Disturbed Interaction of p21-rac with Mutated p67-phox Causes Chronic Granulomatous Disease

By Jeanette H.W. Leusen,*+ Angelique de Klein,* Petra M. Hilarius,* Anders Åhlin,§ Jan Palmblad,§ C.I. Edvard Smith,† Dagmar Dickmann,† Alan Hall,§ Arthur J. Verhoeven,* and Dirk Roos*

From the *Central Laboratory of the Netherlands Red Cross Blood Transfusion Service and Laboratory for Experimental and Clinical Immunology, University of Amsterdam, 1066 CX Amsterdam; †Department of Pediatric, Emma's Children's Hospital, Academic Medical Center, 1105 A2 Amsterdam, The Netherlands; §Department of Pediatrics, Sachs' Children's Hospital and Medicine, The Center for Inflammation Research, Stockholm Söder Hospital, Stockholm, S-11895 Sweden; †Center for Bio Technology, Karolinska Institute, NOVUM, Stockholm, S-14157 Sweden; and **MRC Laboratory for Molecular Cell Biology and Department of Biochemistry, University College London, London WC1E 6BT, UK

Summary

Chronic granulomatous disease (CGD) is characterized by the failure of phagocytic leukocytes to generate superoxide, needed for the intracellular killing of microorganisms. This is caused by mutations in any one of the four subunits of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. In a rare, autosomal recessive form of CGD, a 67-kD cytosolic component of this enzyme (p67-phox) is missing. We here report on a patient with a mutation in the p67-phox gene that leads to expression of a nonfunctional p67-phox protein. The purified granulocytes of this patient failed to produce superoxide and contained about half of the normal amount of p67-phox. Analysis of the cDNA and genomic DNA of this patient showed that the patient is a compound heterozygote for a triplet nucleotide deletion in the p67-phox gene, predicting an in-frame deletion of lysine 58 in the p67-phox protein and a larger deletion of 11-13 kb in the other allele. Interestingly, the 58Lys deletion in p67-phox disrupts the interaction with p21-rac1, a ras-related protein involved in the activation of the NADPH oxidase. In contrast to normal neutrophils, in which p47-phox and p67-phox translocate to the plasma membrane upon cell activation, the cells of the patient did not show this translocation, indicating that an interaction between p67-phox and p21-rac1 is essential for translocation of these cytosolic proteins and activation of the NADPH oxidase. Moreover, this CGD patient represents the first case of a disease caused by a disturbed binding of a ras-related protein to its target protein.

Phagocytic leukocytes use reactive oxygen metabolites to kill ingested microorganisms. The first step in the production of these compounds is the generation of superoxide by the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase enzyme in these cells. This enzyme consists of a flavocytochrome (cytochrome b556), located in the plasma membrane (1), and several cytosolic components that translocate to the plasma membrane upon activation of the oxidase (2, 3). This activation is initiated by attachment of opsonized microorganisms to cell surface receptors and serves to restrict the generation of the reactive oxygen compounds to periods of phagocytosis. During activation, the 47- and 67-kD cytosolic oxidase components (called p47-phox and p67-phox, respectively) couple to the flavocytochrome and probably induce a conformational change in this protein that renders its flavin accessible to NADPH (4). As a result, electrons flow from NADPH via flavin adenine dinucleotide (FAD) and heme to oxygen, thus generating superoxide.

Mutations in either the α or the β subunit of cytochrome b556 in p47-phox or in p67-phox lead to a dysfunction of the NADPH oxidase enzyme (5). Patients with such mutations suffer from chronic granulomatous disease (CGD), characterized by severe recurrent infections. There

1Abbreviations used in this paper: CGD, chronic granulomatous disease; FAD, flavin adenine dinucleotide; GST, glutathione-S-transferase; NADPH, nicotinamide adenine dinucleotide phosphate; PAF, platelet-activating factor; SOD, superoxide dismutase; STZ, serum-treated zymosan.
are at least two other proteins thus far described to be involved in the NADPH oxidase: p40-phox, which was shown to reside in a complex with p67-phox in the cytosol of resting neutrophils (6, 7), and the small GTPase p21-rac1 or p21-na2 (8, 9). This latter protein, unlike p40-phox, is essential for the activity of the NADPH oxidase (10, 11).

