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Research Article

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Respiratory Burst Oxidase and Three of Four Oxidase-related Polypeptides Are Associated with the Cytoskeleton of Human Neutrophils

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Abstract

Resting and phorbol-activated human neutrophils were separated by treatment with Triton X-100 into detergent-extractable and cytoskeleton fractions. Respiratory burst oxidase activity was restricted entirely to the cytoskeleton. The cytoskeleton also contained $\approx$ 15% of the neutrophil cytochrome b_{558}, an oxidase-associated heme protein, as well as most of the oxidase-related cytosolic polypeptide p67^{phox}. In contrast, the components of the oxidase-associated phosphoprotein family p47^{phox} were found almost exclusively in the detergent extract, suggesting that p47^{phox} is needed for oxidase activation but not for O_2^- production by the activated oxidase. Activation of the oxidase had no apparent effect on the distribution of any of these species between the cytoskeleton and the detergent extract. Our results support earlier studies implying that the cytoskeleton participates in an important way in regulating the activity of the O_2^--forming respiratory burst oxidase of neutrophils. (J. Clin. Invest. 1991. 87:1345–1351.) Key words: neutrophil • cytoskeleton • respiratory burst oxidase

Introduction

The exposure of neutrophils to appropriate stimuli activates a metabolic pathway known as the "respiratory burst" whose purpose is the generation of microbicidal oxidants through the partial reduction of oxygen (1). The key to this metabolic pathway is the respiratory burst oxidase, a membrane-bound enzyme that catalyzes the one-electron reduction of oxygen to O_2^- at the expense of NADPH: $O_2 + NADPH \rightarrow O_2^- + NADP^+ + H^+$. The respiratory burst oxidase is dormant in resting neutrophils, but acquires catalytic activity when the cells are incubated with any of a wide variety of stimuli. The O_2^- produced by this oxidase is the precursor of the very complex mixture of microbicidal oxidants generated by activated neutrophils during the respiratory burst.

Recent observations have furnished strong indications that the respiratory burst oxidase and its activating apparatus may be associated in some manner with the neutrophil cytoskeleton, a series of filament networks that define the shape and organelle distribution of the neutrophil and are indispensable for its motor function. Inhibitor studies have suggested that the actin microfilament network in particular plays an important role in regulating the activity of the respiratory burst oxidase. The effect of several microfilament-disrupting agents, including the cytochalasins (2) and the *Clostridium botulinum* C2 iota toxin (3), on the production of O_2^- in response to certain stimuli, most notably the N-formylated chemotactic peptides, clearly implies some sort of relationship between the cytoskeleton and the respiratory burst oxidase. Jesaitis and his associates have published several studies suggesting that this relationship is mediated through effects of the cytoskeleton on the traffic of N-formylated peptide receptors (2), but a more direct connection between the oxidase and the cytoskeleton was not ruled out. Recently, Nathan has found that the delayed but protracted respiratory burst elicited from surface-adherent neutrophils by exposure to tumor necrosis factor can be abolished by dihydrocytochalasin B (4), again implicating the cytoskeleton in the regulation of the respiratory burst.

We have now used more direct methods to examine the association between the respiratory burst oxidase and the neutrophil actin microfilament network. Our experiments were based on the results with microfilament-depolymerizing agents described above, as well as on earlier work with neutrophil phosphoproteins (5–9). Our findings provide further support for a relationship between the actin microfilament system and the respiratory burst oxidase, suggesting that the microfilaments are connected to the oxidase not only functionally, but physically as well.

Methods

Materials. ^{32}P-labelled phosphoric acid (carrier free in 0.02 M HCl) was obtained from ICN Radiochemicals, Div. Radiochemicals, Inc., Irvine, CA. Protease inhibitors (EDTA, diisopropyl fluorophosphate [DFP],[1] leupeptin, and pepstatin), phorbol myristate acetate (PMA), mercaptoethanol, *N, N, N', N'*-tetramethylethylene diamine, DNAse I (type II, bovine pancreas), dihydrocytochalasin B, cytochrome *c* (horse heart type VI), superoxide dismutase (bovine erythrocyte), dimethyl sulfoxide, prestained fumarase (used as a 48K marker for the second dimension of two-dimensional gel electrophoresis) and Triton X-100 were purchased from Sigma Chemical Co., St. Louis, MO. Ampholines and Ficoll-Hypaque were obtained from Pharmacia Fine Chemicals, Pis-

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1. *Abbreviations used in this paper:* CGD, chronic granulomatous disease; DFP, diisopropyl fluorophosphate.

cataway, NJ, and LKB Instruments, Inc., Gaithersburg, MD. SDS, acrylamide, *N,N'*-methylenebisacrylamide, ammonium persulfate, Coomassie brilliant blue R-250, and agarose (ultrapure high grade DNA agarose) were obtained from Bio-Rad Laboratories, Richmond, CA, pI markers from Calbiochem-Behring Corp., San Diego, CA, and Ultrapure® urea from Schwarz/Mann, Spring Valley, NY. Asolectin (Associated Concentrates, Woodside, NY), a preparation of mixed soybean phospholipids, was prepared for use by sonication to clarity (50 W, room temperature, argon atmosphere) at 2 mg lipid/ml in Hanks' balanced salts without Ca^{++} and Mg^{++}. Other materials were the best quality commercially available and were used without further purification.

