A Point Mutation in gp91-*phox* of Cytochrome b_{558} of the Human NADPH Oxidase Leading to Defective Translocation of the Cytosolic Proteins p47-*phox* and p67-*phox*

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Abstract

The superoxide-forming NADPH oxidase of human phagocytes is composed of membrane-bound and cytosolic proteins which, upon cell activation, assemble on the plasma membrane to form the active enzyme. Patients suffering from chronic granulomatous disease (CGD) are defective in one of the following components: p47-*phox* and p67-*phox*, residing in the cytosol of resting phagocytes, and gp91-*phox* and p22-*phox*, constituting the membrane-bound cytochrome b_{558}. In an X-linked CGD patient we identified a novel missense mutation predicting an Asp → Gly substitution at residue 500 of gp91-*phox*, associated with normal amounts of nonfunctional cytochrome b_{558} in the patient's neutrophils. In PMA-stimulated neutrophils and in a cell-free translocation assay with neutrophil membranes and cytosol, the association of the cytosolic proteins p47-*phox* and p67-*phox* with the membrane fraction of the patient was strongly disturbed. Furthermore, a synthetic peptide mimicking domain 491–504 of gp91-*phox* inhibited NADPH oxidase activity in the cell-free assay (IC_{50} about 10 μM), and the translocation of p47-*phox* and p67-*phox* in the cell-free translocation assay. We conclude that residue 500 of gp91-*phox* resides in a region critical for stable binding of p47-*phox* and p67-*phox*. (*J. Clin. Invest.* 1994. 95:2120–2126.) Key words: chronic granulomatous disease • X-linked • missense mutation • human neutrophils • superoxide

Introduction

Neutrophils and other phagocytic cells contain a multi-component electron transfer chain known as the 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 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). Upon cell activation, p47-*phox* and p67-*phox* translocate to the plasma membrane, to form the membrane-bound oxidase (4, 5).

The NADPH oxidase is defective in patients with chronic granulomatous disease (CGD).[1] 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 (reviewed in 6, 7). In the X-linked form, which comprises the majority of cases, the cytochrome *b* heme spectrum is consistently absent (8). X-linked CGD is caused by mutations in the gene encoding gp91-*phox* (9, 10). Virtually all patients with cytochrome *b* defects lack gp91-*phox* and p22-*phox*, regardless of which gene is affected by the mutation (11, 12, 13). Complete absence of both subunits in X-linked CGD is referred to as $X91^0$, whereas a residual amount of cytochrome is designated $X91^-$ (14). Sofar, only two X-linked CGD patients have been described as $X91^+$, with (almost) normal amounts of cytochrome b_{558} present in their neutrophils (15, 16). In one of these patients, a point mutation in the gene encoding gp91-*phox* was found, resulting in an amino-acid substitution of proline into histidine at residue 415 (numbering according to Orkin, 17). This substitution is located in a region of gp91-*phox* that recently has been suggested to be involved in the binding of NADPH (18, 19, 20).

Here, we report a novel missense mutation at residue 500 of the gene encoding gp91-*phox*, which is also associated with a nonfunctional cytochrome b_{558}. The domain of the protein in which the mutation resides, is most probably located on the cytoplasmic side of the membrane (21). We found that the translocation of p47-*phox* and p67-*phox* to the neutrophil membrane fraction of this patient was disturbed. Therefore, the mutated region might play a crucial role in the interaction of cytochrome b_{558} with cytosolic oxidase components.

Methods

Materials. ATP, guanosine 5'-3-*O*-(thio)triphosphate (GTP-γ-S) and NADPH were purchased from Boehringer Mannheim, Mannheim, Germany. Reagents and molecular weight markers for SDS-PAGE were from Bio Rad Laboratories (Richmond, CA). Nitrocellulose sheets for Western blotting were obtained from Schleicher & Schull (Dassel, Germany). Antibodies used in this study were mAbs 449 and 48, directed against p22-*phox* and gp91-*phox*, respectively (13). Rabbit antisera specific for either p47-*phox* or p67-*phox*, were raised against synthetic peptides identical to the last 12 residues of the COOH

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Received for publication 23 August 1993 and in revised form 31 January 1994.