The most common form of CGD (~60% of cases) is X-linked and caused by mutations in the CYBB gene encoding the cytochrome b558 subunit (12). Mutations in the CYBA gene encoding the α subunit of the cytochrome lead to a rare, autosomal form of the disease, found in ~5% of patients (13). The 47-kD component is encoded by the NCF1 gene; mutations in this gene lead to the common autosomal-recessive type of CGD, with an estimated incidence of 30% (14, 15). Finally, another rare autosomal type of CGD is caused by mutations in the NCF2 gene, encoding p67-phox (16). This type of CGD is called A67 CGD and is found in <5% of patients. So far, all reported A67 CGD patients completely lack material immunoreactive with antibodies directed against p67-phox. Only three of these patients have been characterized at the molecular level (17-19). All three patients appeared to be homozygous for a mutation in the NCF2 gene. We here report on two heterozygous mutations in an additional p67-phox-affected CGD patient. Interestingly, one of these mutations apparently leads to the expression of a nonfunctional p67-phox protein. Since p67-phox has been shown to interact with p21-rac1 (20), we investigated the effect of this mutation on this interaction and found it to be impaired.

Clinical History

A girl, born in 1976 in Chile to parents of South American origin, was adopted by Swedish parents in 1977. A medical examination at arrival in Sweden revealed only a calcified lymph gland in the left axillae. At age 2, she had a left-sided lobar pneumonia. In 1980, an abscess in her parotid gland was identified and cultured negative for Pseudomonas aeruginosa. At age 7, she had an abscess in her left axillae, but cultures were negative. Histologic examination showed unsepecific necrotizing inflammation without granuloma formation. The same year she developed sterile pyuria. At age 9, she developed synovitis at the distal tibial bone. The possibility of tuberculosis was considered; however, tuberculosis cultures as well as regular cultures were negative. A biopsy from the tibial bone revealed osteitis but no granuloma. The same year, granulocyte function tests showed no increase in oxidative metabolism. Thus, the diagnosis of CGD was established. She was treated with rifampicin and trimethoprim-sulphamethoxazole for her osteitis and was thereafter put on prophylactic treatment with trimethoprim-sulphamethoxazole. The symptoms in her joints disappeared gradually, and there are no sequelae visible on x-ray photographs. During the years 1986-1990 she suffered from repeated lymphadenitis and a swelling of the parotid gland, despite antibiotic prophylaxis. She was also treated for a Salmonella septicemia. Since 1990, she has been well without antibiotic prophylaxis.

Materials and Methods

Purification of Granulocytes. Blood was drawn in venous tube tubes (Terumo Europe, Leuven, Belgium), and granulocytes were purified as described previously (21). The cells (>90% neutrophils and 2–10% eosinophils) were suspended in incubation medium (132 mM NaCl, 6.0 mM KCl, 1.2 mM Na2HPO4, 1 mM CaCl2, 1 mM MgCl2, 20 mM Hepes, 5 mM glucose, and 0.5% [wt/vol] human serum albumin [pH 7.4]).

Functional Tests. The nitro blue tetrazolium (NBT) slide test was performed as described previously (22). About 400 cells stained with nuclear fast red were examined and scored as formation negative or positive.

NADPH oxidase activity was also measured in the cell-free activation system as previously described (23). In short, purified plasma membranes from sonicated granulocytes (10 μg of protein) and cytosol from sonicated granulocytes (120 μg of protein) were incubated at 28°C in 800 μl of oxidase buffer (pH 7.2) containing 75 mM NaCl, 20 mM Hepes, 170 mM sucrose, 1 mM MgCl2, 0.5 mM EGTA, and 60 μM ferricytochrome c. The assembly of oxidase components was started by the addition of SDS (100 μM). NADPH (250 μM) was added after 3 min, and NADPH oxidase activity was measured by the slope of the absorbance change at 550 nm.

Western Blotting. The presence of NADPH oxidase components was determined on Western blots with antisera specific for the cytosolic components as described (23).