Buffers. The names and compositions of the buffers referred to in the succeeding sections of Methods and elsewhere in the text are given in Table I.

Respiratory burst oxidase activity in neutrophil cytoskeletons and detergent extracts. Neutrophils were suspended in incubation buffer at a concentration of 10^7 cells/ml. 4 ml of this suspension was incubated at 37°C for 5 min, then supplemented with 40 μl PMA solution (20 μg/ml in DMSO) and incubated for an additional 2 min at 37°C to activate the neutrophils, terminating the reaction by immersion in an ice slurry. The suspension was centrifuged at 100 *g* for 10 min at 4°C, the supernatant discarded, and the cells (total 4×10^7 neutrophils) suspended in 2 ml of Triton inhibitor buffer (10–12) and incubated at 0°C for 30 min. Next, 0.3 ml of the cell suspension was reserved for measuring total respiratory burst oxidase activity, while 0.1-ml samples were placed in each of eight screw-cap Eppendorf tubes and centrifuged for 30 s at 4°C. The cytoskeletons in one pair of tubes were immediately resuspended in their supernatants by repeated pipetting, while the detergent extracts from the other tubes were withdrawn and set aside. Cytoskeletons in a second pair of tubes were suspended in 0.1-ml portions of Triton inhibitor buffer, and 0.1 ml of detergent extract was placed in each of a third pair of tubes. Finally, isolated cytoskeletons in a fourth pair of tubes were resuspended in 0.1-ml portions of detergent extract. Respiratory burst oxidase activity was assayed by adding superoxide dismutase (0.1 mg) to one of each pair of tubes to serve as reference, then adding to each tube flavin adenine dinucleotide (FAD) (final concentration 1.0 μM), cytochrome *c* (83 μM), asolectin solution (0.6 ml), and NADPH (0.1 mM, added last) to a final vol of 1 ml, incubating the samples for 10 min at room temperature on a rocking platform, and finally adding superoxide dismutase to the second of each pair of tubes and measuring cytochrome *c* reduction as described elsewhere (13).

Table I. Buffers

Name	Composition
Incubation buffer	Hepes/Tris (20 mM each) pH 7.4 containing 0.12 M NaCl, 5 mM KCl, 10 mM glucose, 0.25 mM $CaCl_2$, and 1 mM $MgCl_2$
Termination buffer	Hepes/Tris (20 mM each) pH 7.4 containing 0.12 M NaCl, 5 mM KCl, and 10 mM glucose
Inhibitor buffer (3)	Termination buffer supplemented with protease inhibitors consisting of 0.5 mM DFP (added as a 10-mM solution in dry ethylene glycol), 20 μM leupeptin, and 18 μM pepstatin (final concentrations)
Triton inhibitor buffer	Inhibitor buffer: 5% (wt/vol) aqueous Triton X-100 = 4:1 (vol/vol)
Lysis buffer	Freshly prepared 9.5 M urea containing 2% (wt/vol) Nonidet P-40, 1.6% (vol/vol) pH 5–7 ampholines, 0.4% (vol/vol) pH 3.5–10 ampholines, and 5% (vol/vol) mercaptoethanol
Sonication buffer	Tris (10 mM) pH 7.4 containing 5 mM $MgCl_2$

