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Two CGD Families with a Hypomorphic Mutation in the Activation Domain of p67phox

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Abstract

Study background: Chronic granulomatous Disease (CGD) is a rare immunodeficiency caused by a defect in the leukocyte NADPH oxidase. This enzyme generates superoxide, which is needed for the killing of bacteria and fungi by phagocytic leukocytes. Most CGD patients have mutations in *CYBB*, the X-linked gene that encodes gp91phox, the catalytic subunit of the leukocyte NADPH oxidase. We report here three autosomal recessive CGD patients from two families with a homozygous mutation in *NCF2*, the gene that encodes p67phox, the activator subunit of the NADPH oxidase.

Methods: Leukocyte NADPH oxidase activity, expression of oxidase components and gene sequences were measured with standard methods. The mutation found in the patients' *NCF2* gene was expressed as Ala202Val-p67phox in K562 cells to measure its effect on NADPH oxidase activity. Translocation of the mutated p67phox from the cytosol of the patients' neutrophils to the plasma membrane was measured by confocal microscopy and by Western blotting after membrane purification.

Results: The exceptional feature of the A67 CGD patients reported here is that the p.Ala202Val mutation in the activation domain of p67phox was clearly hypomorphic: substantial expression of p67phox protein was noted and the NADPH oxidase activity in the neutrophils of the patients was 20-70% of normal, dependent on the stimulus used to activate the cells. The extent of Ala202Val-p67phox translocation to the plasma membrane during cell activation was also stimulus dependent. Ala202Val-p67phox in K562 cells mediated only about 3% of normal oxidase activity compared to cells transfected with the wild-type p67phox.

Conclusion: The mutation found in *NCF2* is the cause of the decreased NADPH oxidase activity and the (mild) clinical problems of the patients. We propose that the p.Ala202Val mutation has changed the conformation of the activation domain of p67phox, resulting in reduced activation of gp91phox.

Keywords: Chronic granulomatous disease; NADPH oxidase; p67phox; NCF2; p67phox activation domain; hypomorphic mutation; p67phox translocation

Abbreviations

Ala: Alanine; Arg: Arginine; Asp: Aspartic acid; CGD: Chronic Granulomatous Disease; CH50: Complement Hemolysis 50%; *CYBA*: Cytochrome *b* alpha; *CYBB*: Cytochrome *b* beta; DHR: Dihydrorhodamine-1,2,3; DFP: Diisopropyl fluorophosphate; FAD: Flavine Adenine Dinucleotide; fMLP: formyl-methionyl-leucyl-phenylalanine; GEF: Guanine nucleotide Exchange Factor; GTP: Guanosine 5'-triphosphate; Lys: Lysine; mAb: Monoclonal antibody; MR: Magnetic Resonance; NADPH: Nicotinamide Adenine Dinucleotide Phosphate (reduced); NBT: Nitro-Blue Tetrazolium; *NCF*: Neutrophil Cytosolic Factor; Nox: NADPH oxidase; O_2^-: Superoxide; pAb: Polyclonal Antibody; PAF: Platelet-Activating Factor; PAGE: Polyacrylamide Gel Electrophoresis; PB1: Phox and Bem-1; PBS: Phosphate-Buffered Salt; phox: Phagocyte oxidase; PI3: Phospho-inositol-3; PMA: Phorbol Myristate Acetate; ROS: Reactive Oxygen Species; S.D.: Standard Deviation; SDS: Sodium dodecylsulphate; STZ: Serum-Treated Zymosan; TPR: Tetratricopeptide; Val: Valine; X-CGD: X chromosome-linked CGD

Introduction

Phagocytic leukocytes protect us against bacteria, yeasts and fungi by ingesting these micro-organisms, followed by intracellular killing, or by attachment and extracellular killing. In this process, release of

stored bactericidal proteins as well as generation of reactive oxygen species (ROS) by the phagocytes is essential [1]. Superoxide (O_2^-), as a precursor of other ROS, is produced by the leukocyte NADPH oxidase. This enzyme consists of two membrane-bound components, glycoprotein (gp)91phox (phox from phagocyte oxidase), also called Nox2, and p22phox, together forming flavocytochrome b_{558}, and three cytosolic proteins called p40phox, p47phox and p67phox. The gp91phox protein is the catalytic subunit; it contains an NADPH binding site, one FAD and two heme prosthetic groups. The p22phox protein stabilizes gp91phox in membranes and also provides a docking site for the cytosolic p47phox subunit. The three cytosolic components form a tight complex that changes its conformation and translocates to the gp91phox/p22phox complex in the plasma membrane upon cell activation, e.g. after contact with micro-organisms [2]. Superoxide production also requires membrane translocation and activation of the small Rho GTPase Rac (preferentially Rac1 in macrophages and Rac2 in neutrophils), which subsequently binds to the tetratricopeptide regions (TPR) in p67phox (Supplementary Figure S1) and to the plasma membrane [2,3]. Following assembly and activation of the cytosolic subunits on flavocytochrome b_{558}, the NADPH binding site of gp91phox becomes available for NADPH in the cytosol. NADPH donates two electrons to gp91phox, which are then transported within the protein to FAD, thereafter to the hemes, and finally to molecular oxygen at the other side of the membrane. Electron transfer requires Rac-activated p67phox binding to gp91phox as well as interactions between Rac and gp91phox [4]. In this way, superoxide is generated within the phagosome or on the cell surface, in close proximity to the ingested or attached micro-organisms.

