The p67-*phox* Cytosolic Peptide of the Respiratory Burst Oxidase from Human Neutrophils

Functional Aspects

Naoki Okamura, Bernard M. Babior, Laura A. Mayo, Paola Peveri, Robert M. Smith, and John T. Curnutte
Department of Molecular and Experimental Medicine, Research Institute of Scripps Clinic, La Jolla, California 92037

Abstract

Most cases of cytosol-defective chronic granulomatous disease are due to the deficiency of a 47-kD protein (p47-*phox*) whose phosphorylation normally accompanies the activation of the respiratory burst oxidase. Recently, a form of chronic granulomatous disease was described in which the failure of O_2^- production was associated with the absence of a 67-kD polypeptide (p67-*phox*) from the cytosol of affected neutrophils. Using neutrophils obtained from a patient with this form of the disease, we examined the function of p67-*phox* in the activation of the oxidase. Our studies showed that in whole p67-*phox*–deficient neutrophils, p47-*phox* was phosphorylated in a normal fashion. In the cell-free oxidase-activating system, the ability of the p67-*phox*–deficient cytosol to support oxidase activation was partly restored by the addition of p47-*phox*–deficient cytosol; the p67-*phox*–deficient cytosol, however, was not complemented by cytosol inactivated with NADPH dialdehyde, an affinity label previously found to block the NADPH-binding component of the oxidase. Despite these differences, the kinetic properties of the p67-*phox*–deficient cytosol closely resembled those of the p47-*phox*–deficient cytosol. Taken together with earlier findings, these results suggest that (*a*) in the neutrophil cytosol, p67-*phox* is at least partly complexed to p47-*phox*; (*b*) it is in the form of this complex that p67-*phox* participates in oxidase activation; and (*c*) p47-*phox* appears to be translocated from the cytosol to the plasma membrane during oxidase activation, but complexation to p67-*phox* is not necessary for this translocation, nor for the accompanying extra protein phosphorylation. (*J. Clin. Invest.* 1990. 85:1583–1587.) neutrophil • superoxide • chronic granulomatous disease • respiratory burst oxidase

Introduction

The respiratory burst oxidase is an enzyme found in the membranes of professional phagocytes (neutrophils, eosinophils, monocytes, and macrophages) that catalyzes the one-electron reduction of oxygen to O_2^- at the expense of NADPH: $2\ O_2 + NADPH \rightarrow 2\ O_2^- + NADP^+ + H^+$. The enzyme is dormant in resting phagocytes, but is activated when the cells are exposed to appropriate stimuli. The O_2^- produced by the oxidase serves as the precursor of a large group of highly reactive oxidants that are used by the phagocytes for the destruction of invading microorganisms (1–4).

In homogenates of activated neutrophils, the O_2^--forming activity resides entirely in the plasma membrane (5). Studies with cell-free preparations from resting neutrophils, however, have shown that the activation of the oxidase is a complex process requiring components not only from the plasma membrane, but from the cytosol as well (6–11). These cytosolic components include a 47-kD protein (p47-*phox*)[1] that undergoes phosphorylation during oxidase activation (12–19), 67-kD peptide (p67-*phox*) of unknown function (17), a protein of unknown size that appears to contain the NADPH-binding site of the oxidase (this NADPH-binding protein is different from p47-*phox* [20]; whether or not it is identical to p67-*phox* is not yet known) (21, 22), and perhaps additional components yet to be characterized (23–25). Some of these components appear to exist in the cytosol in the form of a complex (7, 20). It is likely that during activation, the oxidase is assembled by the transfer of one or more of these cytosolic components to the plasma membrane.

Chronic granulomatous disease (CGD) is an inherited disorder in which phagocytes are unable to make O_2^- because of a defect in one of the components of the respiratory burst oxidase or its activating system (1, 11, 26). Some time ago, it was shown that one type of CGD was associated with a defect in p47-*phox*. In this type of CGD, the expected phosphorylation of p47-*phox* did not take place when ^{32}P-loaded neutrophils were activated with phorbol myristate acetate (13, 15), and p47-*phox* was not detected on Western blots of cytosol from affected neutrophils (17, 27). For several years, this was the only cytosolic abnormality known to be associated with CGD. Quite recently, however, patients have been found whose CGD is associated with a second cytosolic abnormality (17, 24, 25, 27). The abnormality in these patients involves p67-*phox* (17, 27). We have studied neutrophils from one of these patients to obtain information as to how p67-*phox* might participate in the operation of the respiratory burst oxidase.