J. Clin. Invest.

0021-9738/94/05/2120/07 $2.00
Volume 93, May 1994, 2120–2126

1. *Abbreviations used in this paper:* CEA, carcino embryonic antigen; CGD, chronic granulomatous disease; GTP-γ-S, guanosine 5'-3-O-(thio)triphosphate; NBT, nitroblue tetrazolium.

termini. Rabbit antiserum, directed against carcino embryonic antigen (CEA), was purchased from DAKO (Denmark). Goat anti-mouse Ig conjugated to alkaline phosphatase was obtained from Promega (Madison, WI), as were the alkaline phosphatase substrate bromo-chloro-3-indolyl phosphate (BCIP) and the chromogen nitroblue tetrazolium (NBT). Goat anti-rabbit Ig conjugated to horse radish peroxidase was produced within our institute (CLB, Amsterdam, The Netherlands). The chemiluminescense kit with luminol was from Amersham International (Buckinghamshire, England). X-Omat AR diagnostic films were from Eastman Kodak Co., Rochester, NY. Synthetic peptides derived from different domains of gp91-*phox* were produced within our institute (CLB) or purchased from Eurosequence (Groningen, The Netherlands). The peptide FAVHHDEEKDVITG, representing residues 491–504 of gp91-*phox*, and the peptide FAVHHDEEKGVITG, containing the substitution as predicted in patient D.S., were dissolved in distilled water to a concentration of 2 mM. A control peptide GPEALAETLSKQSIS (residues 537–552) was dissolved in DMSO to a concentration of 5 mM.

Clinical history of patient D.S. D.S., a boy, was born after an uneventful delivery in 1987 from non-consanguineous parents. This family is not related to any of the 40 other CGD families in The Netherlands. At the age of 6 mo he suffered from a lobular pneumonia. When he was two years of age he suffered from recurrent bloody stools from which *Campylobacter jejunii* and *Salmonella group C* were cultured, as well as from urinary tract infections from which *Klebsiella pneumoniae* and *E. coli* were grown. His liver and spleen enlarged and at age three liver abscesses were diagnosed on ultrasound. At that time the diagnosis of CGD was made. His liver abscesses were fully cleared by antibiotic and interferon-γ treatment. He is given bactrium and interferon-γ prophylaxis since then.

Classification of CGD patients. Patient R.C. was well characterized in (16) and as an affected son in family 1 in (22). The neutrophil membranes of an $X91^0$ CGD patient served as a negative control in the translocation assay. NBT slide tests were performed on the neutrophils of patient D.S., his mother, and younger sister, and respiratory burst activity after stimulation with opsonized yeast particles or phorbol myristate acetate was determined by the rate of oxygen consumption and chemiluminescence with lucigenin. Cytochrome b_{558} contents were determined by absorption spectroscopy (23) and by immunodetection of neutrophil membranes on Western blot. For immunodetection, 2 μg of membrane fraction were dissolved in SDS sample buffer (125 mM Tris, pH 6.8, 20% (wt/vol) SDS, 10% (vol/vol) β-mercaptoethanol), and loaded on a 10% polyacrylamide gel according to Laemmli (24), in a Bio Rad Mini-Protean II gel apparatus. Western blotting was performed in a Mini Trans-Blot cell according to the manufacturers recommendations. The nitrocellulose was stained for gp91-*phox* and p22-*phox* with monoclonal antibodies 48 and 449 and subsequently with goat anti-mouse Ig conjugated to alkaline phosphatase (13).

Preparation of RNA and DNA. Total RNA was purified from mononuclear leukocytes as described (25) and cDNA was synthesized. The cDNA of the coding region of gp91-*phox* was amplified with PCR in three overlapping fragments as described (16, 26, 27), and subsequently sequenced with the Sequenase Version 2.0 kit (US Biochemical Corp, Cleveland, OH) (28). Genomic DNA was isolated from circulating blood leukocytes (29) of the CGD patient, his sister and 18 control donors.

Mismatch PCR (30) was performed by amplifying exon 12 with sense oligonucleotide primer 5'GCTGTGCACCATGATGAGGAGgAAG3' (the lower-case letter indicates a noncomplementary nucleotide introducing an MboII restriction site in control DNA) and antisense primer 5'TTAGGGTGTTGACTTGCAAT3'. The 101 bp products were digested with MboII (New England Biolabs, Beverly, MA) and electroforesed on a 12% polyacrylamide gel.