Genetic failure of superoxide generation leads to a rare syndrome of recurrent, life-threatening infections called Chronic Granulomatous Disease (CGD) [5]. The most common form of CGD (about 70% of all cases) is due to mutations in *CYBB*, the gene that encodes gp91phox [6]. Since CYBB is located on the X chromosome, this form of CGD is found almost exclusively in males. The autosomal forms of CGD are less common, with mutations in *NCF1* (p47phox) in about 20% of cases and mutations in *CYBA* (p22phox) or *NCF2* (p67phox) each in about 5% of cases [7]. A single patient with mutations in *NCF4* (p40phox) has also been described [8]. Usually, these mutations lead to complete absence of the protein involved, but a few cases are known with diminished expression of gp91phox (resulting in diminished NADPH oxidase activity) or normal expression of completely inactive gp91phox [9]. We describe here three unusual CGD patients from two families with an identical mutation in *NCF2*, leading to diminished to near normal p67phox expression and substantial residual NADPH oxidase activity in their neutrophils. Expression of the mutated p67phox protein in a cellular test system proved this mutation to be the cause of the disease.

Materials and Methods

Cell purification

Blood samples were obtained from healthy controls, patients and their family members by their physician, following the procedures and appropriate consent protocols approved by the Human Subjects Committee of the hospitals involved. Total leukocytes were obtained by lysis of the erythrocytes in the pellet fraction with a non-fixing lysis solution of 155 mM NH_4Cl, 10 mM $NaHCO_3$ and 0.1 mM EDTA. Neutrophils were purified by centrifugation of the leukocyte fraction over a layer of Percoll with a specific gravity of 1.077 g/ml. The cells in the pellet (neutrophils) were suspended in Hepes medium [132 mM NaCl, 6 mM KCl, 1 mM $MgSO_4$, 1.2 mM KH_2PO_4, 20 mM Hepes, 5.5 mM glucose and 0.5% (wt/vol) human albumin (pH 7.4)], and the cells in the ring fraction (mononuclear leukocytes) were used for RNA purification.

NADPH oxidase tests

Oxygen consumption by neutrophils activated with serum-treated zymosan (STZ) or phorbol myristate acetate (PMA) was measured with an oxygen electrode [10]. The dihydrorhodamine-1,2,3 (DHR) test was performed with total leukocytes as described in Köker et al. [11]. This test measures the oxidation of DHR by hydrogen peroxide to the fluorescent compound rhodamine-1,2,3 on a per-cell basis in a flow cytometer by gating of the neutrophils on the basis of forward and side scatter. The nitro-blue tetrazolium (NBT) slide test was performed as described by Meerhof and Roos [12]. This test was performed with purified neutrophils and measures in a semi-quantitative way the reduction of NBT by superoxide to dark blue precipitates in each cell. The formation of superoxide by purified neutrophils was also measured by reduction of ferricytochrome *c* to ferrocytochrome *c*, followed in a spectrophotometer at 550 nm [13]. Finally, the secretion of hydrogen peroxide by purified neutrophils was evaluated in a 96-well plate with Amplex Red (Molecular Probes, Life Technologies, Carlsbad, CA, USA) and horse-radish peroxidase [14]. The resulting resorufin was measured over a period of 30 min in a plate reader (Genios Plus, Tecan, Männedorf, Switzerland) at 590 nm (excitation at 535 nm). The steepest part of the slope was used for calculating the maximal rate of H_2O_2 production.

Expression of NADPH oxidase components

The expression of gp91phox, p22phox, p47phox and p67phox was analyzed in a flow cytometer with permeabilized and fixed blood cells as described [11]. Western blot analysis was performed after SDS-10% PAGE of DFP-treated neutrophil lysates in 2-mercapto-ethanol, transfer to nitrocellulose, blocking with 5% (w/v) milkpowder and incubation with mAb anti-p47phox (Santa Cruz Biotechnology, Santa Cruz, CA, USA; mouse-anti-human p47phox, clone 10, cat. no. SC-17845) and pAb anti-p67phox (Merck Millipore, Billerica, MA, USA; rabbit-anti-human p67phox, cat.no. 07-002). Conjugates were fluorescently labelled (LI-COR Biosciences, Lincoln, NE, USA), detected by scanning with the Odyssey Infrared Imagine System and quantified with Odyssey Application Software V3.0 (LI-COR).

Translocation of p67phox to the membrane in intact neutrophils

Neutrophils (5×10^6/ml, 1 ml) were stimulated with PMA (100 ng/ml) or STZ (1 mg/ml), washed once, and resuspended in 0.5 ml PBS with 0.1 mM diisopropyl fluorophosphate (DFP) for 10 min at 4°C. The cells were centrifuged and the pellet was resuspended in 100 μl of digitonin for 10 min at 4°C; for PMA-stimulated neutrophils a concentration of 150 μM digitonin in PBS was used, and for STZ stimulation a concentration of 300 μM digitonin in PBS. Thereafter, the cells were centrifuged (20 sec 20,000xg), the pellets were resuspended in Laemmli sample buffer and a Western blot was performed as described above. At these concentrations of digitonin, >90% of LDH was released from the cells and less than 5% of the

protease content (data not shown), indicating proper separation of the cytosol and the rest of the cell.