Assay of neutrophil cytoskeletons and detergent extracts for cytochrome b_{558}. Neutrophils (10^8 cells in 10 ml) were prepared in incubation buffer and (where indicated) activated with PMA (final concentration 0.2 μg/ml) exactly as described elsewhere (6). Incubations were terminated after 2 min with 10 ml ice-cold termination buffer supplemented with 10 mM EDTA. The cells were isolated by centrifugation (110 *g*, 4°C, 10 min), suspended in 5 ml inhibitor buffer containing 10 mM EDTA, incubated at least 20 min at 4°C, and reisolated by centrifugation. Cytoskeletons were then prepared by suspending the cells in 5 ml Triton inhibitor buffer and extracting for 30 min on ice. A 1-ml portion of the whole cell lysate was reserved for the determination of cytochrome b_{558}. Detergent extract and cytoskeleton were then separated by centrifugation of the remaining whole cell lysate (15 min, 4°C, Eppendorf). Detergent extract left in the cytoskeleton as initially prepared was removed by suspending the cytoskeleton in 4 ml Triton inhibitor buffer and centrifuging again. The supernatant from this second extraction (designated "residual extract") was reserved for assay of cytochrome b_{558}. The firm pellet of washed cytoskeleton was suspended by trituration for 5–15 min at room temperature in 1 ml Triton inhibitor buffer containing 2 mg/ml DFP-treated DNAse (DNAse I in buffer containing 0.5 mM DFP [added as 10 mM DFP in dry ethylene glycol]) and 2 mM $CaCl_2$ to depolymerize F-actin (14). The amount of cytochrome b_{558} in each sample (i.e., whole neutrophil lysate, initial detergent extract, residual extract, and washed cytoskeleton) was determined by dithionite difference spectroscopy as described elsewhere (15), using a recording spectrophotometer (860; Kontron Elektronik GmbH, Zurich, Switzerland) and measuring absorbances between 410 and 440 nm at 0.5-nm intervals.

Analysis of protein phosphorylation in neutrophil cytoskeletons and detergent extracts. Neutrophils were prepared, loaded with $^{32}P_i$ (0.4 mCi/10^7 cells), and activated with PMA (final concentration 0.2 μg/ml) exactly as described elsewhere (6). Incubations were terminated after 2 min (unless otherwise indicated) with 1 ml ice-cold termination buffer supplemented with 10 mM EDTA, and the cells isolated by centrifugation (Eppendorf microfuge [15,600 *g*], 10 min). The neutrophils were then resuspended in 1 ml inhibitor buffer containing 10 mM EDTA, incubated at least 20 min at 0°C, and reisolated by centrifugation. Cytoskeletons were then prepared by suspending neutrophils in Triton inhibitor buffer at a concentration of 2×10^7 cells/ml and incubating at 0°C for 30 min. The detergent extract was separated from the nonextractable cytoskeleton by centrifugation as described above. Proteins in the detergent extract were precipitated by the method of Wessel and Flügge (16), dried in air, and dissolved in 0.1 ml lysis buffer. The cytoskeleton was washed once with 0.25 ml Triton inhibitor buffer, suspended by sonication at 0°C (model W-220F; Heat Systems-Ultrasonics, Inc., Plainview, NY, microtip, using multiple brief pulses) in 25 μl sonication buffer, then treated to hydrolyze DNA by incubating for 5 min at 37°C after adding 2 μl DFP-treated DNAse. The suspended cytoskeleton was then dissolved by gentle warming after adding 25 mg solid urea followed by 50 μl lysis buffer. After centrifugation (1 min, Eppendorf microfuge), the solutions of cytoskeletal and detergent-soluble proteins were subjected to two-dimensional gel electrophoresis as described elsewhere (6) except that pH 2–4 ampholines were replaced by pH 5–7 ampholines. In preparing each gel, the entire quantity of cytoskeleton or extract solution was used as sample; all gels from a given experiment were subjected to autoradiography for the same length of time.

Immunoblotting. Immunoblots of gp91^{phox} and p22^{phox} were prepared by electrophoretic transfer of proteins from SDS-polyacrylamide 7–18% gradient gels to nitrocellulose as previously described (17, 18). The nitrocellulose transfers were incubated for at least 2 h in blocking buffer (10% goat serum, 3% BSA, 0.5 M NaCl, and 10 mM Hepes, pH 7.4) and then probed for 3 h with 1/1,000 dilutions of affinity-purified polyclonal rabbit anti-p22^{phox} or a polyclonal antibody raised in rabbits against a peptide corresponding to a portion of the gp91^{phox} polypeptide chain in Dulbecco's PBS containing 3% normal goat serum, 1% BSA, and 0.2% Tween 20. The blots were then rinsed with wash buffer (0.25 M NaCl, 0.2% Tween 20, 10 mM Hepes, pH 7.4); incubated for 1

h with 1 μg/ml alkaline phosphatase conjugated goat anti-rabbit IgG (Bio-Rad) in Dulbecco's PBS containing 3% goat serum, 1% BSA, and 0.2% Tween 20; washed again; and developed using the Bio-Rad alkaline phosphatase conjugation substrate kit according to the manufacturer's directions. The stained blots were scanned using a soft laser densitometer (Zeineh, Fullerton, CA), and the data from six separate experiments averaged to accurately quantitate the relative distribution of cytochrome. (Blotting conditions were designed for optimal blocking of nonspecific proteins, but several bands were present on the gp91^{phox} blot that were not eliminated by competition with excess synthetic peptide under conditions that eliminated the gp91^{phox} band [data not shown]).

