Brief Definitive Report

^{156}Pro → Gln Substitution in the Light Chain of Cytochrome b_{558} of the Human NADPH Oxidase (p22-*phox*) Leads to Defective Translocation of the Cytosolic Proteins p47-*phox* and p67-*phox*

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Summary

Src homology 3 (SH3) domains have been suggested to play an important role in the assembly of the superoxide-forming nicotinamide adenine dinucleotide phosphate (NADPH) oxidase upon activation of phagocytes, which involves the association of membrane-bound and cytosolic components. We studied the translocation of the cytosolic proteins to the plasma membrane in neutrophils of a patient with a point mutation in the gene encoding the light chain of cytochrome b_{558}. This mutation leads to a substitution at residue 156 of a proline into a glutamine in a putative SH3 binding domain of p22-*phox* (Dinauer, M., E. A. Pierce, R. W. Erickson, T. Muhlebach, H. Messner, R. A. Seger, S. H. Orkin, and J. T. Curnutte. 1991. *Proc. Natl. Acad. Sci.* 88:11231). In PMA-stimulated neutrophils and in a cell-free translocation assay with neutrophil membranes and cytosol, association of the cytosolic proteins p47-*phox* and p67-*phox* with the membrane fraction of the patient's neutrophils was virtually absent. In contrast, when solubilized membranes of the patient's neutrophils were activated with phospholipids in the absence of cytosol (Koshkin, V., and E. Pick. 1993. *FEBS [Fed. Eur. Biochem. Soc.] Lett.* 327:57), the rate of NADPH-dependent oxygen uptake was observed at a rate similar to that of control membranes. We suggest that the binding of an SH3 domain of p47-*phox* to p22-*phox*, and thus activation of the oxidase, does not occur in the neutrophils of this patient, although under artificial conditions, electron flow from NADPH to oxygen in cytochrome b_{558} is possible.

Neutrophils and other phagocytic cells contain a multicomponent electron transfer chain known as the nicotinamide adenine dinucleotide phosphate (NADPH)[1] oxidase (1). This enzyme is responsible for production of microbicidal oxidants upon activation of phagocytes. For an active NADPH oxidase at least five different proteins are required: two subunits of the membrane-bound cytochrome b_{558}, gp91-*phox* and p22-*phox*, and three cytosolic proteins, p47-*phox*, p67-*phox*, and a low molecular weight GTP-binding protein, either *rac*-1 (in macrophages) or *rac*-2 (in neutrophils) (2, 3). Recently, a fourth cytosolic oxidase component was found: p40-*phox*, which is probably involved in stabilizing p67-*phox* (4, 5). Upon cell activation, p47-*phox* and p67-*phox* translocate to the plasma membrane, to form the active membrane-bound NADPH oxidase (6–8). Both p47-*phox* and p67-*phox* contain two so-called src homology (SH3) domains (9, 10), which are known to be involved in protein–protein interactions (11, 12). In addition, both proteins as well as p22-*phox* possess proline-rich regions that are putative SH3 counter structures (13, 14).

Recently, it has been described that superoxide production can be elicited in solubilized cytochrome b_{558} by a mixture of phospholipids in the absence of cytosolic proteins (15, 16). This suggests that cytochrome b_{558} contains the complete electron transport chain from NADPH to superoxide, and that the cytosolic components merely function as activators.

NADPH oxidase activity is absent in patients with chronic

[1] *Abbreviations used in this paper:* CGD, chronic granulomatous disease; GTP-γ-S, guanosine 5′-3-O-(thio)triphosphate; NADPH, nicotinamide adenine dinucleotide phosphate; SH3, *src* homology.

granulomatous disease (CGD). As a consequence, these patients suffer from recurrent severe bacterial and fungal infections. There are four genetic causes of CGD, reflecting defects in four different components of the NADPH oxidase (for reviews see references 1, 17, 18). When the gene encoding p22-*phox* or gp91-*phox* is affected, both subunits of cytochrome b_{558} are typically absent (19–22).