For immunofluorescence, neutrophils were incubated with PMA (100 ng/ml) or left untreated for 10 minutes at 37°C in suspension. Next, the cells were allowed to adhere for 10 minutes on fibronectin (10 ng/ml)-coated glass covers, followed by a 10-minute incubation with STZ (1 mg/ml) or PMA (100 ng/ml), or left untreated. Thereafter, the cells were fixed with 3.7% (w/v) formaldehyde for 10 minutes and permeabilized with 0.5% (w/v) Triton X-100 for 10 minutes. To visualize p67^{phox} protein, the cells were incubated with the corresponding rabbit-anti-human antibody (Merck) for 30 minutes at room temperature, followed by a 30-minute incubation with a secondary goat-anti-rabbit-Ig ALEXA-568 antibody (Invitrogen). Coverslips were mounted with Vectashield (Vector Laboratories Inc., Peterborough, UK) on microscope slides and imaged with a confocal microscope through a 63x oil-objective (LSM510 META; Carl Zeiss MicroImaging, Inc.).

Expression and functional testing of recombinant p67^{phox} in K562 cells

K562 cells, immature myeloid cell line cells that constitutively express p22^{phox}, were first stably transfected with gp91^{phox} cDNA in pEF-PGKpac [15] and then with p47^{phox} cDNA in pEF-PGKhygro [16]. Cells were selected as individual clones in 2 μg/ml puromycin and 250 μg/ml hygromycin for 3 weeks. A clone with high recombinant gp91^{phox} and p47^{phox} expression (K562-91-47) was used for further studies, and immunoblots were made as described [8]. K562-91-47 cells were transiently transfected with Amaxa by means of Nucleofector Kit V and protocol T-16kit V (Walkersville, MD, USA). Superoxide production by these cells was determined with isoluminol chemiluminescence in the presence of HRP as described [17].

Mutation analysis

Genomic DNA was isolated from total leukocytes by standard procedures and analyzed for mutations in *NCF2* exons and exon-intron boundaries by PCR amplification of each exon with its intronic boundaries, followed by bi-directional sequencing. The PCR conditions were as follows: 50 cycles of 5 s at 95°C, 30 s at 60°C and 15 s at 72°C, and for exon 16 50 cycles of 5 s at 95°C, 30 s at 52°C and 15 s at 72°C. The PCR products were sequenced with the Big dye terminator sequencing kit v1.1 (Applied Biosystems, Foster City, CA, USA).

Total mRNA was purified from the mononuclear leukocyte fraction and converted into cDNA by means of Superscript III first-strand synthesis system for RT-PCR (Invitrogen, Carlsbad, CA, USA).

Case Presentations

Family A, a presumably non-consanguineous Turkish immigrant family with three daughters and one son, originally from the province of Tokat, Turkey, and now living in London, UK

Patient A1 (eldest female sibling), born 1970: This lady presented at age 17 with a 4-year history of recurrent cutaneous abscesses. These were controlled with antibiotics alone, and she managed minor flares of these on her own without the need to seek medical advice. She also has a chronic inflammatory, discoid lupus-like rash on her face. At age 30, she had an episode of peripheral ulcerative keratitis with adjacent conjunctival granulomata. The keratitis itself was non-granulomatous on biopsy. There was a recurrent episode of keratitis at age 35 (during the second trimester of pregnancy). Both episodes responded well to topical steroids and chloramphenicol drops. Since the diagnosis of CGD was made, the patient has been on trimethoprim-sulfamethoxazole and itraconazole prophylaxis. Neutrophil testing (ferricytochrome *c* reduction assay) revealed about 10% of normal NADPH oxidase activity with PMA.

Patient A2 (male sibling), born 1987: The male sibling was diagnosed with CGD at birth by NBT slide testing. He completed all childhood vaccinations without complications, but suffered from recurrent oral ulceration, leg ulcers, folliculitis and skin abscesses throughout childhood, controlled with repeated courses of topical and systemic antibiotics. Levels of all immunoglobulin sub-classes were normal. The patient has been taking trimethoprim-sulfamethoxazole and itraconazole prophylaxis during the last twelve years. At age 19, he had a short episode of what was thought to be inflammatory bowel disease, with diarrhoea and rectal bleeding, although an MR-imaging of the abdomen at age 21 demonstrated no small or large bowel inflammation. He is currently in symptomatic remission with no history of other bacterial infections. Other members of this family did not present with medical problems.

