for 30 min at 4°C as described elsewhere (26), and then washed and stimulated with 1 μg/ml PMA at 37°C for 10 min unless otherwise indicated. Neutrophil membranes and cytosol were then prepared by a published method (19).

Preparation of Membrane Cytoskeletons—Whole membranes from 10⁶ cells were incubated at 4°C for 30–60 min in 250 μl of cytoskeleton buffer (20 mM Tris-HCl, pH 7.4, 3 mM MgCl2, 8% sucrose, and 0.5% Triton X-100 (21) that had been supplemented with protease inhibitors (5 μM E64, 0.25 mM leupeptin, and 1 mM DFP). Triton-insoluble and -soluble fractions were separated by ultracentrifugation at 180,000 × g for 2 h at 4°C as described elsewhere (21).

DNsase I Treatment of Membranes—DNase I (2 mg/ml) or carbonic anhydrase (2 mg/ml) was dissolved in cytoskeleton buffer described above and treated with DFP for at least 15 min. Membranes were treated with the DNase I-containing cytoskeletal buffer as described in the previous section, incubating at 4°C for 2 h unless otherwise noted. Triton-soluble and -insoluble fractions were then separated as described above. Control extractions were carried out with cytoskeleton buffer containing carbonic anhydrase or no additions.

Measurement of O2 Production—To measure O2 production, the fractions were suspended in 0.25 M sucrose at 4°C, and the activity was measured as superoxide dismutase-inhibitable reduction of ferricytochrome c using NADPH as substrate (19, 25). For each assay, the reaction rate shown is the maximum rate of cytochrome c reduction observed during the course of the incubation.

Protein Phosphorylation in Intact Neutrophils—32Ploading, PMA stimulation, and cell fractionation were performed using modifications of previously described methods (15). Neutrophils were washed and resuspended in loading buffer (10 mM HEPES, pH 7.4, 137 mM...
NaCl, 1 mM MgCl₂, 5.5 mM KCl, 5.6 mM glucose, and 0.025% bovine serum albumin). The cells were incubated with ³²P-labeled phosphoric acid (1 mCi/10⁶ ml) for 1 h at 37°C, with gentle agitation every 15 min. The cells were then washed, treated with DFP, and stimulated as described above.

Electrophoresis and Immunoblotting—Neutrophil membranes were extracted with cytoskeleton buffer as described above. Proteins in the Triton-soluble fractions were precipitated by treatment with methanol/chloroform/H₂O as described elsewhere (27). The Triton-insoluble and precipitated Triton-soluble proteins were subjected to SDS-polyacrylamide gel electrophoresis on 12.5% polyacrylamide gels using the Laemmli buffer system (28). For two-dimensional gel electrophoresis, the cytoskeleton buffer used for the extraction was supplemented with phosphatase inhibitors (5 mM EDTA, 5 mM NaF, and 1 mM Na₃VO₄). The first dimension of the two-dimensional procedure consisted of nonequilibrium pH gradient electrophoresis as described by O’Farrell (29), except that before mixing the sample proteins with urea/Nonidet P-40 buffer, they were sonicated in 215 μl of 20 mM Tris-Cl and then dissolved by heating to 100°C for 3 min after adding 25 μl of SDS solution and 10 μl of aqueous mercaptoethanol to achieve final concentrations of 2% (w/v) and 4% (v/v), respectively. Ampholines consisted of 1% pH 3.5–10 ampholines and 1% pH 7–9 ampholines. The separated proteins were electrophoretically transferred onto a nitrocellulose sheet (30), which was blocked as described (31), probed with ammonium sulfate-purified rabbit antibodies raised against synthetic peptides from p47phox and p67phox (22) or with the anti-Rac2 antibody, and finally detected with alkaline phosphatase-labeled goat anti-rabbit immunoglobulin antibodies and the BCIP/NBT detection kit (Bio-Rad). The antibodies were used at the dilutions: anti-p47phox, 1:4000–1:10,000; anti-p67phox, 1:400–1:1000; anti-Rac2, 1:200–1:1000; anti-rabbit immunoglobulin, 1:2000.

Quantification of proteins on immunoblots was accomplished by scanning with an UltraScan XL laser densitometer. Phosphorylated proteins were visualized by autoradiography of the immunoblots.

Protein Concentrations—Protein concentrations were determined using a Bio-Rad kit. The concentrations of the proteins in the neutrophil fractions were as follows (in μg/10⁶ cells ± S.D.): cytosol, 17.3 ± 3.4; whole membranes, 7.8 ± 0.5; cytoskeletons, 3.3 ± 1.0; Triton-soluble membrane fractions, 1.4 ± 0.3.