Isolation and fractionation of leukocytes. Human neutrophils were prepared on four occasions from 50 to 100 ml of citrated blood from CGD patient D.S., and once from 100 ml of heparinised blood from CGD patient R.C. after obtaining informed consent. On each occasion neutrophils from a healthy donor were isolated in parallel. Neutrophils were isolated as described (31). Subsequently, neutrophils were fractionated as described (32). In short, neutrophils were resuspended (60×10^6/ml) in ice-cold Sonication Buffer (10 mM Hepes, 1 mM EGTA, 0.15 M sucrose, 0.5 mM PMSF, 20 μM leupeptin in PBS, pH 7.2). After mild sonication of the neutrophil suspension (three times 15 s at 21 kH frequency and 9 μm peak-to-peak amplitude) and pelleting undisrupted cells and nuclei, 1 ml of supernatant was layered on a discontinuous sucrose gradient consisting of 1 ml of 52% (wt/vol) sucrose, 1 ml of 40% (wt/vol) sucrose, and 1 ml of 15% (wt/vol) sucrose. After centrifugation (100,000 *g*, 60 min), 800 μl of the supernatant (as the source of cytosol), 600 μl of the interface of the 15/40% sucrose layers (as the source of plasma membranes), and 600 μl of the 40/52% interface (as the source of specific granules) were collected and stored at −80°C.

Translocation of cytosolic proteins in intact neutrophils. On one occasion, 40×10^6 cells of patient D.S. 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 (40×10^6/ml) and sonicated in 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 GTPγS and 100 μg/ml PMSF. After centrifugation (10 min, 800 *g*), the sonicate was layered on a sucrose gradient as described above, with 1 mM $MgCl_2$, 40 mM NaCl, 0.5 mM EGTA, and 5 μM GTPγS added to the 15% sucrose layer. After centrifugation (45 min, 100,000 *g*), 400 μl of plasma membranes were harvested. The samples were analyzed on Western blot for the presence of p47-*phox* and p67-*phox* by incubation with rabbit antisera specific for these proteins (13), followed by goat anti-rabbit Ig conjugated to horse radish peroxidase. Detection of bound antibodies was carried out with enhanced chemiluminescence (Amersham).

Superoxide assay. NADPH oxidase activity with neutrophil membranes and cytosol was measured as the SOD-sensitive reduction of cytochrome *c* in a Perkin Elmer spectrophotometer (model Lambda 2; Perkin-Elmer Corp., Norwalk, CT). The contents of six cuvettes measured in parallel were stirred continuously and thermostatted at 28°C. Plasma membranes (5 μg of protein) and cytosol (100 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 (10 μg protein) were mixed with neutrophil cytosol (200 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 (33). NADPH oxidase activity 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 reisolated membranes were analyzed by immunoblot. For this purpose, the nitrocellulose sheet was first incubated with rabbit antisera directed against CEA for detection of membranes, washed, incubated with HRP-labeled goat anti-rabbit Ig, washed, and finally developed with enhanced chemiluminescence (Amersham). The proteins were visualized by exposure to Kodak X-AR film for 30 s. Secondly, the nitrocellulose was stained for both subunits of cytochrome b_{558} as described above. Third, p47-*phox* and p67-*phox* were visualized also by means of enhanced chemiluminescence (Amersham) as described above. In some experiments, the autoradiographs were quantified by densitometry.

Results

Initial laboratory observations on CGD patient D.S. The neutrophils of patient D.S., stimulated with PMA or opsonized

yeast particles, showed no respiratory burst as measured by oxygen consumption and chemiluminescence, indicative of a severe form of CGD. The cells of the mother and a sister of patient D.S. showed impaired oxygen consumption, as compared with control cells, and a mozaic pattern in the NBT-slide test (65 and 56% positive cells for the mother and sister, respectively). These findings are compatible with an X-linked defect in this family.

Because cytochrome b_{558} is typically absent in neutrophils of most patients with X-linked CGD, we measured the cytochrome b_{558} content in the patient's neutrophils by spectral and Western blot analysis. Interestingly, the spectrum of the patient's neutrophils showed a normal heme content (Fig. 1). On the immunoblot of the neutrophil membranes, both subunits of cytochrome b_{558} appeared to be present (Fig. 2).