Family B, Turkish family living in the province of Adana, Turkey, with no obvious relation to family A

The patient in this family is the only sibling, a girl born in 1990, whose parents are first cousins, with no history of early death in the family. She was referred to hospital at 8 years of age with diffuse pustular and eczematous lesions of the scalp skin, which were treated with systemic antibiotics, but without complete cure. CGD was diagnosed by impaired NBT test (all cells weakly positive). T cell function and lymphocyte subsets as well as CH50 were also normal. Her serum immunoglobulin levels were IgG 2380 mg/l, IgA 273 mg/l, IgM 73 mg/l, and IgE 71 mg/l, which is within normal limits. When she was 8 months of age, strabismus in the left eye was noticed after convulsions. During ophthalmological inspection, chorioretinitis and decreased vision in the left eye was diagnosed. She suffered repeatedly from chorioretinitis attacks in both eyes and has severe bilateral uveitis. At present, that has resulted in an almost 75% loss of vision in her left eye. She has been doing well for 10 years on prophylactic trimethoprim-sulfamethoxazole and itraconazole, at half dose during the last five years. Eczematous lesions of the scalp skin disappeared with Fe^{++} supplements. DHR analysis showed 5-10% of normal NADPH oxidase activity after stimulation of her neutrophils with PMA [11].

Results

All three patients had considerable NADPH oxidase activity, measured as oxygen consumption and as hydrogen peroxide release, in neutrophils activated by various stimuli (Figure 1A). This activity was about 50% of control values with unopsonized zymosan, 60-70% with serum-treated zymosan (STZ), 15-25% with phorbol-myristate acetate (PMA) and 20% with formyl-methionyl-leucyl-phenylalanine (fMLP)

in platelet-activating factor (PAF)-primed neutrophils. The difference in residual NADPH oxidase activity in the patients' cells activated with STZ as compared with PMA was highly significant, both in the oxygen consumption assay (p=0.009) and in the H_2O_2 release assay (p<0.0001). For comparison, neutrophils from two "classical" CGD patients (one with a one-nucleotide insertion in *CYBA* and another with a p.Arg102X nonsense mutation in *NCF2*) were also tested, in the same assay run. These neutrophils showed only 6% residual oxidase activity with unopsonized zymosan and 3% or less of control values with the other stimuli (Supplementary Table 1). The parents and sisters of patients A1 and A2 showed normal hydrogen peroxide release from their neutrophils (not shown). The mother of patient B showed normal hydrogen peroxide generation by her neutrophils in the DHR test (not shown). Western blots of neutrophil lysates from all three patients showed substantial expression of p47phox and p67phox (Figure 1B), as well as of gp91phox and p22phox (not shown).
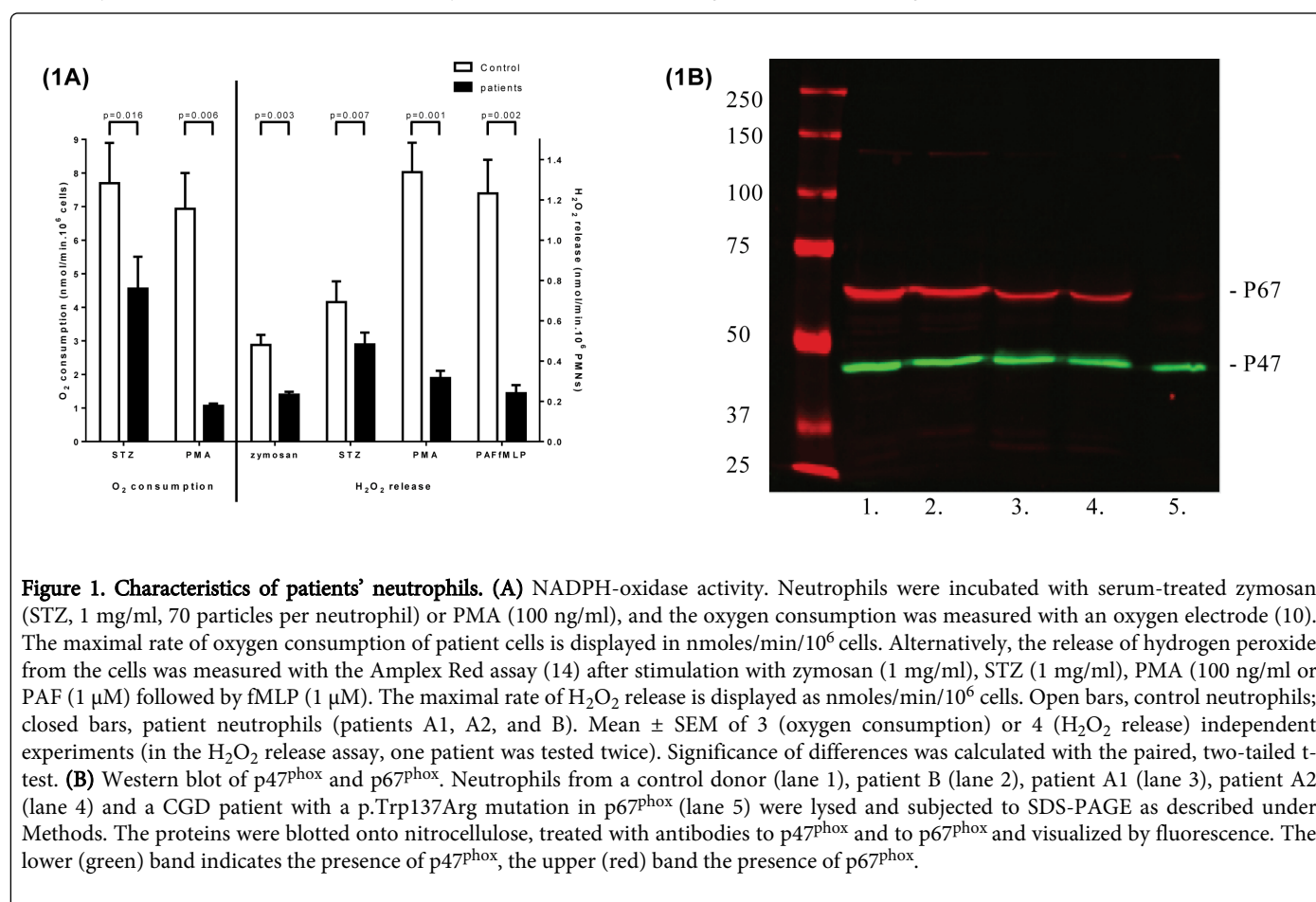