The cytosolic oxidase components p47^{phox} and p67^{phox} were also located by immunoblotting. For this purpose, antibodies were raised against the peptides C[^{14}C]FSTKRKLASAV and [^{14}C]FTDLESTR-REV, the 10 COOH-terminal residues of which are identical to the COOH-terminal decapeptides of p47^{phox} and p67^{phox}, respectively. Both peptides were coupled to rabbit serum albumin and also to ovalbumin (Cappel Laboratories, Cochranville, PA) using standard techniques (19), and the conjugates were used to raise polyclonal antisera in rabbits (20). The resulting antisera were screened for reactivity with the oxidase components by immunoblotting against normal neutrophil cytosol, and for specificity by immunoblotting against cytosols from patients with chronic granulomatous disease (CGD) whose neutrophils lacked either p47^{phox} or p67^{phox} (data not shown). For electrophoresis, cytoskeletons were dissolved in 25 μl inhibitor buffer, digested for 5 min at 37°C with DFP-treated DNAse (1 mg/ml), then supplemented with 25 μl 2× sample buffer, while detergent extract proteins were precipitated as described (16) and dissolved in 25 μl inhibitor buffer plus 25 μl 2× sample buffer. Protein solutions were then applied to 10% polyacrylamide gels and subjected to electrophoresis as described by Laemmli (21). The separated proteins were transferred to nitrocellulose at 50 V for 2 h at 4°C. The nitrocellulose blots were blocked for 1 h with Blotto (22), incubated overnight with antibody (1/10,000 in Blotto) at 4°C, then washed five times with Blotto. Visualization was accomplished by incubating the blots at room temperature for 3 h with alkaline phosphatase conjugated to goat anti-rabbit F(ab')$_2$, washing five times with Blotto, and developing the color with the Bio-Rad alkaline phosphatase conjugation substrate kit as described above.

Protein concentrations in cytoskeleton and detergent extracts. Protein concentrations were determined by the Bradford method (23) using a kit purchased from Bio-Rad. Concentrations of protein in detergent extracts and cytoskeleton were 153.7±5.1 and 116.3±3.8 μg/10^7 cells, respectively (n = 6).

Results

Both the respiratory burst oxidase and cytochrome b$_{558}$ are associated with the neutrophil cytoskeleton. Earlier evidence supporting a relationship between the neutrophil cytoskeleton and the respiratory burst oxidase depended on the demonstration that oxidase activity in whole cells was altered by agents known to inhibit the formation of actin microfilaments. We sought to obtain more direct evidence for such a relationship by measuring respiratory burst oxidase activity in cytoskeleton preparations obtained from PMA-activated human neutrophils. For our studies, cytoskeleton was prepared by extracting the cells with nonionic detergents using techniques similar to those employed in other laboratories (10–12). We found that the treatment of whole activated neutrophils with Triton X-100 brought most of the proteins into solution, but left behind an insoluble residue of detergent-resistant cytoskeleton. By two-dimensional gel electrophoresis, this insoluble residue was found to contain three major components; a protein that from its prominence and its location on the gel is probably actin, and

two basic proteins, one that migrated at $M_r \approx$ 60K and the other at $M_r \approx$ 90K, together with a large number of minor components at low concentration (Fig. 1). The composition of the cytoskeleton fraction from our experiments was similar to that reported by Yassin et al. (11), except that they observed only one major basic component, which migrated at $M_r \approx$ 60K.

Neutrophils treated with PMA are known to make large quantities of O_2^- through the activation of the respiratory burst oxidase. Table II shows the distribution of the oxidase between the cytoskeleton and soluble fractions of PMA-treated neutrophils extracted with Triton X-100. The results indicate that when the cytoskeleton fraction was separated from the detergent-solubilized material by centrifugation, > 95% of the detectable O_2^--forming activity remained with the cytoskeleton. Even if it is assumed that the loss in oxidase activity that occurred during the separation procedure (compare row 1 with row 2 plus row 3 of Table II) was sustained entirely by a (hypothetical) detergent-soluble oxidase pool, cytoskeleton oxidase still would represent a third of the total oxidase activity found in the original Triton-solubilized preparation (Table II, row 2). It appears therefore that much, and probably most, of the O_2^--forming activity in PMA-activated neutrophils is associated with the cytoskeleton.

