

Neutrophil Nicotinamide Adenine Dinucleotide Phosphate Oxidase Assembly Translocation of p47-phox and p67-phox Requires Interaction between p47-phox and Cytochrome b₅₅₈

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Abstract

Two of the cytosolic NADPH oxidase components, p47-phox and p67-phox, translocate to the plasma membrane in normal neutrophils stimulated with phorbol myristate acetate (PMA). We have now studied the translocation process in neutrophils of patients with chronic granulomatous disease (CGD), an inherited syndrome in which the oxidase system fails to produce superoxide due to lesions affecting any one of its four known components: the gp91-phox and p22-phox subunits of cytochrome b₅₅₈ (the membrane-bound terminal electron transporter of the oxidase), p47-phox, and p67-phox. In contrast to normal cells, neither p47-phox nor p67-phox translocated to the membrane in PMA-stimulated CGD neutrophils which lack cytochrome b₅₅₈. In one patient with a rare X-linked form of CGD caused by a Pro → His substitution in gp91-phox, but whose neutrophils have normal levels of this mutant cytochrome b₅₅₈, translocation was normal. In two patients with p47-phox deficiency, p67-phox failed to translocate, whereas p47-phox was detected in the particulate fraction of PMA-stimulated neutrophils from two patients deficient in p67-phox. Our data suggest that cytochrome b₅₅₈ or a closely linked factor provides an essential membrane docking site for the cytosolic oxidase components and that it is p47-phox that mediates the assembly of these components on the membrane. (*J. Clin. Invest.* 1991. 87:352–356.) Key words: respiratory burst • chronic granulomatous disease • superoxide • protein phosphorylation • plasma membrane

Introduction

The respiratory burst oxidase of neutrophils and other phagocytic cells is a multicomponent electron transport system that catalyzes the NADPH-dependent, one-electron reduction of oxygen to produce superoxide (O₂⁻)¹. The system is dormant in

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1. Abbreviations used in this paper: CGD, chronic granulomatous disease; O₂⁻, superoxide.

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resting cells but can be activated by several particulate and soluble stimuli. The dormant oxidase consists of both cytosolic and membrane-bound components, but the active, O₂⁻-generating complex is confined to the plasma membrane (1, 2). This implies that the cytosolic components must either act in a signaling capacity by modifying the membrane components, or that they must become directly associated with the membrane via a translocation process.

In the inherited syndrome chronic granulomatous disease (CGD), phagocytes fail to generate a respiratory burst and produce O₂⁻ (1). The resulting microbicidal defect renders these patients highly susceptible to bacterial and fungal infections. CGD is genetically heterogeneous, resulting from lesions affecting any one of at least four distinct oxidase components (3). In the most common form a genetic modification of the X-chromosome results in the absence of the 91-kD transmembrane glycoprotein subunit of cytochrome b₅₅₈ (termed gp91-phox, for phagocyte oxidase), the terminal electron transporter of the oxidase (4, 5). There is also a secondary loss of the 22-kD cytochrome subunit (p22-phox) (6, 7). In a much rarer autosomal recessive form of CGD, the primary mutation involves the gene encoding this smaller subunit, with an associated loss of gp91-phox (8, 9). These two forms of CGD are therefore characterized by a total absence of cytochrome b₅₅₈. In a rare variant form of X-linked CGD, the neutrophils contain a cytochrome b₅₅₈ that appears quantitatively and spectrally normal, but nonetheless fail to produce O₂⁻ (10). In one family with this form of the disease, the defect has been identified as a missense mutation in the gene encoding the 91-kD subunit, resulting in a nonconservative Pro → His substitution in what is thought to be a junction region between the membrane and cytosolic domains of the protein (11). In the majority of autosomal recessive CGD patients the levels of cytochrome b₅₅₈ are normal. The genetic defects in these cases involve either of the two known cytosolic oxidase components, p47-phox or less commonly p67-phox (12–14).

In intact phagocytic cells a 47-kD protein is phosphorylated at multiple sites in association with activation of the respiratory burst (15–17), and it has recently been confirmed that this phosphoprotein is p47-phox (18–20). Phosphorylated p47-phox has a dual localization in the cytosol and membrane fractions of neutrophils activated with phorbol esters (15, 21, 22). Association of the phosphoprotein with the membrane appears to depend on the presence of cytochrome b₅₅₈, since phosphorylated p47-phox was confined to the cytosol in cytochrome-deficient CGD neutrophils (22). Although these findings strongly supported the idea that translocation of cytosolic oxidase components to the membrane was involved in the activation pro-

cess, they did not rule out the possibility that the dual localization in the activated state represented two pools of p47-phox.