Figure 1. Characteristics of patients' neutrophils. (A) NADPH-oxidase activity. Neutrophils were incubated with serum-treated zymosan (STZ, 1 mg/ml, 70 particles per neutrophil) or PMA (100 ng/ml), and the oxygen consumption was measured with an oxygen electrode (10). The maximal rate of oxygen consumption of patient cells is displayed in nmoles/min/10^6 cells. Alternatively, the release of hydrogen peroxide from the cells was measured with the Amplex Red assay (14) after stimulation with zymosan (1 mg/ml), STZ (1 mg/ml), PMA (100 ng/ml or PAF (1 μM) followed by fMLP (1 μM). The maximal rate of H_2O_2 release is displayed as nmoles/min/10^6 cells. Open bars, control neutrophils; closed bars, patient neutrophils (patients A1, A2, and B). Mean ± SEM of 3 (oxygen consumption) or 4 (H_2O_2 release) independent experiments (in the H_2O_2 release assay, one patient was tested twice). Significance of differences was calculated with the paired, two-tailed t-test. **(B)** Western blot of p47phox and p67phox. Neutrophils from a control donor (lane 1), patient B (lane 2), patient A1 (lane 3), patient A2 (lane 4) and a CGD patient with a p.Trp137Arg mutation in p67phox (lane 5) were lysed and subjected to SDS-PAGE as described under Methods. The proteins were blotted onto nitrocellulose, treated with antibodies to p47phox and to p67phox and visualized by fluorescence. The lower (green) band indicates the presence of p47phox, the upper (red) band the presence of p67phox.

We started DNA analysis by sequencing the exons and intron-exon boundaries of *CYBB*, as well as the first 600 nucleotides of its promoter region, because mutations are known in this gene to cause diminished expression of gp91phox and diminished NADPH oxidase activity [9]. However, no mutations were found in *CYBB* in these patients. We then investigated whether a common dinucleotide deletion in *NCF1* was present, because deficiency of p47phox is known to leave some residual NADPH oxidase activity [18]. However, a gene scan [19] failed to detect this GT deletion in *NCF1*. We then sequenced the relevant parts of *CYBA* (from gDNA) and *NCF1* (from cDNA) but found no mutations or indications for mRNA missplicing. Finally, in *NCF2* we did find a homozygous c.605C>T mutation in all three patients, predicting p.Ala202Val in p67phox (Figure 2A). The parents and sisters of patients A1 and A2 were heterozygotes for the c.605C>T mutation, as were the parents of patient B. In more than 100 healthy controls we did not observe this mutation. To investigate whether the mutant p67phox mRNA was as stable as the wild-type p67phox mRNA, we amplified the relevant part of p67phox cDNA in the mother of patient B and found both cDNA species to be present in

similar amounts (Figure 2B). Moreover, the wild-type and the mutated cDNA amplicon had a similar size, which rules out activation of a cryptic splice site by the mutation.

The question remained whether this mutation in p67phox was really the cause of the diminished NADPH oxidase activity in the neutrophils of the patients. To investigate this, we expressed the mutant p67phox Ala202Val and the wild-type p67phox in K562 cells stably transfected with p47phox and gp91phox and expressing endogenous Rac and p22phox. As shown in Figure 3A, both mutant and wild-type p67phox proteins were expressed in similar amounts in these cells. For comparison, we also expressed p67phoxVal204Ala in the K562 cells, because this mutation, which – like Ala202Val – resides in the p67phox "activation domain" that is critical for activation of electron transport in gp91phox, has been shown in an *in vitro* system to lack all oxidase-activating potency [20,21]. Moreover, an Ala202Asn mutation in p67phox markedly reduces NADPH oxidase activity in a gp91phox-dependent whole cell system stimulated with PMA [21]. The p67phoxVal204Ala protein we used also contained a C-terminal myc

tag, which does not have any effect on the superoxide production supported by p67phox wt-tagged protein [16]. Figure 3B shows that both the p67phox Ala202Val and the p67phox Val204Ala variant were far less effective than the wild-type p67phox in inducing NADPH oxidase activity in PMA-activated K562 cells. In three separate experiments, the Ala202Val variant induced $2.7 \pm 1\%$ (S.D.) of wild-type p67phox-induced oxidase activity, whereas the Val204Ala variant induced $1.0 \pm 0.8\%$ of wild-type p67phox-induced oxidase activity.