Closely associated with the respiratory burst oxidase is a heme protein known as cytochrome b$_{558}$ that is present only in phagocytes (1) and B lymphocytes (18, 24) and is missing in two of the four currently recognized types of CGD, an inherited condition caused by a heterogeneous group of molecular abnormalities affecting the respiratory burst oxidase or its activating
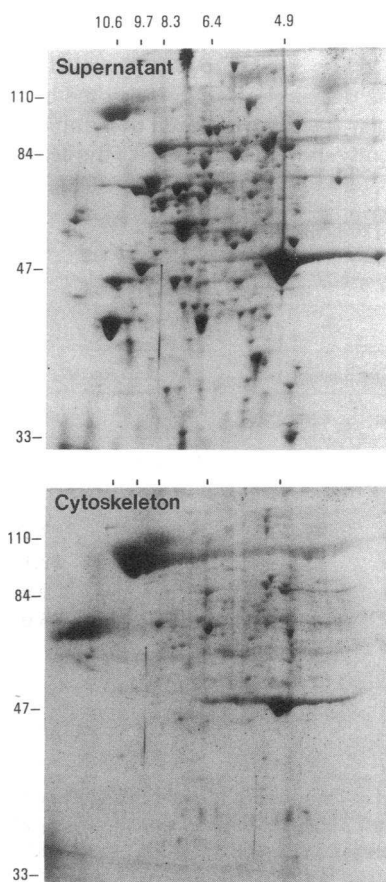

Figure 1. Composition of the detergent-resistant (cytoskeleton) and detergent-extractable fractions from phorbol-activated human neutrophils as determined by two-dimensional gel electrophoresis. Ten million (10^7) neutrophils were used for this experiment. The preparation and extraction of the neutrophils and the electrophoresis of the extractable and cytoskeleton fractions were carried out as described in Methods. The proteins were visualized with Coomassie blue.

Table II. Distribution of Respiratory Burst Oxidase between the Cytoskeleton and the Detergent-soluble Extract of Activated Human Neutrophils

Material	O_2^- production		
	Experiment 1	Experiment 2	Recovery
	nmol/10 min/2 × 10⁶ cell equivalent		%*
Triton-treated cells	10.6	11.7	(100)
Cytoskeleton	2.9	4.1	31.4
Detergent extract	0.1	0.1	0.8
Cytoskeleton + detergent extract	3.7	4.6	37.2

Experiments were carried out as described in Methods using two different neutrophil preparations. The controls for these experiments (row 1) are the Triton-treated cells that were centrifuged and immediately resuspended (see text). This procedure caused the samples to lose 50.1% (Experiment 1) and 39.7% (Experiment 2) of their original O_2^--forming activity as measured in uncentrifuged samples, emphasizing the lability of the respiratory burst oxidase in the buffers employed for the preparation of cytoskeletons. * Average.

apparatus (25). We have found that this cytochrome too is associated with the neutrophil cytoskeleton. This association was demonstrated both spectrophotometrically (Table III) and immunologically (Fig. 2), the latter showing that the association involved both the α- (22-kD) and β- (91-kD) subunits of the cytochrome. In contrast to the respiratory burst oxidase, however, only ~ 15% of the cytochrome was associated with the cytoskeleton. Somewhat surprisingly, this percentage did not change with the state of activation of the cells. It is of interest that the value of 15% is similar to that obtained in earlier studies in which the distribution of cytochrome b₅₅₈ among neutrophil subcellular fractions has been determined. Those studies have consistently shown that ~ 85% of the heme

protein is found in specific granules, which are devoid of oxidase activity, while the remaining 15% is found in plasma membrane fractions, which contain the membrane-associated components of the respiratory burst oxidase (15).

Lack of association of the phosphorylated forms of p47^phox with the cytoskeleton. In prior investigations, we and others have identified a family of closely related (6) 47K phosphoproteins whose states of phosphorylation were shown to be intimately connected with the activation of the respiratory burst oxidase (6, 26–28). Because of (a) the connection between 47K protein phosphorylation and oxidase activation; (b) the finding that the oxidase is associated with the cytoskeleton (see above); and (c) prior work showing that some neutrophil phosphoproteins are situated in the cytoskeleton (8, 9), we felt that it would be of interest to examine the relationship between the oxidase-related 47K phosphoprotein family and the neutrophil cytoskeleton. We therefore loaded neutrophils with ³²Pᵢ, activated them with PMA, then prepared extracts and cytoskeleton fractions that were analyzed for phosphoprotein composition by two-dimensional gel electrophoresis followed by autoradiography. The results showed a PMA-dependent increase in the labelling of four components of the p47^phox family: p47^phox/6.8, p47^phox/7.3, p47^phox/7.8, and p47^phox/8.6 (the members of the p47^phox family are designated by terminology based on their M_r and pI values [6]) (Fig. 3, A, C [arrows]). These p47^phox components were present exclusively in the detergent-soluble extract (Fig. 3, A-D). Their distribution was not altered by incubating the cells for 10 min at 37° with 10 μM dihydrocytochalasin B before adding the activating agent (data not shown). The cytoskeleton too contained a group of $M_r \approx$ 47K phosphoproteins with pI values in the neutral range, but these were not members of the p47^phox family: their labelling did not increase with activation, and they were present in p47^phox deficient cytoskeletons (Fig. 3, E-J) (6, 7, 26, 29).