Superoxide production in the cell-free assay. To localize the cellular defect in NADPH oxidase activity, membrane and cytosolic fractions were prepared from the patient's neutrophils and studied in a cell-free oxidase assay. As depicted in Table I, the membrane fraction of a control donor mixed with cytosol of patient D.S. showed a normal rate of cytochrome *c* reduction, whereas the membrane fraction of the patient mixed with his own or control cytosol showed almost no superoxide production. This demonstrates that the defect in patient D.S. is localized in a membrane-bound component of the NADPH oxidase, i.e., in cytochrome b_{558}.

Genetic analysis of CGD patient D.S. Because of the localization of the patient's oxidase defect in the neutrophil membrane fraction and the mozaic pattern in the NBT slide test of the patient's mother and sister, we hypothesized that the gp91-*phox* cytochrome *b* subunit contains a mutation that results in normal levels of a defective cytochrome b_{558}. To test this hypothesis, we amplified the coding region of the gp91-*phox* cDNA in three overlapping fragments and subsequently sequenced these fragments.

The sequence of patient D.S. showed a point mutation at base pair 1511 of an Adenine into a Guanine, which predicts an amino acid substitution at residue 500 of aspartate into glycine (Fig. 3). This mutation was confirmed on the patient's genomic DNA after PCR amplification of exon 12 and subsequent sequencing (data not shown). To exclude the possibility of a polymorphism, mismatch PCR of genomic DNA of 38 control alleles was performed by introducing an MboII restriction site in the wild-type sequence. The restriction enzyme MboII digested these PCR products completely, whereas the PCR product of the patient's DNA was not cleaved. The PCR fragment of his sister showed a partly digested product, as evidence of her carrier state (results not shown).

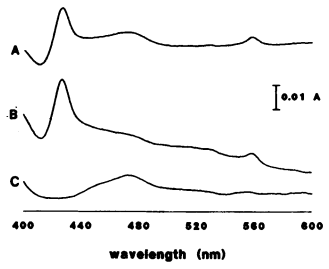

Figure 1. The reduced minus oxidized difference spectrum of neutrophil plasma membranes. Neutrophil membranes (100 μg of protein) were dissolved in PBS containing 1% Triton X-100, and the 400–600-nm range of the oxidized spectrum was determined. After addition of dithionite, the reduced spectrum was measured. The reduced minus oxidized spectra are shown for a control donor (*A*), patient D.S. (*B*), and a known X91^0 patient (*C*).

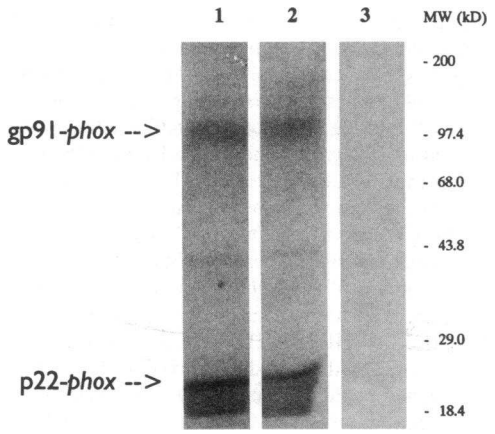

Figure 2. Western blot of neutrophil plasma membranes. 2 μg of plasma membranes were run on a 10% polyacrylamide minigel and blotted onto nitrocellulose. The blot was stained for gp91-*phox* and p22-*phox* with monoclonal antibodies 48 and 449, respectively. Lane *1*, control; lane *2*, patient D.S.; lane *3*, X91^0 patient.

Translocation of p47-phox and p67-phox in neutrophils of patient D.S. To investigate the functional effect of the substitution at residue 500 of gp91-*phox*, we studied the association of the cytosolic proteins with the plasma membranes of PMA-activated neutrophils. For this purpose, neutrophils of patient D.S. and those of a healthy donor were incubated with or without PMA and subcellular fractions were isolated. Fig. 4 shows the Western blot of the plasma membranes of these neutrophils, stained for p47-*phox* and p67-*phox*. The plasma membranes of patient D.S. showed a reduced translocation of p47-*phox* and almost no translocation of p67-*phox*. Estimating the intensity of these bands by densitometry in three different blot experiments showed 45±16 (SEM) percent residual staining for p47-*phox* and 24±7 percent for p67-*phox*, defining the amount of cytosolic proteins in control membranes as 100%. In the same assay, translocation of p47-*phox* and p67-*phox* in neutrophils from an X91^0 CGD patient was virtually absent (results not shown).