Methods

Cytochrome *c* (horse heart, type VI), bovine erythrocyte superoxide dismutase, NADPH, protease inhibitors (EDTA, diisopropyl phos-

Dr. Okamura's present address is Department of Biochemistry, Hiroshima National University, Hiroshima, Japan.

Dr. Smith's present address is Department of Medicine, USCD School of Medicine, San Diego, CA.

Address reprint requests to Dr. J. T. Curnutte, Department of Molecular and Experimental Medicine, Research Institute of Scripps Clinic, La Jolla, CA 92037.

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1. *Abbreviations used in this paper:* CGD, chronic granulomatous disease; *phox*, refers to phagocyte oxidase and is used to designate protein components of the respiratory burst oxidase of phagocytes.

phofluoridate, leupeptin, and pepstatin), phorbol myristate acetate (PMA), EGTA, Pipes, glycerol, sucrose, and dextran (Mr 78,000) were obtained from Sigma Chemical Co., St. Louis, MO. SDS was purchased from Bio-Rad Laboratories, Richmond, CA. Sodium deoxycholate (Ultrol[R] grade) was obtained from Calbiochem-Behring Corp., La Jolla, CA. ^{32}P-labeled phosphoric acid (carrier-free, in 0.02 M HCl) was obtained from ICN Radiochemicals, Irvine, CA. Ficoll-Paque and Percoll were purchased from Pharmacia Fine Chemicals, Piscataway, NJ. Other reagents were the best grade commercially available and were used without further purification.

Methods. Neutrophils were obtained by dextran sedimentation of whole blood followed by hypotonic lysis and Ficoll-Hypaque centrifugation (28) or by leukapheresis (29) followed by diisopropyl phosphofluoridate treatment (30), as previously described. Informed consent was granted by all subjects before neutrophils were procured. Cytosol and solubilized membranes were prepared from resting human neutrophils by a published modification (7, 31) of the method of Borregaard et al. (32). Cytosol obtained from normal and CGD donors contained similar amounts of protein. Phosphorylation of p47-*phox* in intact neutrophils was determined as described by Okamura et al. (15). Complementation studies with cytosols from CGD neutrophils, and experiments to ascertain the effect of these cytosols on the activation of the respiratory burst oxidase in cell-free preparations from resting neutrophils were conducted as described elsewhere (24, 29). The first order rate constant k for the slow step of oxidase activation (a reflection of the lag during which O_2^- production gradually accelerates) was calculated by computer as previously described (23) as was the maximal rate of O_2^- production ($V\infty$, previously referred to as V [23]).

Results

A cytosolic abnormality in a population of CGD neutrophils is detected as a failure of cytosol from those neutrophils to support the activation of the respiratory burst oxidase in a cell-free system containing the cytosol in question, plasma membranes from normal resting neutrophils, Mg^{2+} as an essential cation, and a suitable anionic detergent (usually arachidonate or SDS) as the activating agent (11). It has recently been found that patients with cytosol-defective CGD could be divided into two complementation groups according to the ability of pairwise combinations of the defective cytosols to support oxidase activation in the cell-free system (24, 25). About 34% of all patients with CGD fall into one of these groups, while $\approx$ 4% fall into the other (26, 27). These two complementation groups have been further defined by the observation that p47-*phox* is lacking in neutrophil cytosols from the more common of these two groups, whereas cytosols from the rare group are missing p67-*phox* (27).

The patient we have studied (N.S., previously reported elsewhere [11]) is a member of the rare complementation group. This was demonstrated by the finding that this patient's cytosol partly corrected the functional defect in a cytosol obtained from a CGD patient known to lack p47-*phox*. The results in Table I show that while the amounts of O_2^- produced by cell-free activating systems containing either p47-*phox*–deficient or N.S. cytosol were negligible, a system containing a mixture of the two defective cytosols generated O_2^- at a brisk rate demonstrating complementation. Confirmation was later obtained with an antiserum that recognizes both p47-*phox* and p67-*phox;* Western blots prepared using this antiserum showed that N.S. cytosol contained normal amounts of p47-*phox* but was devoid of detectable p67-*phox* (reference 27, patient 9). Of note in these complementation studies is that the rates of O_2^- production seen with the combination of deficient cytosols

Table I. O_2^- Production by Cell-free Systems Containing Cytosol Inactivated with NADPH Dialdehyde and Cytosols from Patients with p47-phox Deficiency and p67-phox Deficiency, Used Individually and in Pairwise Combinations