Recently it was conclusively demonstrated that both p47-phox and p67-phox translocate to the membrane during activation of the respiratory burst in intact neutrophils (23). A polyclonal antiserum, B-1, which recognizes both components, was used to probe electrophoretic blots of proteins from neutrophil soluble and particulate fractions separated by SDS-PAGE. The two proteins were detected in the particulate fraction of activated, but not resting neutrophils. Analytical subcellular fractionation revealed that they were present in the plasma membrane fraction but not in fractions enriched in either specific or azurophilic granules (23). In an attempt to understand more fully the interactions among the cytosolic oxidase components and their roles in oxidase assembly, we have studied the translocation process in neutrophils from patients with different genetic forms of CGD.

Methods

Preparation of neutrophils. Blood was collected by venipuncture from normal subjects and patients with CGD after obtaining informed consent. Neutrophils were purified by dextran sedimentation, hypotonic lysis of erythrocytes, and centrifugation through Ficoll-Hypaque (24). After pretreatment with 2 mM diisopropylfluorophosphate for 20 min at 4°C and at a density of 5×10^7 cells/ml, the neutrophils were washed and resuspended in phosphate-buffered saline with glucose (2.7 mM KCl, 138 mM NaCl, 7.5 mM D-glucose, 8.1 mM Na₂HPO₄, 1.47 mM KH₂PO₄, pH 7.3) at the same cell density. All materials were obtained from the sources previously cited (24).

Neutrophil activation and fractionation. Neutrophils (3×10^7 cells in 0.6 ml) were preincubated at 37°C for 5 min, then either activated with PMA (100 ng/ml) or treated with an equal volume (1.5 µl) of dimethylsulfoxide, and after 5 min transferred to an ice bath. Subsequent steps are modifications of the method of Clark et al. (23), and were all performed at 4°C. The cells were pelleted by centrifugation for 6 min at 300 g, resuspended in 0.6 ml relaxation buffer (100 mM KCl, 3 mM NaCl, 3.5 mM MgCl₂, 1.25 mM EGTA, 10 mM piperazine diethane sulfonic acid, pH 7.3), and disrupted by a single 5-s burst of sonication, using a microprobe sonicator at low power. This resulted in > 95% breakage. Unbroken cells and nuclei were removed by centrifugation for 5 min at 250 g. The resulting supernatant (S1) was centrifuged for 20 min at 115,000 g in the TLA 100.2 rotor of a Beckman TL 100 ultracentrifuge (Beckman Instruments, Inc., Palo Alto, CA). The high speed supernatant (S2), representing the cytosolic fraction, was removed, and the pellet washed with a further 0.6 ml of relaxation buffer and recentrifuged at 115,000 g for 20 min. The final pellet (P3), referred to here as the membrane fraction, was resuspended in 0.2 ml of relaxation buffer and dispersed with a 2–3-s burst of sonication. In each experiment, neutrophils from normal control subjects were prepared concurrently with those from the patients to allow direct comparison of the immunoblots. The protein content of the fractions was measured using the Pierce bicinchoninic acid assay (Pierce Chemical Co., Rockford, IL).

Neutrophils from a patient with the rare X-linked cytochrome b-positive form of CGD were fractionated using a slightly modified procedure. After activation the cells (5×10^7 in 1 ml) were centrifuged for 3 min at 3,600 g, resuspended in 0.5 ml relaxation buffer, and disrupted with 15–20 0.5-s bursts of sonication. The sonicate was centrifuged for 5 min at 400 g to remove unbroken cells and the supernatant diluted to 16 ml with relaxation buffer. After centrifugation for 20 min at 150,000 g and without further washing, the pellet was resuspended in 0.5 ml relaxation buffer.

Electrophoresis and immunoblotting. Soluble and particulate fractions from resting and activated neutrophils were subjected to SDS-

PAGE on 9% acrylamide gels. The proteins were electrophoretically transferred to nitrocellulose and the blots probed with B-1, a polyclonal antiserum that recognizes p47-phox and p67-phox. Immunoreactive proteins were detected with ¹²⁵I-protein A and autoradiography and quantitated, where indicated, by densitometry and calculation of the area under the curve. These procedures have been described elsewhere (13, 23).