Finally, we studied the translocation of p67phox to the cell membrane after NADPH oxidase activation of neutrophils with two different assays, as described under Methods. The results are shown in Figure 4 and indicate that with PMA as the stimulus, the translocation of the p67phox protein from the cytosol to the membrane was clearly diminished, whereas with STZ, the translocation was close to normal. In control experiments with classical X-CGD neutrophils (without expression of gp91phox) the translocation of p67phox was completely absent with either PMA or STZ (Supplementary Figure S2).
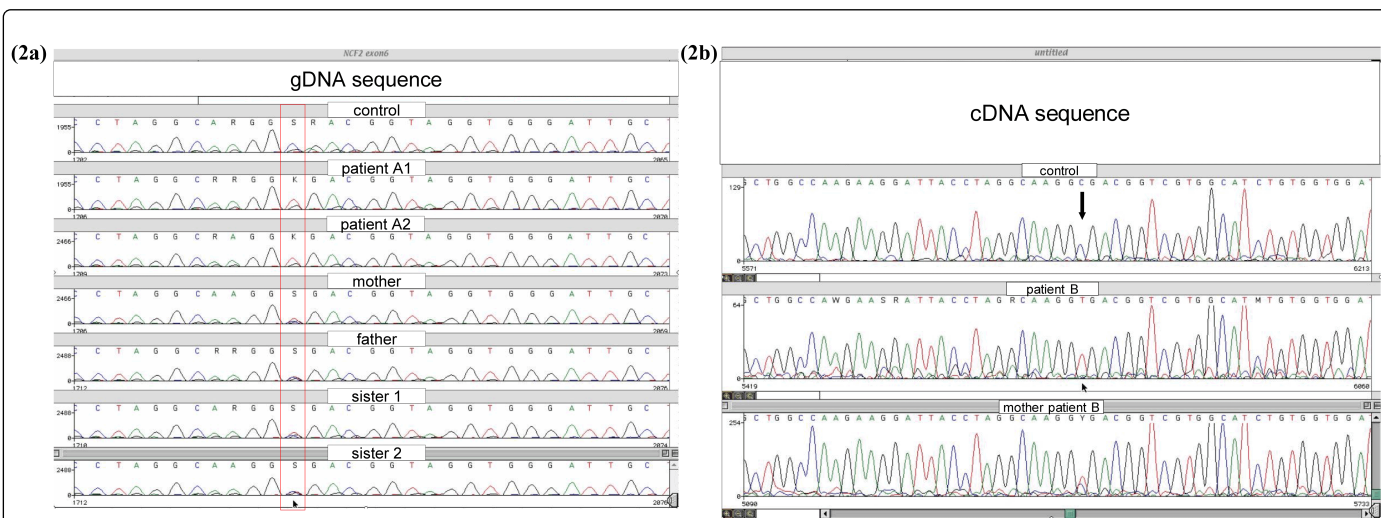

Figure 2: DNA and RNA sequencing of *NCF2* (end exon 6). Figure 2A shows the sequence obtained from genomic DNA of family A, with patients A1 and A2 being homozygous for the c.605C>T mutation and all other family members heterozygous for this mutation. Figure 2B shows the sequence obtained with cDNA of patient B and her mother. The c.605C peak and the c.605T peak in the mother have a similar height, indicating that the mutated c.605T mRNA is as stable as the wild-type c.605C mRNA.
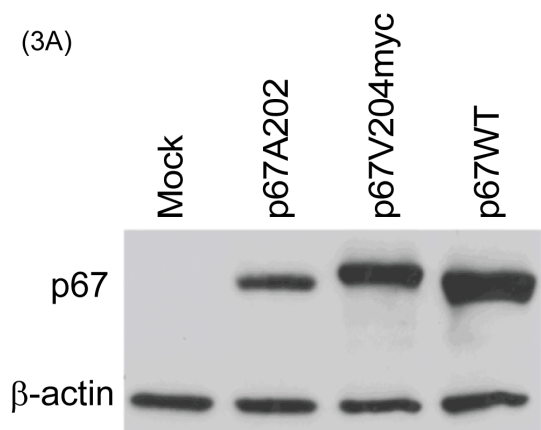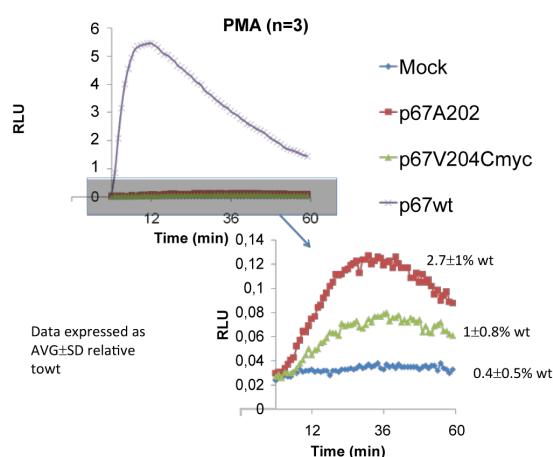