We studied the only CGD patient described thus far with a missense mutation in the cytoplasmic part (23) of p22-*phox* with normal amounts of cytochrome b_{558} (24). This patient has a missense mutation in codon 156 (numbered according to 25), predicting an amino acid substitution of a proline into a glutamine residue. This substitution is located in a proline-rich region of p22-*phox* that could be involved in the binding of an SH3 domain of p47-*phox* or p67-*phox*. Here, we report that for this patient both in PMA-activated neutrophils and in the cell-free assay, hardly any translocation of cytosolic proteins was observed. In addition, when solubilized neutrophil membranes of the patient were stimulated with phospholipids in the absence of cytosol, oxidase activity was observed comparable with that of neutrophil membranes from healthy donors.

Materials and Methods

Materials. Guanosine 5'-3-O-(thio)triphosphate (GTP-γ-S) and NADPH were purchased from Boehringer (Mannheim, Germany). Reagents and molecular weight markers for SDS-PAGE were from Bio-Rad Laboratories (Richmond, CA). Nitrocellulose sheets BA84 for Western blotting were obtained from Schleicher & Schuell (Dassel, Germany). Antibodies used in this study were mAb 449 and 48, directed against p22-*phox* and gp91-*phox*, respectively (22). Rabbit antisera specific for either p47-*phox* or p67-*phox*, were raised against synthetic peptides identical to the last 12 residues of the COOH-termini. Goat antibody against rabbit Ig conjugated to horseradish peroxidase was produced within our institute (Central Laboratory of the Netherlands, Red Cross Blood Transfusion Service). The enhanced chemiluminescence kit with luminol was from Amershan International (ECL; Amersham, Buckinghamshire, UK). X-Omat AR diagnostic films were from Kodak Co. (Rochester, NY). A synthetic peptide resembling region 149–162 of p22-*phox* and a peptide resembling the same region, but containing the same Pro → Gln substitution as the patient were purchased from Eurosequence (Groningen, The Netherlands), and dissolved in distilled water to a concentration of 10 mM. The lipids were obtained from Sigma Chemical Co. (St. Louis, MO) (product numbers appear within parentheses): L-α-phosphatidylcholine type II-S from soybean, 14% (P5638) and L-α-phosphatidic acid, sodium salt, from egg yolk lecithin, 98% (P9511).

Isolation and Fractionation of Leukocytes. Human neutrophils were prepared on one occasion from 100 ml of citrated blood from the CGD patient after obtaining informed consent. Neutrophils from a healthy donor were isolated in parallel. Neutrophils were isolated and fractionated by sonication as described (26, 27).

Translocation of Cytosolic Proteins in Intact Neutrophils. 40×10^6 cells of the patient and of a healthy donor were incubated with PMA (100 ng/ml) or without PMA for 10 min at 37°C. The cells were then resuspended and sonicated in 1 ml of ice-cold oxidase buffer (containing 75 mM NaCl, 10 mM Hepes, 170 mM sucrose, 1 mM $MgCl_2$, 0.5 mM EGTA, 10 μM ATP, and 2 mM azide, pH 7.0) with 5 μM GTP-γ-S and 100 μg/ml PMSF. After centrifugation (10 min, 800 *g*), the sonicate was layered on a sucrose gradient as described before (26), with 1 mM $MgCl_2$, 40 mM NaCl, 0.5 mM EGTA, and 5 μM GTP-γ-S added to the 15% sucrose layer. After centrifugation (45 min, 100,000 *g*), 500 μl of plasma membranes was harvested. For immunodetection, 25 μl of membrane fraction was dissolved in SDS sample buffer (125 mM Tris, pH 6.8, 20% [wt/vol] SDS, 10% [vol/vol] β-ME), and was loaded on a 10% polyacrylamide gel according to Laemmli (28), in a gel apparatus (Mini-Protean II; Bio-Rad Laboratories). Western blotting was performed in a Mini Trans-Blot (Bio-Rad) cell according to the manufacturers recommendations. Detection of proteins was performed as described previously (29). When the blot was stained for two sets of proteins, the nitrocellulose was stripped with 62.5 mM Tris, pH 6.7, 2% SDS, and 100 mM β-ME after the first staining.