Results

The autoradiographs in Fig. 1 are from CGD neutrophils devoid of cytochrome b₅₅₈, aligned with their concurrent normal controls. The top four sets of panels are from patients with X-linked cytochrome-negative CGD (gp91-phox mutation), while the bottom set is from a patient with the much rarer autosomal recessive cytochrome-negative form (p22-phox mutation). In both forms the levels of p47-phox and p67-phox in the cytosol were the same as in the normal controls. In unstimulated control neutrophils both p47-phox and p67-phox were confined to the cytosol, but after 5-min stimulation with PMA the two proteins were also detected in the particulate fraction ([23] and Fig. 1). In marked contrast, in four of the five subjects with cytochrome-negative CGD, neither p47-phox nor p67-phox was detected in the membrane fraction. In one patient (Fig. 1, fourth panel) a small amount of immunoreactive protein was detected in the membrane fraction at the position of p67-phox only, but at a much lower level than was detected in control membranes. This probably does not represent contamination of the particulate fraction with cytosol, as no p47-phox was detected in the same preparation. As far as can be determined, this patient's neutrophils are biochemically and functionally the same as those from other patients with X-linked cytochrome-negative CGD (Curnutte, J. T., unpublished observation). The B-1 polyclonal antiserum predominantly recognizes p47-phox and p67-phox, but also shows minor specificities to other proteins (e.g., at 55 kD) of unknown identity (13, 23). The intensity of these bands varied from experiment to experiment depending upon the exposure time of the autoradiograph.

We also studied translocation of cytosolic components in one patient with X-linked CGD, in which a Pro → His substitution in gp91-phox renders cytochrome b₅₅₈ nonfunctional. Translocation in these neutrophils was completely normal; both p47-phox and p67-phox were detected in the membrane fraction of PMA-stimulated cells (Fig. 2).

Fig. 3 shows the results of translocation experiments with neutrophils from two patients with p47-phox-deficient CGD. The absence of p47-phox was evident in the autoradiographs of the immunoblots of the patients' cytosolic fractions. The levels of p67-phox were the same as in the control. Despite this, there was no translocation of p67-phox to the membrane in either patient. In contrast, in two sisters with p67-phox deficiency, p47-phox was detected in the membrane fractions of PMA-stimulated cells (Fig. 4). By densitometry, the relative intensities of these p47-phox bands for the two patients were 110 and 64, compared with the control band set at 100%. The recovery of protein in the membrane fraction of the second patient's PMA-stimulated neutrophils was low (53% of control; see legend to Fig. 4), wholly accounting for the lower level of translocation.