	Cytosol					
Expt	Normal	p67-*phox*–deficient	p47-*phox*–deficient	p67-*phox*–deficient plus pp47-deficient	Dialdehyde-treated	p67-*phox*–deficient plus dialdehyde-treated
	nmol O_2^-/min per 10^7 cell eq membrane					
1	54	1.3	1.9	17.8	6.6	4.5
2	31	0.7	1.3	11.0	—	—
3	53	0.2	3.0	25.1	4.1	5.4

Experiments were carried out as described in Methods. Incubations contained the cytosols indicated, 2×10^7 cell eq when used individually (except for normal cytosol, which was used at 1×10^7 cell eq/reaction mixture) and 1×10^7 cell eq each, when used pairwise. The total volume of each reaction mixture was 0.75 ml.

were less than half those seen with a system containing a similar quantity of normal cytosol alone. Extending the preincubation of the two deficient cytosols from 3 to 30 min did not result in any further enhancement of O_2^- production.

Similar experiments were carried out to examine the effect of N.S. cytosol on normal cytosol whose ability to support oxidase activation in the cell-free system had been abolished by treatment with NADPH dialdehyde plus $NaCNBH_3$ (21). In Table I, it is seen that the rate of O_2^- production when dialdehyde-treated and N.S. cytosol are combined was less than the rate of O_2^- production when an equivalent amount of dialdehyde-treated cytosol is used alone (the use of double volumes of individual cytosols in the control incubations is necessitated by the high-order dependence of oxidase yield on cytosol concentration [20, 23, 29]). Therefore, as opposed to its ability to complement p47-*phox*–deficient cytosol, N.S. cytosol appeared to have no discernable effect on dialdehyde-inactivated cytosol. This finding is in contrast to the recently reported ability of p47-*phox*–deficient cytosol to restore activity to dialdehyde-treated cytosol (20).

Earlier studies have shown that in the cell-free oxidase activating system, O_2^--forming activity is generated according to strict first-order kinetics (23). The rate constant for the first-order oxidase activation reaction was independent of the cytosol concentration, but the total amount of active oxidase generated in the course of the activation reaction (i.e., the oxidase yield) was found to be proportional to $[\text{cytosol}]^{2.5}$. This result indicated that the cytosol must contain at least three kinetically independent components that participate in the formation of the respiratory burst oxidase. When oxidase was activated at varying concentrations of normal cytosol in the presence of a large fixed excess of cytosol from p47-*phox*–deficient neutrophils, however, the oxidase yield was found to be no longer proportional to $[\text{cytosol}]^{2.5}$, but instead was directly proportional to [cytosol]. This finding suggested that the p47-*phox*–deficient cytosol contained all but one of the kinetically independent components. The results of a similar experiment carried out with p67-*phox*–deficient cytosol are shown in Fig. 1. As with p47-*phox*–deficient cytosol, the presence of excess

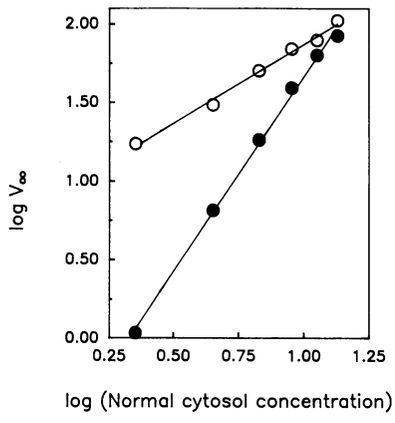

Figure 1. The relationship between the extent of oxidase activation and the concentrations of normal and p67-*phox*–deficient cytosol. Incubations were carried out as described in Methods, using membranes at 3.12×10^6 cell eq/reaction mixture (0.75 ml total volume) and various concentrations of normal cytosol as shown, in the presence of 1.3×10^7 cell eq/reaction mixture of CGD cytosol, or an equal volume of relaxation buffer. Results with normal cytosol in the presence of buffer (*closed symbols*) are representative of data obtained with cytosols prepared from neutrophils from three different normal subjects. Results in the presence of excess p67-*phox*–deficient cytosol (*open symbols*) are representative of three separate determinations, two with one preparation of cytosol and one with another. Nonlinear regression analysis yielded a slope on the log–log plot of 2.56±0.07 for the experiments where normal cytosol was used alone. In this type of plot, the slope is equivalent to the exponent of the relationship $V\infty = [\text{cytosol}]^x$. In contrast, when normal cytosol was assayed with p67-*phox* cytosol, the slope was 0.99±0.03.

p67-*phox*–deficient cytosol in the cell-free activation system altered the dependence of oxidase yield on cytosol concentration from 2.5 order to first order. It appears then that, like p47-*phox*–deficient cytosol, p67-*phox*–deficient cytosol lacks one kinetically independent oxidase component.