RESULTS

Association of p47phox, p67phox, and Rac2 with Membrane Cytoskeleton—Treatment with Triton X-100 separates neutrophil membranes into two fractions: a Triton-insoluble fraction, generally equated with the membrane cytoskeleton, and a Triton-soluble fraction, containing the non-cytoskeleton membrane proteins (21, 32). Analysis of cytoskeletal and non-cytoskeletal fractions by immunoblotting (Fig. 1, top panel) showed that cytoskeleton from resting membranes contained no detectable p47phox or p67phox. Stimulation of neutrophils with PMA, however, caused both proteins to move to the membrane. All of the p67phox that had migrated to the membrane was associated with the cytoskeleton (Fig. 1, bottom panel); the proportion of membrane-bound p47phox associated with the cytoskeleton, however, was relatively small, most appearing in the non-cytoskeletal fraction.

The presence of p47phox and p67phox in the Triton-insoluble fraction could reflect either their physiological association with the cytoskeleton or their insolubility in cytoskeleton buffer. To distinguish between these possibilities, the cytoskeleton was dissolved with DNase I (32, 33). DNase I will solubilize proteins associated with the cytoskeleton but should not affect proteins that are intrinsically insoluble in cytoskeleton buffer. Fig. 1 (bottom panel) shows that p47phox and p67phox were solubilized by DNase I (DNase) but not by carbonic anhydrase (C-Anh), a protein similar in size to DNase I, or by protein-free buffer (—). This result suggests that in activated neutrophils, both p47phox and p67phox are associated with the membrane cytoskeleton.

An activated GTP-binding protein is required for O₂ production by the respiratory burst oxidase (6, 7, 11, 34, 35). In human neutrophils, Rac2 has been shown to fulfill this requirement (6, 18). Consistent with its postulated role in oxidase activation, Rac2 was found in the cytosol to the plasma membrane when neutrophils were stimulated with PMA (Fig. 2, lanes 1–3). The release of Rac2 by treatment with DNase I confirmed that in stimulated neutrophils this protein, like p47phox and p67phox, is associated with the membrane cytoskeleton (Fig. 2, lanes 4–7).

An earlier study with p47phox-deficient neutrophils had...
shown that p47phox is required for the transfer of p67phox from the cytosol to the plasma membrane during oxidase activation (37). We have found that p47phox is also needed for the transfer of Rac2 to the plasma membrane. When normal neutrophils were activated with PMA, p47phox and Rac2 migrated as expected from cytosol to the plasma membrane (Fig. 3, left). When p47phox-deficient neutrophils were similarly treated, however, Rac2 remained in the cytosol (Fig. 3, right).

Kinetics and Extent of Transfer of Cytosolic Proteins to Membrane Cytoskeleton during Oxidase Activation—We compared the kinetics of activation of the respiratory burst (15) with the rate of transfer of p47phox, p67phox, and Rac2 from cytosol to the cytoskeleton in PMA-stimulated neutrophils. Fig. 4 shows that the transfer of all three proteins was well under way by 2 min and was virtually complete by 5 min. A good temporal correlation, therefore, exists between protein transfer and the activation of O2; production in PMA-treated neutrophils.

At the concentration of PMA employed in these studies, the neutrophil respiratory burst is fully activated (15). Table I shows, however, that <15% of the cytosolic oxidase components had migrated to the cytoskeleton. The role of the components remaining in the cytosol during oxidase activation is not clear. They may provide a reserve to maintain O2; production over a long period as previously activated oxidase molecules lose their function or to reactivate oxidase molecules that have been reversibly turned off.

Association of Respiratory Burst Oxidase Activity with Membrane Cytoskeleton—When incubated with NADPH, plasma membranes from PMA-treated neutrophils produce O2; via the respiratory burst oxidase. Table II shows the distribution of oxidase activity between the cytoskeleton and membrane. Confirming earlier results (21), we found that the membrane cytoskeleton contained >90% of the O2;-forming activity of whole membranes from PMA-activated neutrophils.

The association of p47phox, p67phox, and Rac2 with the membrane cytoskeleton may help establish a productive interaction between these subunits and other oxidase components (e.g. cytochrome b566). It was, therefore, of interest to examine the relationship between oxidase activity and cytoskeletal integrity. Dissolution of activated neutrophil cytoskeletons with DNase I resulted in a loss of oxidase activity (Table II) that was nearly complete after 2 h (Fig. 5). No effect was seen when DNase I was added 5 min before starting the reaction with NADPH, indicating that DNase I does not interfere with the catalytic activity of the oxidase. The effects of DNase I appeared to be related to its F-actin depolymerizing ability since competition with G-actin greatly decreased the DNase I-induced losses of p47phox, p67phox, and Rac2 (Fig. 6), as well as oxidase activity (Table III), from the membrane cytoskeleton.