Relationship between the cytoskeleton and the cytosolic oxidase components p47^phox and p67^phox as evaluated by immunoblotting. Confirmation that the cytoskeleton was essentially

Table III. Distribution of Cytochrome b₅₅₈ between the Cytoskeleton and the Detergent-soluble Extract of Resting and Activated Human Neutrophils

Sample	Cytochrome b₅₅₈			
	Experiment 1		Experiment 2	
	Resting	Activated	Resting	Activated
	pmol/10⁷ cell equivalent			
Whole neutrophil suspension	81.6	63.2	89.6	79.2
Initial extract	84.9	75.0	75.5	82.1
Residual extract	ND	ND	ND	0.9
Cytoskeleton	11.7	12.6	10.2	16.0
	Percentage of total			
Cytoskeleton	12.1	14.3	11.9	16.3

Experiments were conducted as described in Methods using two different neutrophil preparations. Total recoveries of cytochrome b₅₅₈ in the subcellular fractions averaged 111% and 131% for resting and activated cells, respectively. ND, not detected.
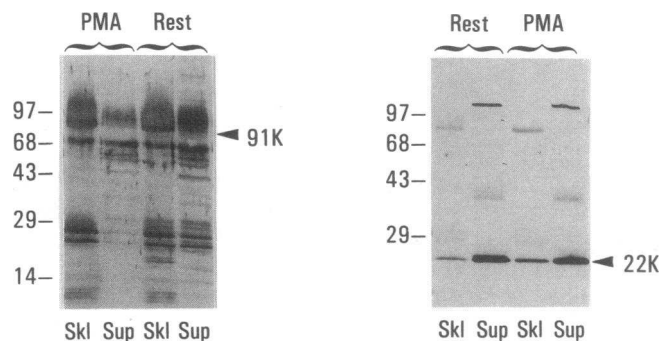