Kinetic studies have suggested a minimum oxidase activation sequence consisting of two stages: (*a*) an early stage that precedes the addition of activating agent, during which components from the cytosol combine reversibly with membrane-bound elements to form a primary activation complex; and (*b*) a late stage characterized by a slow step during which the primary activation complex is irreversibly converted to the catalytically active respiratory burst oxidase. The rate of this slow step is independent of cytosol concentration (23), suggesting that cytosolic components outside of those needed to form the primary activation complex have no effect on the slow step.

The slow activation of the oxidase is reflected in a characteristic lag in O_2^- production seen with the cell-free oxidase-activating system. If the activating detergent is added to a cell-free system containing normal cytosol in the absence of only substrate and the activation reaction is allowed to proceed for 3–4 min before starting O_2^- formation with the addition of NADPH, no lag will be seen because under these conditions the oxidase will be almost fully activated before the NADPH is added. If, however, the activation is performed with a cytosol deficient in a component necessary for the formation of the primary activation complex (that precedes the slow activation step), the lag will not be eliminated by preincubation. (In this type of experiment, normal cytosol has to be added along with NADPH in order to supply all the cytosolic factors necessary for full oxidase activity.) Using this approach with neutrophil cytosol from two CGD patients in the p47-*phox* deficiency complementation group, we showed previously that p47-*phox* is a component needed for the primary activation complex (29). We used the same approach to ascertain whether p67-*phox* was also required for the primary activation complex. The results (Table II) showed that preincubating a cell-free system containing p67-*phox*–deficient neutrophil cytosol with activating detergent had little effect on the lag compared with the typical lag observed when normal cytosol is preincubated without detergent (e.g., rate constants of 0.94 and 0.98 min^{-1}, respectively, in Experiment 1). In contrast, the lag was almost totally abolished by a similar preincubation of a cell-free system containing normal cytosol and detergent (as evidenced by the exceedingly high rate constants > 100 min^{-1}). Including p67-*phox*–deficient cytosol with the normal cytosol did not interfere with the abolition of the lag. It therefore appears that, like p47-*phox*, p67-*phox* is required for the formation of the primary activation complex.

A characteristic abnormality of the cytosol-defective CGD neutrophils of the p47-*phox* deficiency complementation group is their failure to phosphorylate a family of proteins upon stimulation with PMA (13, 15). Because of the close functional relationship between p47-*phox* and p67, as demonstrated by their participation in the formation of the primary activation complex, it was of interest to determine whether a defect in phosphorylation was also seen in the p67-*phox*–deficient neutrophils. Fig. 2 shows the p47-*phox* phosphorylation patterns in resting and phorbol-activated neutrophils from the p67-*phox*–deficient patient, a normal control, and a patient with p47-*phox*–deficient CGD. A comparison of these patterns shows that in the p67-*phox*–deficient neutrophils, all isoforms of p47-*phox* appear to be present.

Table II. Failure of Cytosol from p67-phox–deficient Neutrophils to Eliminate the Lag in O_2^- Production Caused by the Slow Activation of the Respiratory Burst Oxidase in the Cell-free Oxidase-activating System

	k		*V*∞	
Cytosol present in preincubation	Expt 1	Expt 2	Expt 1	Expt 2
	min^{-1}		*nmol O_2^-/min per 10^7 cell eq. memb.*	
Normal	>100	>100	64.9	45.9
p67-*phox*–deficient	0.94	0.72	23.1	21.9
p67-*phox*–deficient plus normal	>100	>100	60.1	55.3
Normal (SDS omitted until addition of NADPH)	0.98	0.73	46.6	42.0

Reaction mixtures containing test cytosol were preincubated for 3 min at 25°C with SDS, the activating detergent. O_2^- production was then initiated with NADPH, and the reactions were followed spectrophotometrically at 550 nm. The incubation initially containing p67-*phox*–deficient cytosol (55 μl) received 55 μl normal cytosol along with NADPH; 55 μl buffer was added along with NADPH to those incubations that initially contained either normal cytosol (55 μl) or a mixture of normal and p67-*phox*–deficient cytosols (55 μl each). The total volume of each reaction mixture was 0.375 ml. Further experimental details are given in Methods. To quantify the lag, the first order rate constant *k* was calculated by computer as described in Methods as was the maximal rate of O_2^- production ($V\infty$, previously referred to as *V*[23]).

