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

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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 ^{58}Lys deletion in p67-*phox* disrupts the interaction with p21-*rac*1, 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-*rac*1 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)[1] oxidase enzyme in these cells. This enzyme consists of a flavocytochrome (cytochrome b_{558}), 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 the 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 b_{558} 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

[1] *Abbreviations 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.

J. Exp. Med. © The Rockefeller University Press • 0022-1007/96/10/1243/07 $2.00
Volume 184 October 1996 1243–1249

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-*rac*1 or p21-*rac*2 (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 b_{558} β 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 hetereozygous 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-*rac*1 (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 revealed growth of *Pseudomonas cepacia*. At age 7, she had an abscess in her left axillae, but cultures were negative. Histologic examination showed unspecific 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 biospy 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 sequalae 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 venoject citrate tubes (Terumo Europe, Leuwen, 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 mM KCl, 1.2 mM Na_2HPO_4, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 20 mM Hepes, 5 mM glucose, and 0.5% (wt/vol) human serum albumin (pH 7.4).

Functional Tests. The nitro blue tetrazdium (NBT) slide test was performed as described previously (22). About 400 cells stained with nuclear fast red were examined and scored as formazan 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 $MgCl_2$, 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 10^7 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).

Amplication 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 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′-CTGGTAAAGGCCTCTGCTTCAGTCATGTTCTTC-3′ (sense) and 5′-GATGAATTCTAATCATGTCCCTGGTGG-3′ (antisense). The product was digested with StuI and EcoRI (underlined nucleotides) and ligated into the StuI/EcoRI-digested pGEX-p67*wt* plasmid. The introduced deletion was confirmed by sequencing. Expression of GST fusion

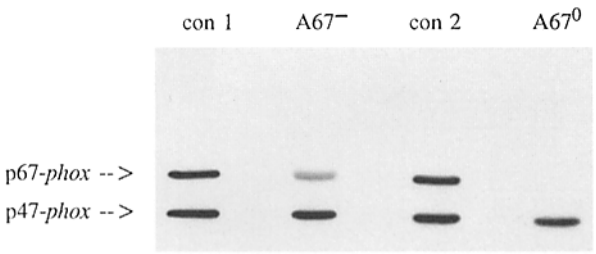

Figure 1. Immunoblot analysis. Blood was drawn from healthy donors and patients after informed consent had been obtained, and granulocytes were purified and fractionated as described previously (28, 32). Granulocyte cytosol (20 μg of protein) from either healthy donors (*con1* and *con2*), from the patient (*A67⁻*) or from a classical p67-*phox*–deficient patient (*A67⁰*) were separated on a 10% polyacrylamide gel and subsequently blotted onto nitrocellulose. Staining with polyclonal antibodies against the COOH-terminal regions of these proteins revealed a diminished amount of p67-*phox* in the patient's granulocytes.

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-*phox*Δ^{58}Lys were spotted onto nitrocellulose. The blot was then incubated with [^{32}P]αGTP–loaded p21-*rac*1 as described (27). Binding was visualized by subsequent exposure of the blot to hyperfilm-ECL (Amersham Corp., Arlington Heights, IL) for 1 h.

Translocation of p47-phox and p67-phox in Intact Granulocytes. Cells were stimulated with PMA (100 ng/ml) or serum-treated zymosan (STZ; 1 mg/ml) for 7 min and fractionated as described (24). Subsequently, supernatants and plasma membranes were immunoblotted as described (28).

Translocation of p47-phox and p67-phox in the Cell-free System. 1 μg GST–p47-*phox*, 1 μg GST–p67-*phox*, and a fraction containing p21-*rac* (obtained from 240 μg of cytosolic protein) (29) were mixed with plasma membranes (40 μg). After activation with SDS and GTPγS and centrifugation over sucrose gradients, plasma membranes were collected as described (24). One-fifth of the membrane fractions was assayed for superoxide production, and the remainder was TCA precipitated and processed for Western blotting.