Preparation of RNA and DNA. RNA was isolated from mononuclear leukocytes as described previously (17). Genomic DNA was isolated from circulating leukocytes (24).

Northern Blotting. RNA corresponding to 107 monocytes (10 μg) was submitted to electrophoresis in 1.2% (wt/vol) agarose gels in the presence of formaldehyde and was blotted onto Genescreen Plus membrane filters (NEN-Dupont, Boston, MA). Blots were hybridized with a p67-phox cDNA probe containing the total coding region and labeled by random priming.

Southern Blotting. Analysis of genomic DNA by Southern blot was performed after treatment with EcoRI or HindIII essentially as described (25). For hybridization, a full-length p67-phox cDNA was used (17).

Amplification and Sequencing of DNA. For analysis of mRNA sequences, first-strand cDNA was synthesized from RNA. The p67-phox cDNA coding region was amplified by PCR in six overlapping fragments as described by (17). The sequence of the oligonucleotide primers used for this PCR are also given in reference 17. The PCR product was purified with the GeneClean II kit (BIO 101, Inc., Vista, CA) to remove the primers and nucleotides. 200 ng of the purified DNA samples was annealed with 40 ng of one of the primers used for amplification by first being heated for 3 min at 100°C and then being chilled on ice water in the presence of 10% DMSO. Direct sequence analysis was performed with the sequenase version 2.0 kit (United States Biochemical Corp., Cleveland, OH). In genomic DNA, the mutations were identified in a similar way.

Analysis of p21-rac1 Binding to Glutathione-S-Transferase (GST) Fusion Proteins. To introduce the deletion of amino acid 58 in p67-phox we used a wild-type p67-phox construct in pGEX-2T (Pharmacia, Uppsala, Sweden) as a template in a PCR with oligonucleotide primers 5′-CTGGTAAGGGCGCTCTCCTCAGTC-3′ (sense) and 5′-GATGAAATCTAATTCA-TGTCCCCTGGTG-3′ (antisense). The product was digested with StuI and EcoRI (underlined nucleotides) and ligated into the StuI/EcoRI-digested pGEX-p67phox plasmid. The introduced deletion was confirmed by sequencing. Expression of GST fusion protein
proteins was performed in Escherichia coli DH5α as previously described (26). Protein expression was compared by gel electrophoresis and protein staining. We found that the mutated p67-phox was poorly expressed, ∼10-fold less than wild-type p67-phox. GST alone, GST–p47-phox, GST–p67-phox, or GST–p67-phoxΔ50 Lys were spotted onto nitrocellulose. The blot was then incubated with 32P-labeled p21-rac as described (27). Binding was visualized by subsequent exposure of the blot to hyperfilm-ECL (Amersham Corp., Arlington Heights, IL) for 1 h.

Identification of CGD Type.

The patient was identified as a CGD patient by lack of superoxide generation by her resting granulocytes, but not by addition of recombinant p47-phox. On Western blots, p47-phox was clearly present in the cytosol of the patient’s resting granulocytes, but p67-phox was also detected, albeit at a lower level (Fig. 1). Densitometric scanning of the blots showed 46 ± 9% (mean ± SD, n = 3) of the normal amount of p67-phox. Thus, the patient suffers from the autosomal form of CGD leading to subnormal amounts of p67-phox, also called the A67G CGD subtype.