The foregoing results suggest that cytoskeletal integrity is important for oxidase activity and that cytoskeletal p47phox, p67phox, and Rac2 are part of the active oxidase, whereas the non-cytoskeletal portion of p47phox is not. These findings are compatible with the idea that one or both of those proteins...
Phosphorylated Forms of p47phox in Membranes and Membrane Cytoskeletons from Resting and Activated Neutrophils—

The activation of the respiratory burst oxidase in intact neutrophils is associated with the phosphorylation of 6-8 serine residues in p47phox (14-16). Both partly and fully phosphorylated forms of p47phox are routinely observed in activated neutrophils, the various phosphorylated forms appearing as a string of radioactive spots at M, = 47,000 in two-dimensional gels prepared from 32P-loaded cells. To determine whether a relationship exists between the level of phosphorylation of p47phox and its attachment to the cytoskeleton, cytoskeletal and non-cytoskeletal fractions from neutrophil membranes were subjected to two-dimensional gel electrophoresis, detecting p47phox by immunoblotting with an antibody against its C-terminal decapeptide.

To verify that the antibody recognized all the phosphorylated forms of p47phox, neutrophils were loaded with 32P, stimulated with PMA, and analyzed by two-dimensional gel electrophoresis, visualizing the p47phox isoforms by both autoradiography and immunoblotting. The immunoblot showed that activated neutrophils contained multiple p47phox forms spanning a pI range of ≈4 to >8 (Fig. 7, top band). Every 32P-labeled p47phox isoform (Fig. 7, bottom band) was detected by the antibody. The antibody also detected some non-labeled isoforms that were not phosphorylated at all or because they contained phosphate that did not turn over during the course of labeling. Alternatively, they could

| Preparation                  | O₂ production (nmol/min/5 × 10⁶ cell eq) |
|------------------------------|-----------------------------------------|
| Intact membranes             |                                         |
| Resting                      | 0.13 ± 0.07                             |
| Active                       | 2.45 ± 0.46                             |
| Active plus DNase I          | 2.45 ± 0.34                             |
| Active plus carbonic anhydrase| 2.46 ± 0.33                             |
| Extracted membranes          |                                         |
| Buffer only                  |                                         |
| Cytoskeleton                 | 2.06 ± 0.32                             |
| Triton-soluble               | 0.22 ± 0.09                             |
| DNase I                      |                                         |
| Triton-insoluble*            | 0.24 ± 0.09                             |
| Triton-soluble               | 0.15 ± 0.07                             |
| Carbonic anhydrase           |                                         |
| Cytoskeleton                 | 2.14 ± 0.41                             |
| Triton-soluble               | 0.43 ± 0.27                             |

*Designated as "Triton-insoluble" rather than "cytoskeleton" because the cytoskeletons had been solubilized by the DNase I treatment.

p47phox and its attachment to the cytoskeleton, cytoskeletal and non-cytoskeletal fractions from neutrophil membranes were subjected to two-dimensional gel electrophoresis, detecting p47phox by immunoblotting with an antibody against its C-terminal decapeptide.
detected autoradiographically and by immunoblotting after electrophoretic transfer of proteins from two-dimensional gels onto nitrocellulose. Activated neutrophils that had been loaded with $^{32}$P (1.25 x $10^7$ cells in each sample) were subjected to two-dimensional gel electrophoresis. The separated proteins were transferred electrophoretically to a nitrocellulose sheet, and the p47phox isoforms on the nitrocellulose sheet were visualized, first by immunoblotting (top band) and then by autoradiography (bottom band), as described under "Experimental Procedures." The locations of the pI markers are indicated above the immunoblot.

![Image](Fig. 7. Isoforms of p47phox from $^{32}$P-loaded neutrophils as detected autoradiographically and by immunoblotting after electrophoretic transfer of proteins from two-dimensional gels onto nitrocellulose. Activated neutrophils that had been loaded with $^{32}$P (1.25 x $10^7$ cells in each sample) were subjected to two-dimensional gel electrophoresis. The separated proteins were transferred electrophoretically to a nitrocellulose sheet, and the p47phox isoforms on the nitrocellulose sheet were visualized, first by immunoblotting (top band) and then by autoradiography (bottom band), as described under "Experimental Procedures." The locations of the pI markers are indicated above the immunoblot.)

![Image](Fig. 8. Isoforms of p47phox in cytoskeleton and non-cytoskeletal fractions of activated neutrophil membranes. The cytoskeleton (105 cell eq) and the non-cytoskeletal fraction (5 x 107 cell eq) were prepared from activated neutrophil membranes and analyzed by two-dimensional gel electrophoresis followed by immunoblotting as described under "Experimental Procedures." The results shown are representative of three separate experiments.)

have contained $^{32}$P in quantities too small to detect. In any case, phosphorylation did not prevent the antibody from recognizing p47phox.