Figure 3: Expression and function of p67phox in K562 cells. K562 cells containing all NADPH oxidase components except p67phox were transfected with cDNA encoding p67phoxAla202Val (p67A202), p67phoxVal204Ala with a myc-tag (p67V204myc) or p67phoxAla202 (p67wt). A representative Western blot in Figure 3A shows that all proteins were expressed in similar amounts in the cells. Figure 3B shows that upon activation with PMA, the mutated proteins did not support the oxidase activity in K562 cells, in contrast to the wild-type protein.
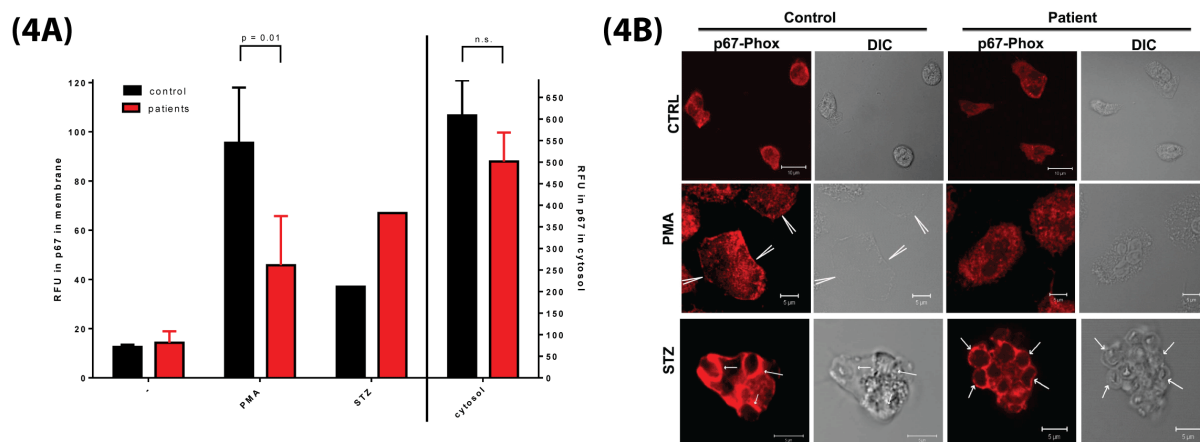
Figure 4: Translocation of p67phox to the cell membrane in intact neutrophils. **(A)** Quantification of p67phox translocation in stimulated human neutrophils. Human neutrophils (5×10^6/ml) were stimulated with PMA (100 ng/ml) for 10 min or with STZ (1 mg/ml) for 20 min. Separation of cytosol and the rest of the cells, followed by Western blot analysis of p67phox was performed as described under Materials and Methods. The amount of p67phox was quantified by means of fluorescently labeled conjugates, and detected by scanning with the Odyssey Infrared Imagine System and Odyssey Application Software V3.0. Black bars, control neutrophils; Red bars, patient neutrophils (patient A1, A2, and B). Mean ± SEM of 3 independent experiments for PMA. With STZ only the cells of patient B were tested. Significance of differences was calculated with the paired, two-tailed t-test. **(B) Visualization of p67phox translocation in stimulated human neutrophils.** Neutrophils from a control donor and from patient B were incubated with PMA (100 ng/ml) or left untreated for 10 minutes at 37°C in suspension. The cells were then allowed to adhere on fibronectin-coated glass covers, followed by a 10-minute incubation with STZ (1 mg/ml) or left untreated. Thereafter, the cells were fixed with formaldehyde and permeabilized with Triton X-100. To visualize p67phox protein, the cells were incubated with rabbit-anti-human-p67phox, followed by incubation with a secondary goat-anti-rabbit-Ig ALEXA-568-labeled. Coverslips were mounted with Vectashield on microscope slides and imaged with a confocal microscope through a 63x oil-objective. Note that with PMA, p67phox translocates to the plasma membrane of control neutrophils (arrows), but much less so to the plasma membrane of patient neutrophils. In contrast, translocation of p67phox to the phagosomal membrane surrounding internalized STZ (arrowheads) is similar in control and patient neutrophils.

Discussion

CGD patients with residual expression of NADPH oxidase components as well as residual NADPH oxidase activity are rare. Only four patients in three families have been described with low expression of p67phox and/or low NADPH oxidase activity [22-24]. The first of these had a deletion of one amino acid (Lys58) on one allele of *NCF2* and an undefined large deletion on the other allele [22]. The Lys58-deleted protein was expressed to a certain extent (tested on Western blot with a polyclonal antibody against p67phox), but whether this was a normal expression (from one allele) or diminished expression could not be decided. The Lys58 deletion is in the fourth TPR domain and destroyed the interaction with Rac and the translocation of p67phox to the membrane in PMA- or STZ-activated neutrophils [22]. The NADPH oxidase activity in the neutrophils of this patient was completely absent with all stimuli tested. However, in the so-called cell-free system with recombinant proteins and neutrophil membranes, SDS and GTPγS did induce the translocation of these cytosolic proteins, although the NADPH oxidase activity was still absent.