Identification of the Mutations On Northern blot, the size and the amount of p67-phox mRNA of the patient was apparently normal (not shown). The p67-phox mRNA of the patient was converted to cDNA and amplified in six overlapping fragments. Electrophoresis on agarose gel showed that the amplified fragments from the patient had a size similar to that of fragments obtained from normal control cells. Direct sequencing of the first fragment with primer 67–109 (17) revealed a triplet nucleotide deletion between position 170 and 174, predicting deletion of a lysine at amino acid position 58. This mutation appeared to be confined to one allele, because the normal sequence was also detectable, albeit faintly. Therefore, we amplified genomic DNA from the patient with primer 67–27 (on exon 2) and primer 67–28 (on intron 2) and directly sequenced this fragment with primer 67–30 (also on intron 2; see reference 17). Again, the AGA 170–172 or GAA 171–173 or AAG 172–174 deletion was found, this time with the normal sequence at equal intensity (data not shown; a schematic representation is given in Fig. 2 A). Analysis of the other amplified cDNA fragments from this patient revealed no other mutations. However, Southern blot analysis of nonamplified genomic DNA with a full-length p67-phox cDNA probe after treatment with restriction enzyme EcoRI showed a much weaker stained 5-kb band than in the control situation (Fig. 2 C). In addition, after treatment with HindIII, an abnormal extra band of ∼13 kb was found (Fig. 2 B). These results are compatible with an 11–13-kb deletion in the NCF-2 gene. Thus, the patient seems to be a compound heterozygote for a 50 Lys deletion and a larger deletion in p67-phox. Family members of this patient were not available for study.

Translocation of p47-phox and p67-phox in Intact Granulocytes. Apparently, the p67-phox with the predicted in-frame deletion of 50 Lys was expressed in the patient’s cells, because a band of normal size was detected on Western blot (Fig. 1). The lower expression of the protein is abnormal: carriers of A67G have (almost) normal amounts of p67-phox protein in their neutrophil cytosol (unpublished results). The product from the patient’s other allele with the large deletion was not expressed, because no protein of lower size was detected. To analyze the functional defect in this patient, we studied the translocation of p67-phox and p47-phox in the granulocytes activated with PMA or STZ. As shown in Fig. 3, PMA in normal cells induced a fair translocation of both p47-phox and p67-phox, and STZ (a more physiological stimulus) induced a mitigated translocation, probably caused by a less stable assembly of NADPH oxidase. However, in the patient’s cells, both proteins failed to translocate to the plasma membrane and were retained in the supernatant with both stimuli. In granulocytes of p67-phox-deficient patients, it has been found that p47-phox can translocate on its own (30, 31). Nevertheless, we found <1% interaction of p47-phox with the plasma mem-
Figure 2. DNA analysis. (A) DNA sequence of genomic DNA and cDNA of one of the patient's alleles compared with the control sequence. Uppercase letters indicate coding sequences and lowercase letters indicate intron sequences. The arrow indicates the triplet nucleotide and the amino acid present in the control sequence but missing in the patient. Amino acid and nucleotide numbering is according to reference 16. The triplet nucleotide deleted in the patient may be AGA 170-172, GAA 171-173, or AAG 172-174. (B and C) restriction fragment length polymorphism analysis of genomic DNA from the patient and from control donors. The DNA was digested with HindIII (B) or EcoRI (C) and electrophoresed in 1.2% (wt/vol) agarose. After Southern blotting, the fragments were hybridized with a full-length p67-phox DNA probe labeled by random priming.

Translocation of p47-phox and p67-phox in the Cell-free System. In the patient described here, the lack of translocation of both cytosolic phox proteins could be the consequence of a disturbed binding of the mutated p67-phox to cytochrome b558. To investigate this possibility, we tested the translocation of mutated p67-phox as a fusion protein to GST in the cell-free system. As shown in Fig. 4, p47-phox and p67-phox translocate to the plasma membrane, using either wild-type or mutated p67-phox. The absence of translocation with membranes of an X-linked CGD patient shows the dependence of this translocation on the presence of cytochrome b558. However, the membranes binding this mutated p67-phox did not support superoxide production upon addition of NADPH (see legend to Fig. 4).

Analysis of p21-rac1 Binding to GST Fusion Proteins. To explain the functional defect of p67-phoxΔ86Lys causing CGD in this patient, we studied the p21-rac1 binding to p67-phox in a dot-blot assay. It has been shown that the
small GTPase p21-rac1 can specifically interact with p67-phox (20, 27), the first 199 amino acids of p67-phox being important for this interaction. As shown in Fig. 5, GTP-loaded p21-rac1 bound to the wild-type GST–p67-phox but not to the 58Lys-deleted form of GST–p67-phox. The presence of wild-type and mutated p67-phox was confirmed by immunostaining with an antibody directed against the COOH-terminus of p67-phox. Under the same experimental conditions, binding of GTP-loaded p21-rac1 could not be detected in either the wild-type or mutated form of p67-phox (data not shown). These results suggest that 58Lys of p67-phox is in a putative binding site for p21-rac1, or, alternatively, is important for maintaining the NH2 terminus in a proper conformation for p21-rac1 binding.