Table I. Superoxide Production in the Cell-free Assay with Mixed Plasma Membrane and Cytosolic Fractions of Control and Patient D.S. Neutrophils

Source of membranes	Source of cytosol: Control	Source of cytosol: Patient D.S.
	($\mu M\ O_2^-/min$)	
Control	7.54±1.26*	7.49±1.02
Patient D.S.	0.15±0.05	0.13±0.06

Neutrophil membranes (5 μg) and cytosol (100 μg) were incubated at 28°C in Oxidase Buffer as described in Methods in the presence of 60 μM cytochrome *c*. After 2 min of incubation, SDS (100 μM) and GTPγS (10 μM) were added. After another 5 min, NADPH (250 μM) was added, and the rate of cytochrome *c* reduction was determined by the change of absorbance at 550 nm.
* Mean±SEM of three separate experiments.

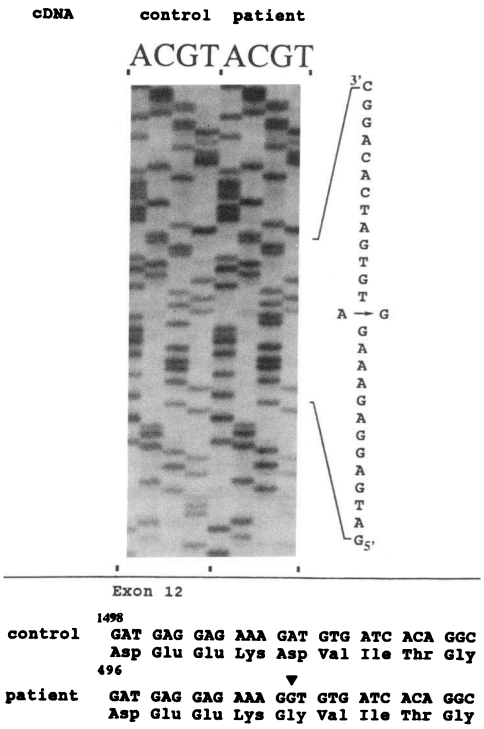

Figure 3. Sequence ladder of the mutated region in patient D.S. cDNAs of patient D.S. and a control donor were amplified with PCR and sequenced as described in Methods. The arrowhead points to the mutated base pair at position 1511. The lower part of the figure shows the consequence of the mutation for the amino acid sequence.

Effect of gp91-phox point mutations ^{500}Asp- > Gly and ^{415}Pro- > His on NADPH oxidase assembly. We also studied the membranes of patient D.S. for their ability to bind p47-*phox* and p67-*phox* in a cell-free translocation assay. In this study we included another X91$^+$ patient, which previously has been described to carry a point mutation in gp91-*phox*, predicting a transversion of a proline into a histidine at residue 415 (16). The neutrophil membranes of this patient R.C. have been shown to contain the cytosolic oxidase components after stimulation with PMA of intact cells (34). The results of the cell-free translocation assay are shown in Table II: control membranes preincubated with control cytosol showed significant superoxide production after reisolation, whereas both patients' membrane fractions preincubated with control cytosol showed no superoxide production. The membrane fraction of an X91^0 patient also showed no O_2^- production in this assay. The reisolated membranes were also analyzed on an immunoblot to determine the association of cytosolic oxidase components. Fig. 5 *A* demonstrates the presence of membranes in all samples, as indicated by the membrane marker CEA. Fig. 5 *B* shows the same blot stained for the membrane-bound oxidase components gp91-*phox* and p22-*phox*. As expected, these components were present in all control samples and in the samples of patients D.S. and R.C. but not in the membrane fraction of X91^0 neutrophils.