Superoxide Assay. NADPH oxidase activity with neutrophil membranes and cytosol was measured as the superoxide dismutase (SOD)-sensitive reduction of cytochrome *c* in a spectrophotometer (model Lambda 2; Perkin Elmer Corp., Norwalk, CT). The contents of six cuvettes measured in parallel were stirred continuously and were thermostatted at 28°C. Plasma membranes (10 μg of protein) and cytosol (200 μg of protein) were incubated in 0.8 ml of oxidase buffer and 60 μM cytochrome *c*. After 2 min of incubation, oxidase assembly was induced by addition of SDS (100 μM) and GTP-γ-S (10 μM). After 5 min, NADPH (250 μM) was added and the rate of cytochrome *c* reduction was measured at 550 nm.

Translocation of Cytosolic Proteins in the Cell-free System. Neutrophil plasma membranes (20 μg protein) were mixed with neutrophil cytosol (400 μg protein) in 1 ml of oxidase buffer. Subsequently, SDS (100 μM) and GTP-γ-S (10 μM) were added. After 10 min at room temperature, the mixture was loaded on a discontinuous sucrose gradient as described (30). NADPH oxidase activity of one-fifth part of the reisolated membranes was measured without cytosol in the presence of SDS (100 μM) and GTP-γ-S (10 μM). After 2 min, NADPH (250 μM) was added and the rate of cytochrome *c* reduction was measured at 550 nm. In addition, the supernatant (50 μl) and four-fifths part of the reisolated membranes were analyzed by immunoblot for the presence of p47-*phox*, p67-*phox*, and cytochrome b_{558} subunits.

Oxygen Consumption in Plasma Membranes without Cytosol According to Koshkin and Pick (15). 6 μg of membrane protein (in 30 μl) was solubilized with 10 μl of buffer A (50 mM Na-phosphate, pH 7.4, 1 mM EGTA, 1 mM $MgCl_2$, 2 mM NaN_3, 1 mM dithiotreitol, and 20% glycerol) with 80 mM 1-O-Octyl-β-D-glucopyranoside. Subsequently, the membranes were reconstituted with 4 μg of L-α-phosphatidylcholine and L-α-phosphatidic acid, diluted eightfold in assay buffer (65 mM K,Na-phosphate buffer, pH 7.0, 1 mM $MgCl_2$, 1 mM EGTA, 2 mM NaN_3, 1 μM flavin adenine dinucleotide [FAD], and 30 μM SDS) and allowed to stand on ice for 30 min. Oxygen consumption was measured with an oxygen electrode after addition of 250 μM NADPH.

Results

Translocation of p47-phox and p67-phox in Neutrophils of the CGD Patient. We studied the only CGD patient described thus far with a missense mutation in the cytoplasmic tail of p22-*phox* leading to normal amounts of nonfunctional cytochrome b_{558} (24). This patient has a mutation that predicts a Pro → Gln substitution at residue 156 of p22-*phox*. To investigate the functional effect of this substitution, we studied the association of the cytosolic proteins with the plasma membranes of PMA-activated neutrophils. For this purpose, neutrophils of the patient and those of a healthy donor were