For the past two years, conflicting data have been published about the function of p21-rac proteins in the assembly of NADPH oxidase. It has been unclear whether or not p21-rac becomes stably associated with the plasma membrane upon cell activation (33–35), and whether translocation of p21-rac is dependent or independent of p47-phox and p67-phox (36–40). The only aberration we observed in the neutrophils of this CGD patient was the inability of its mutant p67-phox protein to interact with p21-rac1, whereas interaction with p21-rac2 (as reported by Dorseuil et al. [41]) could not be demonstrated even with the wild-type p67-phox. Our results seem therefore to corroborate the results of Dusi et al. (42), who reported that the translocation of p21-rac1, but not that of p21-rac2, is dependent on the presence of p47-phox and p67-phox. The interaction between p21-rac1 and p67-phox may not only be mandatory for proper NADPH oxidase assembly (as indicated by the disturbed translocation of cytosolic components in the neutrophils of this patient), but also for induction of catalytic activity: the mutated p67-phox translocated in the cell-free system (i.e., under artificial conditions) did not support oxidase activity.

**Figure 3.** Translocation of p47-phox and p67-phox in intact granulocytes. Cells were stimulated with PMA (100 ng/ml) or STZ (1 mg/ml) for 7 min and fractionated. Subsequently, supernatants and plasma membranes were immunoblotted as described in Fig. 1.

**Figure 4.** Translocation of p47-phox and p67-phox in the cell-free system. 1 μg of GST–p47-phox, 1 μg of GST–p67-phox, and a fraction containing p21-rac (obtained from 240 μg of cytosolic protein) were mixed with plasma membranes (40 μg). After activation with SDS and GTPγS and centrifugation over sucrose gradients, plasma membranes were collected. One fifth of the membrane fractions were assayed for superoxide production, and the rest were processed for Western blotting. (Lanes 1 and 3) Recombinant p47-phox and p67-phox-wt with normal or Xb° membranes (0.40 and 0.35); (lanes 2 and 4) recombinant p47-phox and p67-phoxΔ58Lys with normal or Xb° membranes (0.40 and 0.38). The rate of superoxide production of each membrane preparation is given between brackets (in nmol/10 μg of membrane protein per min).

**Figure 5.** Analysis of p21-rac1 binding to GST fusion proteins. 3 μg of GST, GST–p47-phox, GST–p67-phox, or GST–p67-phoxΔ58Lys were spotted onto nitrocellulose (left lane). For comparison, increasing amounts of GST–p67wt were spotted in the adjacent (right) lanes. The blot was then incubated with [32P]GTP-loaded p21-rac1 (left). Binding was visualized by subsequent exposure of the blot to film for 1 h. Afterward, the presence of p67-phox was verified by immunostaining with an antibody against the COOH terminus of p67-phox visualized by enhanced chemiluminescence (right).
We thank Dr. T. Leto (National Institutes of Health, Bethesda, MD) for providing the GST vector encoding recombinant p21-rac2.

This study was supported by grants from The Netherlands Foundation for Preventive Medicine, the Swedish Medical Research Council, Stiftelsen Samariten, the Swedish Children's Cancer Association, and the Funds of Karolinska Institute.

Address correspondence to Dirk Roos, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service and Laboratory for Experimental and Clinical Immunology, University of Amsterdam, Plesmanlaan 125, 1066 CX Amsterdam, The Netherlands.

Received for publication 21 June 1996 and in revised form 9 July 1996.

References

1. Segal, A.W., I. West, F. Wientjes, J.H. Nugent, A.J. Chavan, B. Haley, R.C. Garcia, H. Rosen, and G. Scrace. 1992. Cytochrome b_{245} is a flavocytochrome containing FAD and the NADPH-binding site of the microbicidal oxidase of phagocytes. Biochem. J. 284:781–788.