Results and Discussion

Identification of CGD Type. The patient was identified as a CGD patient by lack of superoxide generation by her purified granulocytes after activation with PMA in the NBT slide test. Likewise, her cells did not show any superoxide dismutase (SOD)-sensitive cytochrome *c* reduction with several other stimuli tested (fMLP, STZ, platelet-activating factor [PAF]), indicating a total lack of NADPH oxidase activity. In the cell-free activation system, the cytosol of the patient's granulocytes showed only 3% of normal activity, whereas the membranes of these cells had normal activity. The cytosolic activity of the patient's cells was fully restored by addition of recombinant p67-*phox* protein, 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 A67$^+$ 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 compatibile with an 11–13-kb deletion in the NCF-2 gene. Thus, the patient seems to be a compound heterozygote for a ^{58}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 ^{58}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 A67^0 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-

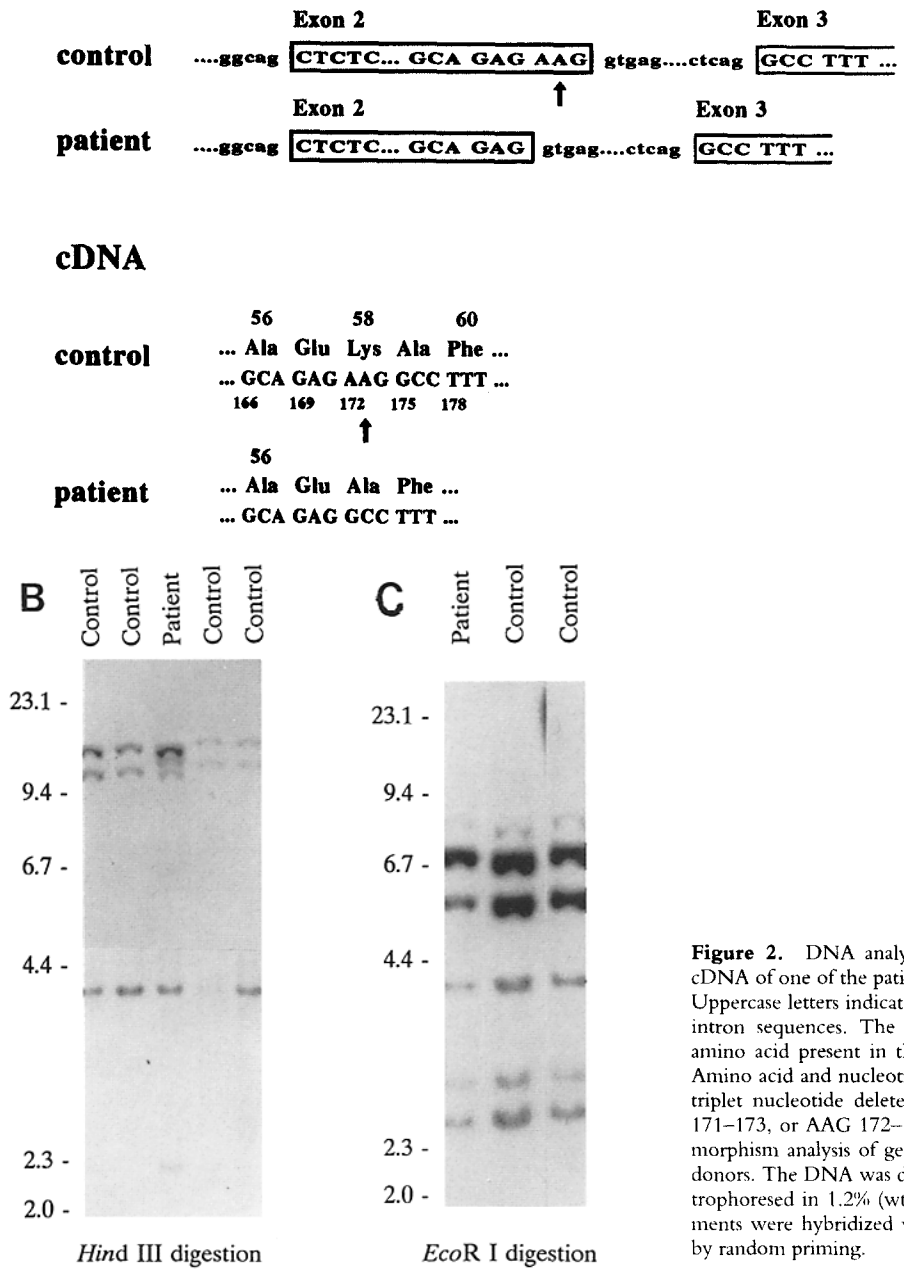

Figure 2. DNA analysis. (*A*) DNA sequence of genomic DNA and cDNA of one of the patient's alleles compared with the control sequence. Uppercase letters indicate coding sequences and lowercase letters indicate intron sequences. The arrow indicates the triplet nucleotide and the amino acid present in 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.