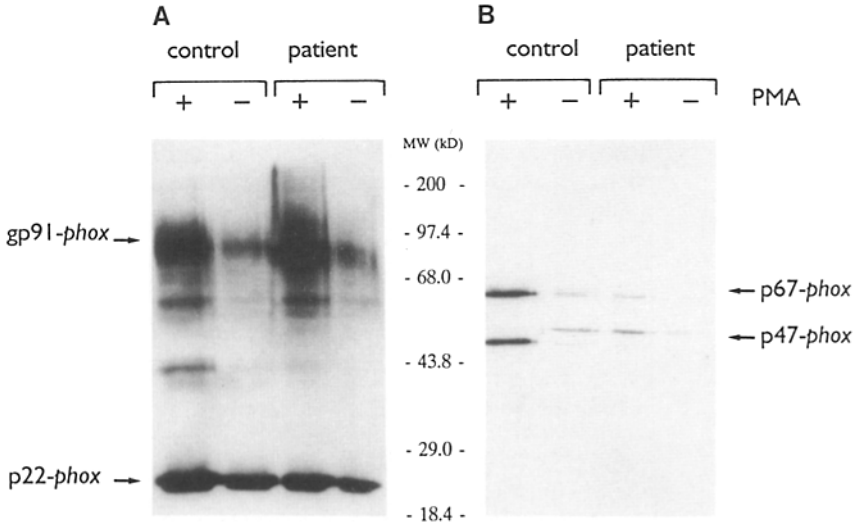

Figure 1. Western blot analysis of plasma membranes of activated neutrophils. 40 × 10^6 cells were incubated with or without 100 ng of PMA per ml for 7 min at 37°C as indicated. Fractionation of the cells was performed as described in Materials and Methods. Plasma membrane proteins of 2 × 10^6 cells were separated by 10% PAGE and blotted onto nitrocellulose. (*A*) The blot was stained for both subunits of cytochrome b_{558} with mAbs 48 (against gp91-*phox*) and 449 (against p22-*phox*). (*B*) The same blot stained with rabbit antisera against p47-*phox* and p67-*phox*. The samples were obtained from the A22$^+$ CGD patient or control donor as indicated.

incubated with or without PMA, and subcellular fractions were isolated. Fig. 1 shows the Western blot of the plasma membranes of these neutrophils, stained for cytochrome b_{558} or p47-*phox* and p67-*phox*. The intensity of the bands of cytochrome b_{558} in this plasma membrane fraction was increased when the cells were treated with PMA, because cytochrome b_{558} is recruited from the specific granules to the plasma membrane upon cell activation (Fig. 1 *A*). Fig. 1 *B* clearly shows a strongly reduced translocation of both cytosolic oxidase components to the neutrophil membranes of the patient upon stimulation with PMA.

Effect of Point Mutation ^{156}Pro → Gln in p22-phox on NADPH Oxidase Assembly. We also studied the neutrophil membranes of the CGD patient for their ability to bind p47-*phox* and p67-*phox* in a cell-free translocation assay. Reisolated control membranes preincubated with control cytosol produced 788 ± 24 (mean ± SEM, n = 3) nmol of superoxide/min × mg membrane protein, whereas the patient's neutrophil membrane fraction preincubated with control cytosol produced only 2 ± 1 nmoles of superoxide/min × mg membrane protein.

The supernatant of the sucrose gradient and the reisolated membranes were also analyzed on an immunoblot to determine the translocation of cytosolic oxidase components. Fig. 2 *A* shows a decreased amount of cytosolic proteins in the supernatant of only the control experiment. Fig. 2 *B* demonstrates the presence of equal amounts of the membrane-bound oxidase components gp91-*phox* and p22-*phox* in the reisolated membrane fraction of the control and the patient. When the same blot was treated with antibodies directed against the cytosolic oxidase components p67-*phox* and p47-*phox* (Fig. 2 *B*), the control membranes showed a significant association with p47-*phox* and p67-*phox*. In contrast, the neutrophil membranes of the patient did not contain detectable amounts of cytosolic oxidase proteins. Also, when the incubation mixture of control plasma membranes and cytosol was