Figure 2. Association of the 91K (left) and 22K (right) subunits of cytochrome b₅₅₈ with the neutrophil cytoskeleton as determined by immunoblotting. Each track contained 10⁷ cell equivalent of cytoskeleton (skl) or detergent extract (sup) from resting (rest) or PMA-activated (PMA) normal human neutrophils. Fractions were prepared and immunoblotting was performed as described in Methods. Similar results were obtained in a second experiment using neutrophils from a different subject. The proportions of cytochrome b₅₅₈ associated with the cytoskeletons from resting and activated neutrophils were 10.2±2.8 and 16.0±3.0% (mean±SD), respectively, as determined from scans of the autoradiograms of the 22K subunits (Zeineh 10/2D laser scanner).
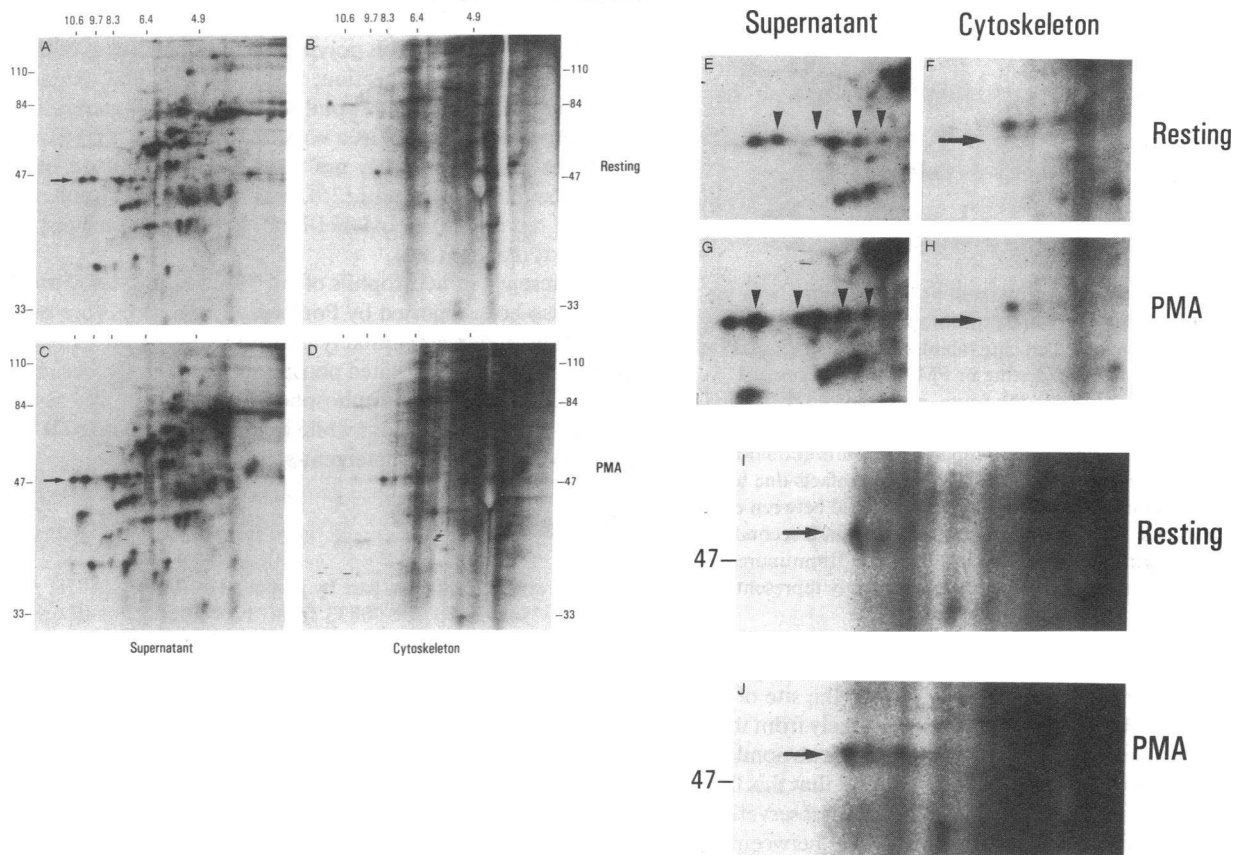
Figure 3. The phosphorylated p47^{phox} components of the respiratory burst oxidase in normal ^{32}P$_i$-loaded human neutrophils are restricted to the detergent-extractable fractions. (*A-D*) autoradiograms of complete two-dimensional gels of cytoskeletal and detergent-soluble proteins from ^{32}P$_i$-loaded neutrophils. The ^{32}P-labelled p47^{phox} family members in the detergent-extractable fractions (*supernatant*) are indicated by the arrows. (*E-H*), details of the areas showing the p47^{phox} family. (*Arrowheads*) Locations of the p47^{phox} family members in the detergent-extractable fractions. (*Arrow*) Corresponding regions in the cytoskeletal fractions. (*I-J*) Cytoskeletal fractions from resting and activated neutrophils from a p47^{phox}-deficient individual showing the same ≈ 47K phosphorylated proteins present in cytoskeletons from normal subjects.

The experiment was carried out as described in the text. Similar results were obtained in a second experiment using neutrophils from a different normal subject.

free of p47^{phox} was obtained by immunoblotting. In preparations obtained from both resting and activated neutrophils, immunoreactive p47^{phox} was restricted almost entirely to the detergent extract (Fig. 4); comparison with standards (shown in the two right-hand tracks of the blot) suggests that the cytoskeleton contained < 5% of the total amount of p47^{phox} in the cell. These observations further indicate that to the extent that the cells contained unphosphorylated p47^{phox}, this unphosphorylated material was also separate from the cytoskeleton.

A 67K protein, p67^{phox}, has been identified as a fourth polypeptide component of the respiratory burst oxidase (30). Immunoblots with an antibody that recognizes this polypeptide have shown that in both resting and activated neutrophils, almost all the detectable p67^{phox} is associated with the cytoskeleton (Fig. 5). The fraction of p67^{phox} associated with the cytoskeleton does not seem to depend in a major way on the state of activation of the cell.

Discussion

As discussed in the introduction, the finding that the activity of the respiratory burst oxidase is related to the neutrophil cytoskeleton has been anticipated by earlier work from other

groups.[2] We have now found that the respiratory burst oxidase appears to be attached to the cytoskeleton, as are three of the four oxidase polypeptides discovered through studies with neutrophils from patients with CGD (31, 32). These findings constitute perhaps the most straightforward evidence to date in favor of a role for the cytoskeleton in the respiratory burst. The interaction between the oxidase and the cytoskeleton is probably the factor that allows the oxidase to be activated locally rather than globally in response to particulate stimuli (33). Other functions of the cytoskeleton-oxidase interaction, including a possible role in priming and deactivation, remain to be determined.