2. Clark, R.A., B.D. Volpp, K.G. Leidal, and W.M. Nauseef. 1990. Two cytosolic components of the human neutrophil respiratory burst oxidase translocate to the plasma membrane during cell activation. J. Clin. Invest. 85:714–721.

3. Ambruso, D.R., B.G.J.M. Bolscher, P.M. Stokman, A.J. Verhoeven, and D. Roos. 1990. Assembly and activation of the NADPH:O_{2} oxidoreductase in human neutrophils after stimulation with phorbol myristate acetate. J. Biol. Chem. 265:924–930. [Erratum: J. Biol. Chem. 1990, 265:19370–19371].

4. Taylor, W.R., D.T. Jones, and A.W. Segal. 1993. A structural model for the nucleotide binding domains of the flavocytochrome b_{245} beta-chain. Protein Sci. 2:1675–1685.

5. Roos, D. 1994. The genetic basis of chronic granulomatous disease. Immunol. Rev. 138:121–157.

6. Wientjes, F.B., J.J. Hsuan, N.F. Totty, and A.W. Segal. 1993. P40-phox, a third cytosolic component of the activation complex of the NADPH oxidase to contain src homology 3 domains. Biochem. J. 296:557–561.

7. Tsunawaki, S., H. Mizunari, M. Nagata, O. Tatsuzawa, and T. Kuratsuji. 1994. A novel cytosolic component, p40-phox, of the respiratory burst oxidase associates with p67-phox and is absent in patients with chronic granulomatous disease who lack p67-phox. Biochem. Biophys. Res. Commun. 199:1378–1387.

8. Abo, A., E. Pick, A. Hall, N. Totty, C.G. Teahan, and C.G. Segal. 1991. Activation of the NADPH oxidase involves the small GTP-binding protein p21^{ras}. Nature (Lond.). 353:668–670.

9. Kraus, U.G., P.G. Heyworth, T. Evans, J.T. Curnutte, and G.M. Bokoch. 1991. Regulation of phagocyte oxygen radical production by the GTP-binding protein Rac. Science (Wash. DC). 254:1512–1515.

10. Abo, A., A. Boyhan, I. West, A. Trasher, and A.W. Segal. 1992. Reconstitution of the neutrophil NADPH oxidase activity in the cell-free system by four components: p67-phox, p47-phox, p21^{ras} and cytochrome b_{245}. J. Biol. Chem. 267:16767–16770.

11. Dorsey, O., A. Vazquez, P. Lang, J. Bertoglio, G. Gacon, and G. Leca. 1992. Inhibition of superoxide production in B lymphocytes by rac antisense oligonucleotides. J. Biol. Chem. 267:20540–20542.

12. Royer-Pokora, B., L.M. Kunkel, A.P. Monaco, S.C. Goff, P.E. Newburger, R.L. Baehner, F.S. Cole, J.T. Curnutte, and S.H. Orkin. 1986. Cloning the gene for an inherited human disorder—chronic granulomatous disease—on the basis of its chromosomal location. Nature (Lond.). 322:32–38.

13. Dinauer, M.C., E.A. Pierce, G.A. Bruns, J.T. Curnutte, and S.H. Orkin. 1990. Human neutrophil cytochrome b_{245} light chain (p22-phox). Gene structure, chromosomal location, and mutations in cytochrome-negative autosomal recessive chronic granulomatous disease. J. Clin. Invest. 86:1729–1737.

14. Volpp, B.D., W.M. Nauseef, J.E. Donelson, D.R. Moser, and R.A. Clark. 1989. Cloning of the cDNA and functional expression of the 47-kilodalton cytosolic component of the human neutrophil respiratory burst oxidase. Proc. Natl. Acad. Sci. USA. 86:7195–7199. [Erratum: Proc. Natl. Acad. Sci. USA. 86:9563].

15. Lomax, K.J., T.L. Leto, H. Nunoi, J.L. Gallin, and H.L. Malech. 1989. Recombinant 47-kilodalton cytosol factor restores NADPH oxidase activity in chronic granulomatous disease. Science (Wash. DC). 245:409–412. [Erratum: Science (Wash. DC). 244:987].

