Brief Definitive Report

156Pro → Gln Substitution in the Light Chain of Cytochrome b₅₅₈ 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 b₅₅₈. 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, K. W. Erickson, T. Muhlebach, H. Messner, K. 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 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 b₅₅₈ is possible.

Neutrophils and other phagocytic cells contain a multi-component electron transfer chain known as the nicotinamide adenine dinucleotide phosphate (NADPH) 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 b₅₅₈, 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 b₅₅₈ by a mixture of phospholipids in the absence of cytosolic proteins (15, 16). This suggests that cytochrome b₅₅₈ 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 granulomatous disease (CGD), a genetic disorder characterized by recurrent bacterial infections. The defective production of reactive oxygen species in CGD patients is due to mutations in genes encoding the cytosolic components of the NADPH oxidase. In the study presented here, we investigated a patient with CGD who had a point mutation in the gene encoding the light chain of cytochrome b₅₅₈ (p22-phox). This mutation leads to a substitution at residue 156 of a proline into a glutamine in a putative SH3 binding domain of p22-phox. 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 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 b₅₅₈ is possible.

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 phospholipid vesicles 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 Amersham 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 Eu-sequence (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): 1-α-phosphatidylcholine type II-S from soybean, 14% (P5638) and 1-α-phosphatidic acid, sodium salt, from egg yolk lecithin, 98% (PP511).

**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-Protein 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 SOD (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, SOD (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 SOD (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 Assay According to Koskin 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 Na$_3$-dithiothreitol, and 20% glycerol) with 80 μM 1-O-Octyl-3-β-D-glucopyranoside. Subsequently, the membranes were reconstituted with 4 μg of t-α-phosphatidylcholine and t-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 Na$_3$-dithiothreitol, and 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

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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 b558 or p47-phox and p67-phox. The intensity of the bands of cytochrome b558 in this plasma membrane fraction was increased when the cells were treated with PMA, because cytochrome b558 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 164 Pro → C14 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 nmole 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 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 IC₅₀ (concentration giving 50% inhibition) lower than 30 μM (29, 31). However, this peptide did not show a potent inhibitory effect: the IC₅₀ 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 b₅₅₈ might still be possible, despite the mutation of p22-phox. Recently, it has been described that a 156Pro → Gln substitution in p22-phox shows a similar rate of oxygen consumption as control membranes, indicating an intact electron flow in the mutated cytochrome b₅₅₈ of this patient under these experimental conditions.

**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 b₅₅₈ only p22-phox contains such a putative SH3-binding domain, comprising residue 149–162. Possibilities 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 b₅₅₈, by the use of neutrophils from a CGD patient with a 156Pro → 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 b₅₅₈ (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 156Pro → 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 b₅₅₈ in this patient is capable of a normal electron transport. This oxygen consumption is indeed cytochrome b₅₅₈ dependent, as was proven by the very low activity in neutrophil membranes from cytochrome b₅₅₈-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 b₅₅₈.

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 (X911) with a 508Asp → Gly mutation in gp91-phox with normal amounts of nonfunctional cytochrome b₅₅₈. Relipidated neutrophil membranes of this patient showed a strongly decreased oxygen consumption, comparable with that of membranes of cytochrome b₅₅₈-deficient CGD patients (results not shown).

The mutations found in these two CGD cytochrome b₅₅₈-

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

| Source of plasma membranes | O₂/min × mg membrane protein | nmol |
|---------------------------|-------------------------------|------|
| Controls                  |                               | 189 ± 5* |
| X911 patient 1           |                               | 62   |
| X911 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 O₂/min. X911 are X-linked CGD patients, affected in gp91-phox, lacking cytochrome b₅₅₈. A22* is the patient discussed in this report, with the autosomal inherited defect in p22-phox.

* Mean ± SEM of three separate experiments.
positive patients indicate at least two binding sites for p47-phox in cytochrome b589. 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 b589 induced by neutrophil activation remains to be elucidated.

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