These experiments show that in resting neutrophils ~ 15% of the cytochrome b$_{558}$ is found in the cytoskeleton. This figure does not change during the activation of the oxidase, indicating that oxidase activation is not accompanied by the transfer of

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2. While this manuscript was under review, Quinn et al. reported the association of oxidase activity and cytochrome b$_{558}$ with the membrane cytoskeleton of human neutrophils (31). These investigators found that all the oxidase activity was associated with the membrane cytoskeleton, and that cytochrome b$_{558}$ was distributed evenly between the membrane cytoskeleton and the detergent extract.
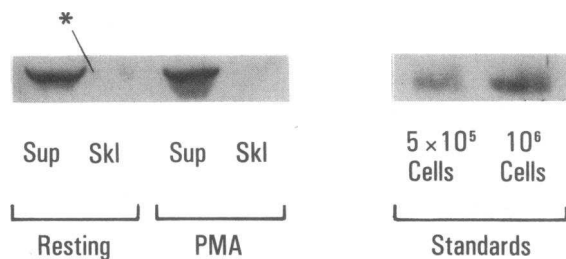
Figure 4. The cytosolic oxidase component p47^{phox} is absent from the neutrophil cytoskeleton as determined by immunoblotting. The four left-hand tracks contained 10^7 cell equivalent of detergent extract (*sup*) or cytoskeleton (*skl*) from resting or PMA-activated normal human neutrophils as indicated in the figure, while the two right-hand tracks contained 5×10^5 and 10^6 cell equivalents of resting detergent extract, respectively. Fractions were prepared and immunoblotting was performed as described in Methods. To avoid artifacts due to leakage from adjacent tracks, an empty track was placed between each pair of sample tracks. Similar results were obtained in a second experiment using neutrophils from a different donor. *Immunoreactive p47^{phox} in an empty track. This material probably represents a leak from the far left track.

cytochrome b$_{558}$ from other parts of the cell to the site of the active enzyme (i.e., the cytoskeleton). It seems likely from these observations that only a small fraction of the neutrophil cytochrome b$_{558}$ is associated with the oxidase, and that this fraction is already in place in the resting cell. These observations and the known distribution of cytochrome b$_{558}$ between the plasma membrane ($\approx$ 15%) and the specific granules ($\approx$ 85%) suggest that it is the cytochrome in the plasma membranes that is related to the oxidase. These considerations raise a question as to what the function of the specific granule cytochrome b$_{558}$ might be.

The foregoing results also indicate that whereas the O$_2^-$-forming activity from activated neutrophils is restricted almost entirely to the cytoskeleton, the p47^{phox} family is found exclusively in the detergent-soluble supernatant. O$_2^-$ production in the cytoskeleton therefore takes place in the absence of p47^{phox}. It appears that p47^{phox}, while necessary for the activation of the respiratory burst oxidase, is not a component of the enzyme
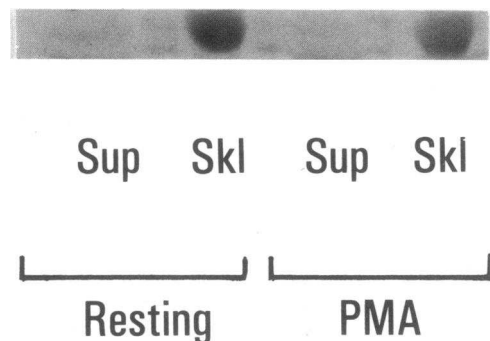

Figure 5. Association of p67^{phox} with the neutrophil cytoskeleton as determined by immunoblotting. Each track contained 5×10^6 cell equivalent of detergent extract (*sup*) or cytoskeleton (*skl*) from resting or PMA-activated normal human neutrophils. Fractions were prepared and immunoblotting was performed as described in Methods. Similar results were obtained in a second experiment using neutrophils from a different donor.

per se. Conversely, the cytoskeletal location of p67^{phox} is compatible with the idea that this polypeptide is a constituent of the oxidase itself. In this connection, it is of interest that oxidase purified from activated neutrophil membranes (34) contained a 67K component, as well as a $\approx$ 48K component that from the present work is probably not p47^{phox} but may be an oxidase-associated flavoprotein (35), and a $\approx$ 32K component that could represent the NADPH-binding subunit of the respiratory burst oxidase (36).

The presence in neutrophils of a 47K phosphorylated protein has also been reported by Pontremoli et al. (8). From our observations, it appears unlikely that this phosphoprotein is a member of the oxidase-related phosphoprotein family, because in their experiments the unphosphorylated protein was associated with the cytoskeleton, while in our experiments p47^{phox} was always found in the detergent-soluble fraction.

Acknowledgments

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