16. Leto, T.L., K.J. Lomax, B.D. Volpp, H. Nunoi, J.M. Scheller, W.M. Nauseef, R.A. Clark, J.L. Gallin, and H.L. Malech. 1990. Cloning of a 67-kD neutrophil oxidase factor with similarity to a noncatalytic region of p60c-src. Science (Wash. DC). 248:727–730.

17. de Boer, M., P.M. Hilarius-Stokman, J.-P. Hossle, A.J. Verhoeven, N. Graf, R.T. Kenney, R. Seger, and D. Roos. 1994. Autosomal recessive chronic granulomatous disease with absence of the 67-kD cytosolic NADPH oxidase component: identification of mutation and detection of carriers. Blood. 83:531–536.

18. Tanugi-Cholley, L.C., J.P. Issartel, J. Lunardi, F. Freycon, F. Morel, and P.V. Vignais. 1995. A mutation located at the 5’ splice junction sequence of intron 3 in the p67-phox gene causes the lack of p67-phox mRNA in a patient with chronic granulomatous disease. Blood. 85:242–249.

19. Nunoi, H., M. Iwata, S. Tatsuzawa, Y. Onoe, S. Shimizu, S. Kanegasaki, and I. Matsuda. 1995. A 6 dinucleotide insertion in a patient with chronic granulomatous disease lacking 67-kD protein. Blood. 86:329–333.

20. Diekmann, D., A. Abo, C. Johnston, A.W. Segal, and A. Hall. 1994. Interaction of Rac with p67-phox and regulation of phagocytic NADPH oxidase activity. Science (Wash. DC). 265:531–533.

21. Roos, D., and M. de Boer. 1986. Purification and cryo-
preservation of phagocytes from human blood. *Methods Enzymol.* 132:225–243.

22. Meerhof, L.J., and D. Roos. 1986. Heterogeneity in chronic granulomatous disease detected with an improved nitroblue tetrazolium slide test. *J. Leukoc. Biol.* 39:699–711.

23. Verhoeven, A.J., J.H.W. Leusen, G.C.R. Kessels, P.M. Hilarius, D.B. de Bont, and R.M. Liskamp. 1993. Inhibition of neutrophil NADPH oxidase assembly by a myristoylated pseudosubstrate of protein kinase C. *J. Biol. Chem.* 268:18593–18598.

24. Sambrook, J., E. Fritsch, and T. Maniatis. 1989. Molecular Cloning: A Laboratory Manual. 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

25. Bolscher, B.G.J.M., M. de Boer, A. de Klein, R.S. Weening, and D. Roos. 1991. Point mutations in the beta-subunit of cytochrome b558 leading to X-linked chronic granulomatous disease. *Blood.* 77:2482–2487.

26. Smith, D.B., and K.S. Johnson. 1988. Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione S-transferase. *Gene (Amst.)* 67:31–40.

27. Prigmore, E., S. Ahmed, A. Best, R. Kozma, E. Manser, A.W. Segal, and L. Lim. 1995. A 68-kDa kinase and NADPH oxidase component p67-phox are targets for Cdc42Hs and Rac1 in neutrophils. *J. Biol. Chem.* 270:10717–10722.

28. Leusen, J.H.W., M. de Boer, B.G.J.M. Bolscher, P.M. Hilarius, R.S. Weening, H.D. Ochs, D. Roos, and A.J. Verhoeven. 1994. A point mutation in gp91-phox of cytochrome b558 of the human NADPH oxidase leading to defective translocation of the cytosolic proteins p47-phox and p67-phox. *J. Clin. Invest.* 93:2120–2126.

29. Leusen, J.H.W., K. Fluiter, P.M. Hilarius, D. Roos, A.J. Verhoeven, and B.G.J.M. Bolscher. 1995. Interactions between the cytosolic components p47-phox and p67-phox of the human NADPH oxidase that are not required for activation in the cell-free system, *J. Biol. Chem.* 270:11216–11221.

30. Kleinberg, M.E., H.L. Malech, and D. Rotrosen. 1990. The phagocyte 47-kilodalton cytosolic oxidase protein is an early reactant in activation of the respiratory burst. *J. Biol. Chem.* 265:15577–15583.