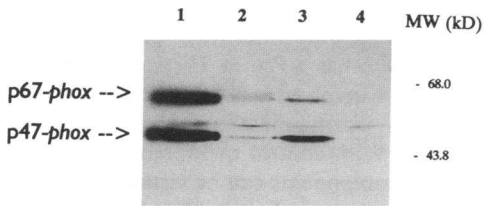

Figure 4. Western blot analysis of plasma membranes of activated neutrophils. 40×10^6 cells were incubated with or without 100 ng/ml PMA for 10 min at 37°C. Fractionation of the cells was performed as described in Methods. 10% PAGE was conducted for the plasma membranes with comparable amounts of cytochrome b_{558} run on the gel and blotted onto nitrocellulose. The blot was stained with rabbit antisera against p47-*phox* and p67-*phox*. Lane *1*, control with PMA; lane *2*, control without PMA; lane *3*, patient D.S. with PMA; lane *4*, patient D.S. without PMA.

Table II. Superoxide Production of Reisolated Membranes in the Cell-free Assay

Source of plasma membranes	μM O_2^-/min
Control	3.55±0.77*
Patient D.S.	0.02±0.01
Patient R.C.	0.01±0.01
X91^0 patient	0.00±0.00

Patients' or control neutrophil membranes (10 μg) were incubated with control cytosol (200 μg) in Oxidase Buffer at room temperature. After 10 min of incubation in the presence of SDS (100 μM) and GTPγS (10 μM), membranes were reisolated from each incubation mixture by sucrose gradient centrifugation. NADPH oxidase activity was determined in 1/5 part of the membrane fractions by incubation in oxidase buffer in the presence of SDS (100 μM), GTPγS (10 μM), and NADPH (250 μM) by the rate of cytochrome *c* reduction.
* Mean±SEM of three separate experiments.

In Fig. 5 *C* the same blot was treated with antibodies directed against the cytosolic oxidase components p67-*phox* and p47-*phox*. All control samples showed a significant association of p47-*phox* and p67-*phox* with the neutrophil membrane fraction. However, the neutrophil membranes of patient D.S. did not contain detectable amounts of cytosolic oxidase proteins, whereas the neutrophil membranes of patient R.C. did show a normal amount of p47-*phox* and p67-*phox*. The reisolated membranes of the X91^0 patient did not contain these cytosolic oxidase components, confirming the requirement of cytochrome *b* for translocation of p47-*phox* and p67-*phox* (34, 35, 36). Also, translocation of p47-*phox* and p67-*phox* was found to be fully dependent on the presence of SDS and GTP-γ-S during preincubation (results not shown).

The effect of peptide 491–504 of gp91-phox on NADPH oxidase assembly. To consolidate the importance of the gp91-*phox* domain mutated in patient D.S. for NADPH oxidase assembly, studies with synthetic peptides were performed. We used a peptide that mimicked domain 491–504, and a peptide of the same region with the same substitution as expected to be present in gp91-*phox* of patient D.S. Fig. 6 *A* shows the effect of these peptides on the NADPH oxidase activity in the cell-free assay. NADPH oxidase activity was inhibited by the wild-type peptide in a dose-dependent fashion, with an IC_{50} of about 10 μM. The mutated peptide was at least ten times less potent. Also, a peptide derived from domain 537–552 of gp91-*phox* did not cause a significant inhibition at concentrations up to 100 μM (results not shown). For comparison, we tested a syn-

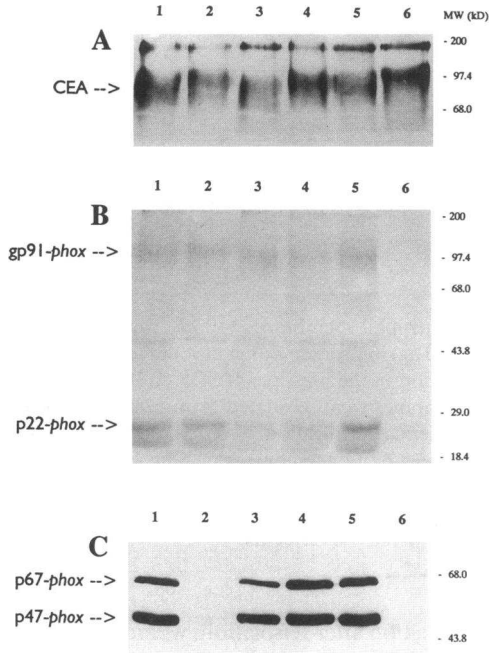

Figure 5. Western blot analysis of neutrophil membranes reisolated from the cell-free activation system. The reisolated membranes were prepared as described in the legend of Table II. $^4/_5$ parts of these membrane samples were precipitated with 10% (wt/vol) trichloroacetic acid and resuspended in sample buffer as described in Methods. After 10% PAGE, the proteins were blotted onto nitrocellulose. The blot was stained with a rabbit antiserum directed against the membrane marker carcino embryonic antigen (*A*), with mAbs 449 and 48 (*B*), or with rabbit antisera against p47-*phox* and p67-*phox* (*C*). Lane *1*, control 1; lane *2*, patient D.S.; lane *3*, control 2; lane *4*, patient R.C.; lane *5*, control 3; lane *6*, X91^0 patient. The results shown are representative of three independent experiments.