CYTOCHROME b-DEFICIENT CGD

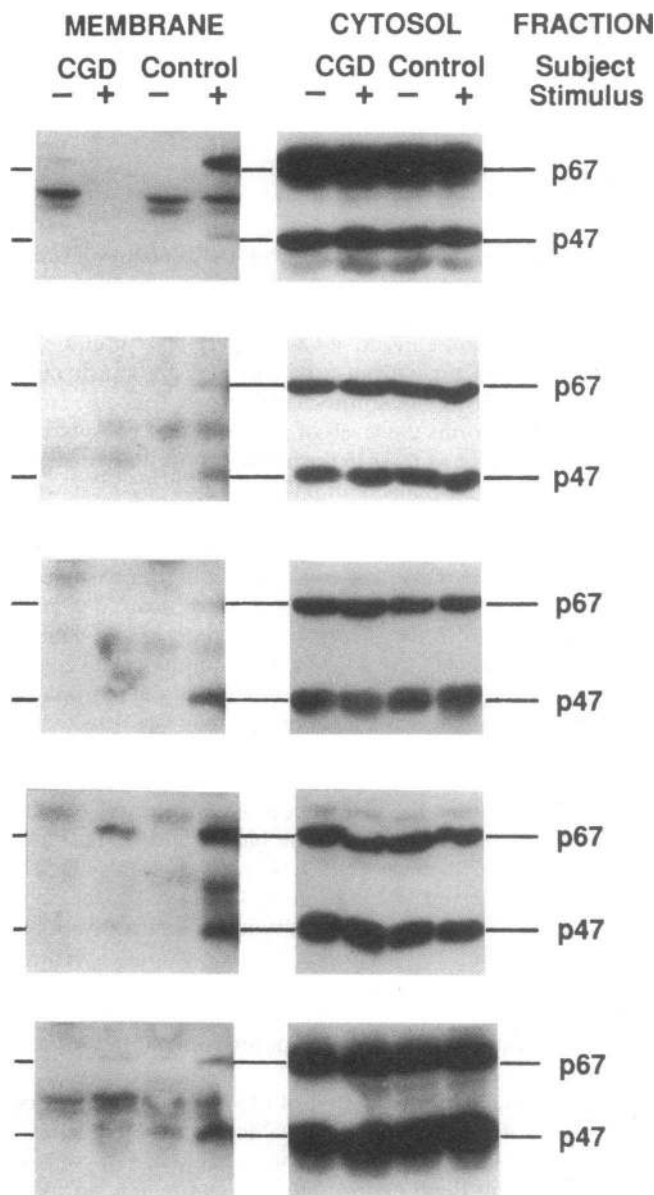


Figure 1. Failure of translocation of p47-phox and p67-phox to the membrane in cytochrome b_{558} -negative CGD neutrophils. The CGD fractions in the top four pairs of panels were prepared from the neutrophils of patients with X-linked cytochrome-negative CGD and those in the bottom panels from a patient with the autosomal recessive cytochrome-negative variant form. In each panel fractions prepared in parallel from normal control neutrophils are shown for comparison. Membrane and cytosol fractions ($\sim 1.5 \times 10^7$ and 5×10^6 cell equivalents, respectively) from resting (-) and PMA-activated (+) neutrophils were subjected to SDS-PAGE. The amount of protein loaded on the gel from patients' neutrophil fractions never differed by > 20% from the corresponding control value. The resolved proteins were electrophoretically transferred to nitrocellulose. The blots were blocked, probed with B-1 (an antiserum that recognizes p47-phox and p67-phox), and exposed to ^{125}I -protein A as described in Methods. The immunoreactive proteins were detected by autoradiography. The locations of the p47-phox and p67-phox bands are indicated.

CYTOCHROME b MISSENSE MUTATION

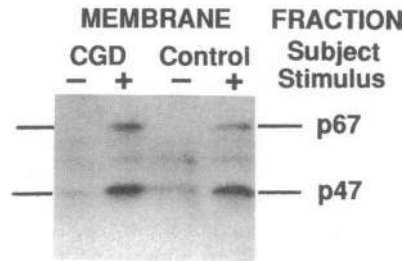


Figure 2. Translocation of p47-phox and p67-phox in an X-linked CGD patient with a missense mutation in the gp91-phox subunit of cytochrome b_{558} . With the exception of the modifications in the preparation of the membrane fraction from the CGD and control neutrophils (for details see Methods), the procedures used in

this experiment were the same as those in Fig. 1. Only the particulate (membrane) fractions are shown in this figure.

Discussion

We have demonstrated that the absence of cytochrome b_{558} , whether it is due to a primary defect in the p22-phox or the gp91-phox subunit, results in the failure of two cytosolic oxidase components (p47-phox and p67-phox) to undergo stimulus-dependent translocation to the plasma membrane. This finding indicates that cytochrome b_{558} , or a closely linked factor (e.g., rap 1) (25), serves as the membrane docking site for the components. The ability of antibodies to the cytoplasmic carboxy-terminal domain of gp91-phox, and the synthetic peptides themselves, to block O_2^- generation in a cell free system, suggest that this cytochrome subunit mediates the interaction with cytosolic components (26). Translocation of p47-phox and p67-phox was normal in a variant X-linked form of CGD in which cytochrome b_{558} is present but does not transfer elec-

p47 - DEFICIENT CGD

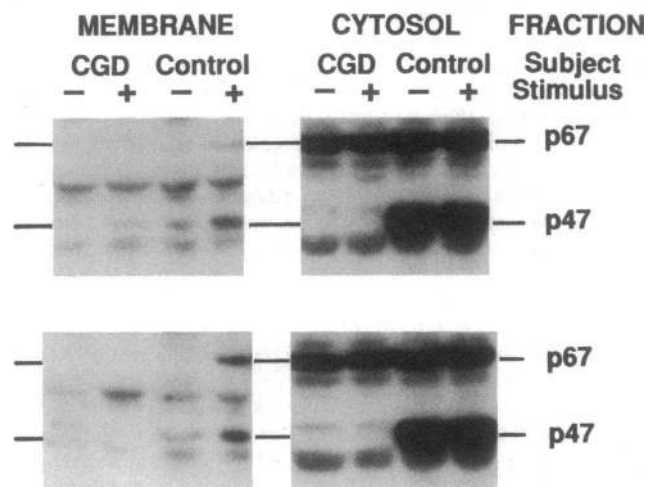


Figure 3. Absence of translocation of p67-phox to the membrane in p47-phox-deficient CGD patients. The amount of protein loaded on the gel from patients' neutrophil fractions did not differ by > 18% from the corresponding control value. Experimental details are the same as in Fig. 1. A similar result was obtained with neutrophils from one other patient with p47-phox deficiency analyzed in a separate experiment.