Using this antibody, we examined the state of phosphorylation of p47phox molecules in the cytoskeletal and non-cytoskeletal fractions from activated neutrophil membranes. The results (Fig. 8) showed that the locations of the p47phox isoforms varied with their levels of phosphorylation. Acidic isoforms (i.e. the more completely phosphorylated isoforms) were principally found in the membrane cytoskeleton (those with the most acidic pI values residing exclusively in the cytoskeleton where the O$_2$-forming activity is located). In contrast, the most basic isoforms were found only in the non-cytoskeletal membrane fraction, while isoforms with intermediate pI values were located in both fractions. These data suggest that the expression of respiratory burst oxidase activity in intact neutrophils requires the relatively complete phosphorylation of p47phox.

**Discussion**

Our experiments showed that when neutrophils are activated by PMA, the oxidative components p47phox and p67phox and the GTP-binding protein Rac2 migrate from the cytosol to the membrane cytoskeleton. Membrane-bound p67phox and Rac2 are found exclusively in the membrane cytoskeleton, but membrane-bound p47phox is distributed between the membrane cytoskeleton and the non-cytoskeletal fraction of the plasma membrane. When neutrophils deficient in p47phox were activated with PMA, neither p67phox nor Rac2 was transferred to the membrane; this finding indicates that in whole neutrophils, Rac2 behaves like a dedicated component of the respiratory burst oxidase. Antisense experiments suggested that Rac2 serves a similar function in B lymphocytes (38).

Data are available that pertain to specific interactions between individual components of the respiratory burst oxidase. Mutual interactions among p47phox, p67phox, and cytochrome b$_{558}$ are indicated by the findings that in cytochrome b$_{558}$-deficient neutrophils neither p47phox nor p67phox migrates from the cytosol to the plasma membrane when the cells are activated and that p67phox does not translocate when p47phox-deficient neutrophils are activated (37). Our results show that p47phox is required for the transfer of Rac2 from the cytosol to the cytoskeleton when neutrophils are activated with PMA but do not indicate whether Rac2 interacts with p47phox, p67phox, or both.

In the activated neutrophil plasma membrane, the most fully phosphorylated forms of p47phox are restricted to the membrane cytoskeleton, while the less phosphorylated forms are located only in the non-cytoskeletal region of the membrane. These results suggest that the transfer of p47phox from cytosol to plasma membrane occurs when a certain level of phosphorylation is achieved but that association of p47phox with the membrane cytoskeleton requires further phosphorylation. These conclusions support earlier evidence for a multistage phosphorylation of p47phox during oxidase activation (16, 40–42).

A prior study suggested that in the cytosol from resting neutrophils, all of the p67phox was present in a complex of $M_r$ = 240,000 that also contained some, but not all, of the p47phox (19). The present study showed that in activated neutrophils, all of the membrane-bound p67phox, but only some of the p47phox, was associated with the cytoskeleton. It may be that only the 240,000 complex, which contains both p47phox and p67phox, is translocated to the membrane cytoskeleton where the active oxidase is located and that free p47phox, though susceptible to phosphorylation and transferable to the plasma membrane, is not incorporated into the active oxidase. Measurements showing three times as much p47phox as p67phox in neutrophil cytosol (43) are consistent with this interpretation.

Previous work from this laboratory suggested that p67phox was attached to the cytoskeleton in both resting and activated neutrophils, while p47phox was never associated with the cytoskeleton (22). With regard to p67phox, the present findings are consistent with the earlier observations because the earlier study dealt with the entire cellular cytoskeleton, while the present study is concerned only with the membrane cytoskeleton. The failure to detect cytoskeletal p47phox in the earlier study was probably due to the very limited amount of p47phox that is transferred to the cytoskeleton during oxidase activation and to the blending of the $^{32}$P signals from the most acidic isoforms of p47phox into the background of the autoradiogram. Our present results agree with those of Clark and co-workers (23) who reported that some p47phox is transferred to the neutrophil cytoskeleton during oxidase activation.

All of the O$_2$-generating activity of activated neutrophil membranes was found in the membrane cytoskeleton. This activity was eliminated, and p47phox, p67phox, and Rac2 were released by dissolving the cytoskeleton with DNase I. These results suggest that the activity of the respiratory burst oxidase depends on the integrity of the membrane cytoskeleton and indicate that the cofractionation of p47phox, p67phox, and Rac2 with the cytoskeleton was due to an association between these proteins and the cytoskeleton rather than to the insolubility of these proteins in the Triton-containing cytoskeleton buffer.

Earlier experiments showed that cytochalasin D had no...
effect on the interaction between the respiratory burst oxidase and the cytoskeleton (21, 22). Cytochalasins depolymerize some of the F-actin in neutrophils but spare the cortical F-actin that lies directly beneath the plasma membrane (44).

Those earlier observations are, therefore, compatible with our finding that the respiratory burst oxidase is associated with the membrane cytoskeleton.

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