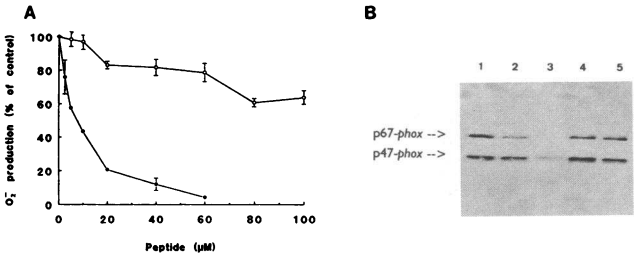

Figure 6. Effect of peptide 491–504 on NADPH oxidase activity in the cell-free assay and in the translocation assay. (*A*) Dose–response curve. A synthetic peptide containing the amino acids 491–504 of gp91-*phox* (*closed circles*) or a peptide containing the mutation of patient D.S. (*open circles*) was added to the cell-free system, 2 min before addition of SDS and GTPγS. NADPH oxidase activity was expressed as percentage of the superoxide production of control samples with only solvent added. The results shown are mean±SEM from three separate experiments. (*B*) Translocation of p47-*phox* and p67-*phox*. Neutrophil membranes (10 μg of protein) and cytosol (200 μg of protein) were preincubated in the presence of solvent (1%, vol/vol) (lane *1*), 10 μM wild type peptide (lane *2*), 30 μM wild-type peptide (lane *3*), 30 μM mutated peptide (lane *4*), or 100 μM mutated peptide (lane *5*). The membranes were activated and reisolated as described in the legend of Table II. NADPH oxidase activity in one fifth of the reisolated membranes (in μM O_2^-/min) amounted to 5.10 (lane *1*), 3.57 (lane *2*), 0.26 (lane *3*), 5.72 (lane *4*), and 4.29 (lane *5*). The remaining material was processed for Western blotting as described in Fig. 4.

thetic peptide resembling domain 556–569 of gp91-*phox*, which previously has been proposed (37, 38) to be important for the binding of the cytosolic oxidase components. Inhibition by this peptide proved to be less efficient than the 491–504 peptide under the same experimental conditions (IC_{50} about 40 μM, results not shown). Subsequently, peptide 491–504 was tested for inhibition in the translocation assay of cytosolic components (Fig. 6 *B*). The peptide clearly inhibited the association of p47-*phox* and p67-*phox* with the membrane fraction. 30 μM of peptide, added during the preincubation of neutrophil membranes and cytosol, resulted in a reduction of translocation of p47-*phox* and p67-*phox* of 82 and 93%, respectively, as determined by densitometry. In contrast, at a concentration of 100 μM the mutated peptide caused an inhibition of only 27 and 38% in translocation of p47-*phox* and p67-*phox*, respectively.

Discussion

We have identified a novel missense mutation in the gene encoding the large subunit of the neutrophil cytochrome b_{558} heterodimer in a patient with X-linked, cytochrome b_{558}-positive CGD (X91$^+$). The mutation is located in exon 12 and predicts a non-conservative amino acid substitution at residue 500 of aspartate into glycine. The mutation is situated in the cytoplasmic tail of the protein (21), which is thought to be important for binding of the cytoplasmic proteins p47-*phox* and p67-*phox* (36). This position is not associated with binding of NADPH or FAD, as predicted by homology with ferrodoxin-NADP$^+$ reductase (18, 19, 20), but might be located in a loop that prevents access of NADPH to the cleft that contains FAD (39).