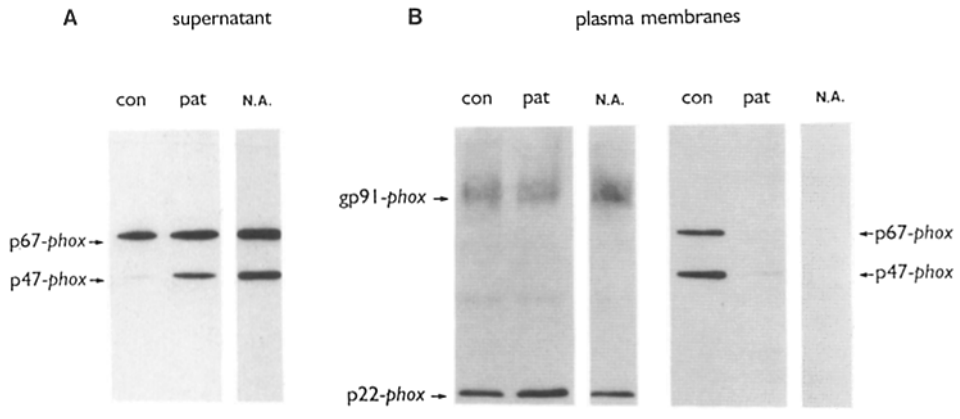

Figure 2. Western blot analysis of neutrophil membranes reisolated from the cell-free activation system. Plasma membranes were mixed with cytosol and activated with SDS and GTP-γ-S in the first two lanes as described in Materials and Methods. After centrifugation through a 15% (wt/vol) sucrose layer, the supernatant and membrane fractions were harvested. 80% of the membrane fraction was precipitated with 10% (wt/vol) TCA and redissolved in SDS sample buffer. After 10% PAGE, both supernatant and membrane proteins were blotted onto nitrocellulose. The supernatant (*A*) was stained with a mixture of two rabbit antisera directed against p47-*phox* and p67-*phox*. The membrane fraction (*B*) was stained with a mixture of mAbs 449 and 48 (*left*), or with rabbit antisera against p47-*phox* and p67-*phox* (*right*). The source of plasma membranes in the first two lanes is indicated; (*con*) control donor, (*pat*) A22$^+$ CGD patient. In lanes 3 (not activated [N.A.]), SDS and GTP-γ-S were omitted from the incubation mixture with control membranes. The results shown are representative of three independent experiments.

not activated with SDS and GTP-γ-S, the reisolated membranes contained no detectable amounts of p47-*phox* and p67-*phox* (lane *N.A.*).

To consolidate the importance of the putative SH3 binding domain 149–162 of p22-*phox* in the assembly of the NADPH oxidase, we explored the effect of a synthetic peptide resembling this region in the cell-free assay. Other peptides have recently been shown to affect this process efficiently with an IC_{50} (concentration giving 50% inhibition) lower than 30 μM (29, 31). However, this peptide did not show a potent inhibitory effect: the IC_{50} amounted to about 100 μM (results not shown). Furthermore, a peptide containing the same substitution as the patient, inhibited the cell-free assay to the same extent.

Oxygen Consumption in Neutrophil Plasma Membranes without Cytosol. If, indeed, residue 156 of p22-*phox* is required for the interaction with p47-*phox*, it is conceivable that electron flow in cytochrome b_{558} might still be possible, despite the mutation of p22-*phox*. Recently, it has been described that relipidation of solubilized cytochrome b_{558} can elicit NADPH-dependent superoxide (O_2^-) production in the absence of cytosolic oxidase proteins (15), suggesting that cytochrome b_{558} contains the complete electron-transporting apparatus of the NADPH oxidase, and that the cytosolic components merely function as activators (15). Therefore, we tested the neutrophil membranes of the patient for oxygen consumption in the absence of cytosol. For this purpose, membranes obtained from control donors or CGD patients were solubilized and activated with phospholipids in the presence of FAD and NADPH according to previous protocols (15). The results are shown in Table 1. The neutrophil membranes of the control donors exhibited a significant oxygen consumption, whereas the neutrophil membranes of two cytochrome b_{558}-deficient CGD patients showed a very low oxygen consumption, thus confirming a cytochrome b_{558}-dependent mechanism. Plasma membranes from the patient described in this study, with a ^{156}Pro → Gln substitution in p22-*phox*, showed a similar rate of oxygen consumption as control membranes, indicating an intact electron flow in the mutated cytochrome b_{558} of this patient under these experimental conditions.