The second patient had a missense mutation in *NCF2* that caused replacement of aspartic acid by valine at position 108 in p67phox [23]. This Asp108Val replacement is between the third and fourth TPR region in p67phox and left substantial residual NADPH oxidase activity (15-20% of normal) in the patient's PMA-activated neutrophils (tested in the DHR assay). In a flow cytometric assay with permeabilized

neutrophils, p67phox was undetectable with a monoclonal antibody. The authors speculate that the mutation may have changed the conformation of p67phox, rendering it undetectable with the monoclonal antibody used, but still able to interact to some extent with gp91phox for inducing some NADPH oxidase activity.

In the last family, two brothers were recognized as CGD patients when they were already in their fifties [24]. They had a splice site mutation in *NCF2* that gave rise to an in-frame deletion of exons 11 and 12 (amino acids 309-342, PB1 domain). The neutrophils from these patients showed 10-15% of normal NADPH oxidase activity after stimulation with PMA in the DHR assay and in the lucigenin-enhanced chemiluminescence assay. This result was reproduced in K562 cells that contained all NADPH oxidase components except p67phox transduced with the Δexon11_12p67phox cDNA. The authors speculate that the p67phox protein with the exon11_12 deletion was to some extent expressed and functional in the patients' phagocytes.

The hypomorphic mutation in the three patients described in this article is in the so-called Activation Domain of p67phox (Supplementary Figure S1). This stretch of twelve amino acids (199-210) is essential for the oxidase-inducing capacity of p67phox[20]. In a cell-free oxidase system, it was found that mutations in this domain do not affect binding of p67phox to p47phox or to Rac but do inhibit the oxidase activity [20]. Alanine-202 is highly conserved in p67phox from humans, mouse, chicken, frog, fish and lancelet, as well as in Noxa1 of humans, mouse and fish and in fungal NoxR [21].

Mutation of alanine-202 in p67phox into leucine inhibits the cell-free oxidase activity induced by arachidonic acid by about 50%, and a Val204Ala mutant totally blocks this activity. This last mutant associates with the membrane (presumably with gp91phox) as well as does the wild-type p67phox [20]. Direct interaction of p67phox with gp91phox was shown by Dang et al. [25,26] by overlay techniques and GST pull-down assays, but the Activation Domain of p67phox was not necessary for this reaction. Thus, the binding of p67phox to gp91phox is probably mediated by a site in p67phox different from the Activation Domain, but the induction of oxidase activity in gp91phox is strictly dependent on this domain. The site in gp91phox interacting with the Activation Domain of p67phox is not known.

The findings in our patients corroborate these notions and extend the findings to intact, primary phagocytes. We found partial inhibition of oxidase activity in the patients' intact neutrophils with Ala202Val p67phox, as was found with the recombinant Ala202Leu variant of p67phox in the cell-free system [20]. Remarkably, much more oxidase activity was induced in the patients' neutrophils with zymosan or STZ than with the soluble activators PMA or PAF/fMLP. This correlates with the normal translocation of p67phox to the membrane after neutrophil activation with STZ and clearly diminished translocation after activation with PMA. It suggests that the Ala202Val mutation in p67phox affects the translocation and – perhaps as a result – also the proper assembly or activation of the NADPH oxidase complex following translocation of the cytosolic components to flavocytochrome b_{558} in the plasma membrane. This reduced translocation of Ala202Val-p67phox might be due to reduced binding of p67phox to gp91phox, which would be in contrast to the conclusion drawn by Dang et al. from studies with purified neutrophil and recombinant proteins that the Activation Domain of p67phox does not interact with gp91phox [25,26]. Unfortunately, the 3D structure of the Activation Domain of p67phox is unknown [27-30]. However, it is known that different stimuli induce different activation pathways in neutrophils, especially with respect to the synthesis of various lipid products needed for assembly of an active oxidase complex [31-34]. The type or amount of lipid mediators generated in STZ-activated neutrophils may have been sufficient for almost normal oxidase activation by Ala202Val-p67phox, in contrast to the situation in PMA-activated neutrophils. Since lipid mediator generation in K562 cells may be different from neutrophils, this may also explain the low oxidase activation by Ala202Val-p67phox in transfected K562 cells as compared to the patients' neutrophils. Alternatively, since the signal transduction pathway induced by PMA (protein kinase C activation leading to p47phox phosphorylation) is different from that induced by STZ (tyrosine phosphorylation of PI3 kinase leading to GEF and Rac activation), the translocation of p67phox to gp91phox and subsequent activation of gp91phox may be differently affected by mutations in p67phox. Thus, the p47phox-dependent pathway induced by PMA may be more sensitive to mutations leading to conformational changes in p67phox than the Rac-dependent pathway induced by STZ.

Our patients raise the question how much NADPH oxidase activity is required to be able to lead a normal life. The high residual oxidase activity in the patients' neutrophils may have protected the patients to a certain extent from the full-blown CGD symptomatology. Indeed, their clinical problems were mild in comparison to those of oxidase-null CGD patients. On the other hand, it should be taken into account that we tested the neutrophil NADPH oxidase activity in *in vitro* assay systems, with strong stimuli. *In vivo*, more subtle stimuli may be encountered, with which the mutant p67phox may be unable to properly activate the NADPH oxidase. Thus, high residual NADPH

oxidase activity *in vitro* is no guarantee for protection against pathogenic infections *in vivo*, but it may help in ameliorating the symptoms [35] and increase the chance of survival [36].

Acknowledgements

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