In this study, we have investigated the translocation of p47-*phox* and p67-*phox* to the plasma membrane in activated neutrophils of patient D.S. and of a healthy donor. Whereas the activated control membranes showed a significant association of both cytoplasmic proteins (Fig. 4, lane *1*), the activated membranes of patient D.S. showed a strongly diminished association of p67-*phox* and a reduced association of p47-*phox* (Fig. 4, lane *3*). This residual staining might be indicative for translocation induced by PMA that is, however, not stabilized by proper protein–protein interactions.

We also performed cell-free translocation experiments. The neutrophil membrane fraction of patient D.S. was compared to the membrane fraction of the only other X91$^+$ patient known with a point mutation, who has a Pro → His substitution at residue 415, predicted to be involved in NADPH binding (18). In this translocation assay, plasma membranes were reisolated after in vitro activation. With control membranes, the associated cytosolic oxidase components can be visualised (Fig. 5 *C*, lanes *1*, *3*, and *5*), whereas membranes of an X91^0 patient show no associated cytosolic proteins (Fig. 5 *C*, lane *6*). Our results clearly show a normal translocation of p47-*phox* and p67-*phox* to the membranes with the ^{415}Pro → His mutation (confirming the findings in intact cells) (34) but no translocation to the membranes with the ^{500}Asp → Gly mutation (Fig. 5 *C*, lanes *4* and *2*, respectively). This indicates an important contribution of residue 500 to the binding of at least p47-*phox*, because this protein is thought to interact first with cytochrome

b_{558} (40). The p47-*phox* contains many basic residues, that might be important for the interaction with cytochrome b_{558}, because this interaction can be blocked by positively charged synthetic peptides (33). In patient D.S. an acidic residue (Asp), that could be an important counterpart for the basic domains of p47-*phox,* is changed into a non-charged amino acid (Gly). However, it cannot be excluded that the ^{500}Asp → Gly substitution causes an alteration in the secundary structure in this region of the protein, and therefore leads to a nonfunctional cytochrome b_{558}.

To confirm the importance of the mutated region of gp91-*phox* in patient D.S., we studied the effect of a synthetic peptide identical to domain 491–504 of gp91-*phox* and a peptide of the same region but with the Asp → Gly substitution at residue 500. A clear inhibitory effect of the wild-type peptide on oxidase activity in the cell-free assay was observed (Fig. 6 *A*). This effect can be explained by the inhibited translocation of p47-*phox* and p67-*phox,* as demonstrated in Fig. 6 *B*. In contrast, the mutated peptide was much less effective in both assays. These results indicate that the found mutation resides in a domain of gp91-*phox* that is exposed on the outside of the protein, and provide additional evidence for the hypothesis that residue ^{500}Asp is involved in the binding of cytosolic oxidase components. The relevance of region 488–497 of gp91-*phox* is indicated by an X91$^+$ patient (15), who has a deletion of these 10 amino acids due to a splicing abnormality, leading to a non-functional cytochrome b_{558}. Interestingly, the ten deleted amino acids are close to the missense mutation at residue 500 we identified in patient D.S. and there is substantial overlap between residues deleted in the patient of ref. 15 and those used in the blocking peptide (residues 491–504). Previously, Malech and colleagues (37, 38) have suggested a critical role for residues 559–565 of the gp91-*phox* for the interaction with the cytosolic oxidase components. Evidently, the results of those studies are not incompatible with the data presented here, but it is of interest to note that under identical experimental conditions peptide 491–504 was more potent in inhibiting NADPH oxidase assembly than peptide 556–569.

Taylor et al. (39) have constructed a model of gp91-*phox,* in analogy to NADP$^+$-ferridoxin reductase, in which residues 414–504 form a loop that shuts off the cleft that contains the FAD moiety. These investigators speculate that this protein loop may move when the NADPH oxidase is activated, thus allowing NADPH to bind and deliver its electrons to FAD. Our results are in accordance with this model, and, in addition, suggest that direct binding of p47-*phox* and/or p67-*phox* to cytochrome b_{558} causes the activating events.

To test the effect of specific mutations in gp91-*phox* (including the ^{500}Asp → Gly mutation presented here) on NADPH oxidase assembly, expression systems with mutated cDNAs are currently developed in our laboratory.

Acknowledgments

We thank Dr. T. W. Kuijpers for his help in the transfer of blood from patient R.C.

This study was supported by the Netherlands Organization for the Advancement of Pure Research (NWO) (grant 900-503-110).

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