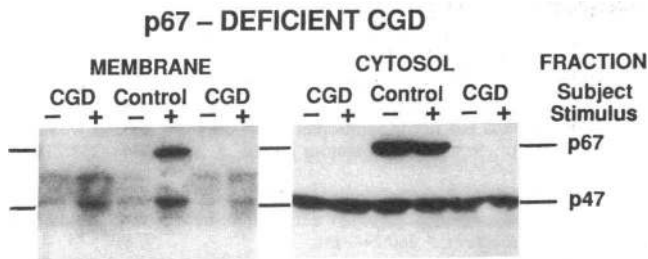


Figure 4. Translocation of p47-*phox* to the membrane in p67-*phox*-deficient CGD patients. The neutrophils from the two patients in this experiment were processed simultaneously together with those from a single control subject. The amounts of protein (μg) loaded per lane (reading from left to right) were as follows: *Membrane*: 175, 139, 180, 135, 163, 72; *Cytosol*: 135, 121, 124, 124, 126, 112. Experimental details are the same as in Fig. 1. A similar result was obtained with a different p67-*phox*-deficient patient analyzed in a separate experiment.

trons as a result of a single amino acid substitution. This is the only form of CGD studied in which translocation of both proteins was normal, and as such demonstrates that the absence of translocation in other forms of CGD is not a secondary effect of the failure to generate O_2^- .

Our results are compatible with earlier observations on the phosphorylation of p47-*phox* in human neutrophils (17). After two-dimensional electrophoresis of ^{32}P -labeled phosphoproteins from stimulated neutrophils, p47-*phox* appeared as a chain of six spots, indicating that it contains at least six phosphorylation sites (10), all of which are on serine residues (16). Moreover, the primary amino acid sequence of p47-*phox* contains six serines in the arginine-rich COOH-terminal domain that exhibit features of target sites of protein kinases (27, 28). In normal neutrophils activated with PMA, phosphorylated p47-*phox* was present in both the cytosol and membrane fractions (15, 21, 22). In classic X-linked cytochrome b-negative CGD however, the phosphorylated p47-*phox* was confined to the cytosol (22). In addition, two-dimensional electrophoresis revealed that in this form of CGD only four of the six phosphorylation sites had acquired the ^{32}P label, suggesting that the two remaining phosphorylation reactions occurred after translocation of the protein to the membrane, in a process that depended upon cytochrome b_{558} (10, 16).

The results from our translocation experiments coupled with the previous phosphorylation studies just discussed, suggest the following model as one way to activate the oxidase. Upon neutrophil stimulation the initial steps in phosphorylation of p47-*phox* occur in the cytosol. The protein then translocates to the membrane, binds to a site formed by cytochrome b_{558} , and is phosphorylated at its two remaining sites. In cytochrome-negative CGD the initial phosphorylation steps take place, but translocation and further phosphorylation are precluded by the absence of cytochrome b. In X-linked cytochrome-positive CGD, in which phosphorylation of p47-*phox* was normal at all six sites (10), translocation of p47-*phox* and p67-*phox* is normal. This indicates that the Pro \rightarrow His substitution does not interfere with the association of cytosolic components with cytochrome b or the subsequent phosphorylation of p47-*phox*. It is important to point out however, that our data do not necessarily imply a causal relationship between

p47-*phox* phosphorylation and either oxidase activation or translocation of cytosolic components, simply that they can occur in parallel. Several studies have shown the O_2^- generation can be activated without apparent p47-*phox* phosphorylation (reviewed in reference 17). Under these conditions other signals clearly must trigger translocation of the cytosolic components and activation of the oxidase.

Our finding that translocation of p67-*phox* is wholly dependent on the presence of p47-*phox* supports the view that cytosolic components of the NADPH oxidase exist in the form of complexes (13, 24, 29). Translocation of p47-*phox*, on the other hand, occurred in the absence of p67-*phox*, suggesting that the former protein is the ligand that fits into the membrane docking site formed by the cytochrome and that p67-*phox* follows by virtue of its association with p47-*phox*. However, it is also conceivable that p67-*phox* translocates independently of p47-*phox* and recognizes a site formed by the cytochrome/p47-*phox* complex. In p67-*phox* deficiency, p47-*phox* retains the ability to bind to the membrane, either alone or as part of a smaller complex. The recent demonstration that phosphorylation of p47-*phox* was normal (i.e., occurred at all sites) in p67-*phox*-deficient neutrophils (29), is compatible with our translocation data. How the membrane complex of cytochrome b_{558} , phosphorylated p47-*phox*, p67-*phox*, and perhaps other components is converted to a catalytically active enzyme system remains to be determined.

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