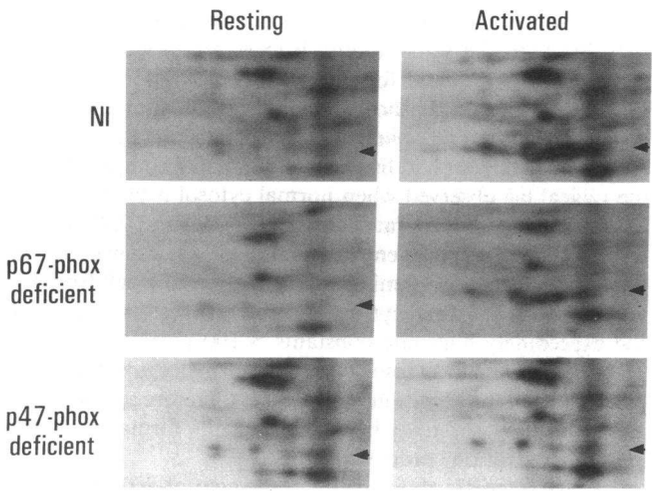

Figure 2. Phosphorylation of p47-*phox* by p67-*phox*–deficient, p47-*phox*–deficient, and normal neutrophils activated with PMA. Phosphorylation of p47-*phox* during activation of the respiratory burst was carried out in intact neutrophils as previously described (15, 16). Whole cell lysates were examined by two-dimensional electrophoresis and autoradiography for the presence of members of the p47-*phox* family. The corresponding regions are shown from autoradiographs obtained from normal, p47-*phox*–deficient or N.S. (p67-*phox*–deficient) neutrophils; the results are shown from studies performed on resting cells and on cells activated by exposure to PMA. The position of p47-*phox* is indicated by the arrows; the more basic region of each gel is to the left.

Discussion

Previous studies have suggested that the activation of the respiratory burst oxidase in the cell-free system (and presumably in the intact neutrophil as well) begins with the binding of a group of cytosolic components to a specific site on the plasma membrane (possibly the 91-kD subunit of cytochrome b_{558} (16, 18, 33)) to form the primary activation complex. Since the p67-*phox*–deficient cytosol fails to abolish the lag, p67-*phox* probably participates as a component of this activation complex. The cytosolic protein p47-*phox* is also included among these early components (29), and there may be others yet to be identified. At some point in the activation sequence, the NADPH-binding component of the oxidase, located in the cytosol of neutrophils when they are at rest (21), must be transferred to the plasma membrane to assemble the active enzyme.

In the intact cell, though probably not in the cell-free oxidase-activating system (7), the binding of the early cytosolic components to the membrane is accompanied by the further phosphorylation of an already partially phosphorylated p47-*phox* (13, 15, 34). The finding that the phosphorylation of p47-*phox* by p67-*phox*–deficient neutrophils is normal suggests that p67-*phox* is not needed for either the initial partial phosphorylation of p47 or for the extra protein phosphorylation that accompanies translocation to the membrane. By inference, it therefore appears that p47-*phox* can translocate to a membrane-docking site independent of p67-*phox*. It is possible that p47-*phox* attaches directly to the plasma membrane–docking site, or that it is part of a complex that can form in the absence of p67-*phox* and attach to the plasma membrane–docking site through a binding region remote from p67-*phox* and possibly from p47-*phox* as well. These results show that at least some of the steps involved in the activation of the respiratory burst oxidase can take place in the absence of p67-*phox*.

As to the question of a complex, lines of evidence supporting the existence of such an entity are beginning to accumulate. In aggregate, the evidence suggests that such a complex would have to contain p47-*phox* and p67-*phox*, as well as the NADPH-binding component (if it is not identical to either of the foregoing). Included among these lines of evidence are reports from several groups that the fractionation of neutrophil cytosol on a gel filtration column yields a relatively large component ($\approx$ 240 kD [7]) that by itself is able to support oxidase activation in the cell-free system. We have reported the inability of the combinations of p47-*phox* and p67-*phox*, p47-*phox* and NADPH dialdehyde–inactivated cytosol (20), and NADPH dialdehyde–inactivated cytosol and p67-*phox*–deficient cytosol (this study) to generate as much oxidase activity as is generated by one half as much normal cytosol (by protein content or cell-equivalents). These results can be explained by the occurrence of all three components in a complex into which exogenous components fail to exchange, or exchange only poorly. The postulation of such a complex provides an alternative to the straightforward notion that the complementation results obtained with the NADPH dialdehyde–treated cytosol mean that p67-*phox* is the NADPH-binding component.

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