Table 1. *Oxygen Consumption of Solubilized Membranes Activated with Phospholipids*

Source of plasma membranes	O_2/min × mg membrane protein
	nmol
Controls	189 ± 5*
X91^0 patient 1	62
X91^0 patient 2	19
A22$^+$ patient	220 ± 12*

Patients' or control neutrophil membranes (6 μg) were solubilized with octylglucoside and incubated with phosphatidylcholine/phosphatidic acid on ice for 30 min. Oxygen consumption was measured in 500 μl after addition of 250 μM of NADPH. Values were corrected for oxygen consumption in the absence of membranes, which was 54 ± 7 nmol O_2/min. X91^0 are X-linked CGD patients, affected in gp91-*phox*, lacking cytochrome b_{558}; A22$^+$ is the patient discussed in this report, with the autosomally inherited defect in p22-*phox*.
* Mean ± SEM of three separate experiments.

Discussion

It is well established that so-called SH3 domains are involved in protein–protein interactions (11, 12). Recently, the counter structure for these SH3 domains has been identified as proline-rich stretches found in various proteins (14). P47-*phox* and p67-*phox* of the NADPH oxidase each contain two SH3 domains and one putative SH3 binding domain. In the membrane-bound cytochrome b_{558} only p22-*phox* contains such a putative SH3-binding domain, comprising residue 149–162. Possibly, residues 176–195 represent another SH3-binding domain. In this report we studied the importance of the proline-rich region of 149–162 in p22-*phox* in the association of the cytosolic oxidase components with cytochrome b_{558}, by the use of neutrophils from a CGD patient with a ^{156}Pro → Gln substitution of p22-*phox* (24).

Upon PMA stimulation, the translocation of both p47-*phox* and p67-*phox* was hardly detectable with the neutrophils derived from the patient, whereas translocation could clearly be demonstrated in neutrophils from control donors (Fig. 1). Also in an in vitro translocation assay, the membrane fraction of the patient failed to support association of these cytosolic oxidase components (Fig. 2). These results indicate an important contribution of residue 156 of p22-*phox* to the binding of one or more cytosolic components. Most likely, binding of p47-*phox* is disturbed, because p47-*phox* is thought to interact first with cytochrome b_{558} (32, 33). As a consequence, p67-*phox* binding is also absent, because translocation of this latter protein is dependent on the presence of p47-*phox* (8).

To assess the consequence of the ^{156}Pro → Gln in p22-*phox* for electron transfer within the cytochrome, solubilized membranes of control neutrophils and patient neutrophils were incubated with phospholipids. Both showed a significant oxygen consumption upon addition of NADPH (Table 1), indicating that the mutated cytochrome b_{558} in this patient is capable of a normal electron transport. This oxygen consumption is indeed cytochrome b_{558} dependent, as was proven by the very low activity in neutrophil membranes from cytochrome b_{558}-deficient CGD patients. Therefore, the dysfunction of the NADPH oxidase in the neutrophils from the A22$^+$ CGD patient is caused by a defective activation mechanism rather than by a defective cytochrome b_{558}.

Recently, we have described another CGD patient with a defective translocation of p47-*phox* and p67-*phox* (29). This patient was characterized as an X-linked CGD patient (X91$^+$) with a ^{500}Asp → Gly mutation in gp91-*phox* with normal amounts of nonfunctional cytochrome b_{558}. Relipidated neutrophil membranes of this patient showed a strongly reduced oxygen consumption, comparable with that of membranes of cytochrome b_{558}-deficient CGD patients (results not shown).

The mutations found in these two CGD cytochrome b_{558}-

positive patients indicate at least two binding sites for p47-*phox* in cytochrome b_{558}. First, a region around residue 500 of gp91-*phox*, with which p47-*phox* interacts possibly through electrostatic forces. Second, the proline-rich region 149–162 of p22-*phox* to which p47-*phox* binds via its SH3 domains. Possibly, the first interaction enables the second interaction to occur, explaining the different effects of peptides resembling these domains. Evidently, the exact mechanism of binding of p47-*phox* to cytochrome b_{558} induced by neutrophil activation remains to be elucidated.

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