31. Uhlinger, D.J., S.R. Tyagi, L. Inge, and J.D. Lambeth. 1993. The respiratory burst oxidase of human neutrophils. Guanine nucleotides and arachidonate regulate the assembly of a multicomponent complex in a semi–recombinant cell-free system. *J. Biol. Chem.* 268:8624–8631.

32. Leusen, J.H.W., B.G.J.M. Bolscher, P.M. Hilarius, R.S. Weening, W. Kaulfersch, R.A. Seger, D. Roos, and A.J. Verhoeven. 1994. 152Pro→Gln substitution in the light chain of cytochrome b558 of the human neutrophil NADPH oxidase (p22-phox) leads to defective translocation of the cytosolic proteins p47-phox and p67-phox. *J. Exp. Med.* 180:2239–2334.

33. Sawai, T., M. Asada, H. Nuno, I. Matsuda, S. Ando, T. Sasaki, K. Kaibuchi, Y. Takai, and K. Katayama. 1993. Combination of arachidonic acid and guanosine 5′-O-(3-thiotriphosphate) induce translocation of rac p21s to membrane and activation of NADPH oxidase in a cell-free system. *Biochem. Biophys. Res. Commun.* 195:264–269.

34. Kreck, M.L., D.J. Uhlinger, S.R. Tyagi, K. Leigh Inge, and J.D. Lambeth. 1994. Participation of the small molecular weight GTP-binding protein Rac1 in cell-free activation and assembly of the respiratory burst oxidase. Inhibition by a carboxyl-terminal Rac peptide. *J. Biol. Chem.* 269:4161–4168.

35. Le Cabec, V., H. Möhn, G. Gacon, and I. Maridonneau-Parini. 1994. The small GTP-binding protein rac is not recruited to the plasma membrane upon NADPH oxidase activation in human neutrophils. *Biochem. Biophys. Res. Commun.* 198:1216–1224.

36. Quinn, M.T., T. Evans, L.R. Loetterle, A.J. Jesaitis, and G.M. Bokoch. 1993. Translocation of Rac correlates with NADPH oxidase activation. Evidence for equimolar translocation of oxidase components. *J. Biol. Chem.* 268:20983–20987.

37. El Benna, J., J.M. Ruedi, and B.M. Babior. 1994. Cytosolic guanine nucleotide-binding protein Rac2 operates in vivo as a component of the neutrophil respiratory burst oxidase transfer of Rac2 and the cytosolic oxidase components p47-phox and p67-phox to the submembrane actin cytoskeleton during oxidase activation. *J. Biol. Chem.* 269:6729–6734.

38. Heyworth, P.G., B.P. Bohl, G.M. Bokoch, and J.T. Curranne. 1994. Rac translocates independently of the neutrophil NADPH oxidase components p47-phox and p67-phox. Evidence for its interaction with flavocytochrome b558. *J. Biol. Chem.* 269:30749–30752.

39. Philips, M.R., A. Feoktistov, M.H. Pilinger, and S.B. Abramson. 1995. Translocation of p21ras from cytosol to plasma membrane is neither necessary nor sufficient for neutrophil NADPH oxidase activity. *J. Biol. Chem.* 269:11514–11521.

40. Dorseuil, O., M.T. Quinn, and G.M. Bokoch. 1995. Dissociation of rac translocation from p47-phox/p67-phox movements in human neutrophils by tyrosine kinase inhibitors. *J. Leukoc. Biol.* 58:108–113.

41. Dorseuil, O., L. Reibel, G.M. Bokoch, J. Camonis, and G. Gacot. 1996. The Rac target NADPH oxidase p67phox interacts preferentially with Rac2 rather than Rac1. *J. Biol. Chem.* 271:83–88.

42. Dusi, S., M. Donini, and F. Rossi. 1996. Mechanisms of NADPH oxidase activation: translocation of p40phox, Rac1 and Rac2 from the cytosol to the membranes in human neutrophils lacking p47phox or p67phox. *Biochem. J.* 314:409–412.

1249 Leusen et al.