brane of another p67-*phox*–deficient patient, both in PMA-activated granulocytes and in the cell-free system (J. Leusen, unpublished results). Also, in CGD patients with a mutation in either the light or the heavy subunit of cytochrome b_{558}, we found virtually no translocation of either p47-*phox* or p67-*phox* (28, 32).

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 b_{558}. To investigate this possibility, we tested the translocation of mutated p67-*phox* as a fusion protein to GST in the cell-free sytem. 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 b_{558}. 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-*phox*Δ^{58}Lys causing CGD in this patient, we studied the p21-*rac*1 binding to p67-*phox* in a dot–blot assay. It has been shown that the

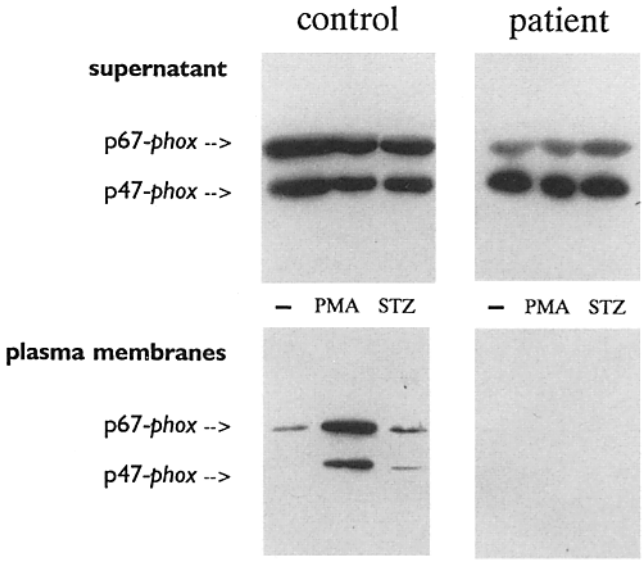

Figure 3. Translocation of p47-*phox* and p67-*phox* in intact granulocytes. Cells were stimulated with PMA (100 ng/ml) or STZ (1 mg/ml) for 7 min and fractionated. Subsequently, supernatants and plasma membranes were immunoblotted as described in Fig. 1.

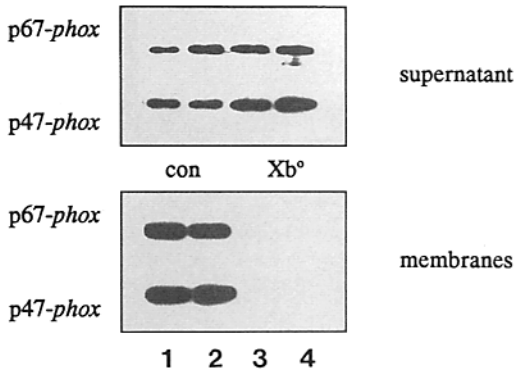

Figure 4. Translocation of p47-*phox* and p67-*phox* in the cell-free system. 1 μg of GST–p47-*phox*, 1 μg of GST–p67-*phox*, and a fraction containing p21-*rac* (obtained from 240 μg of cytosolic protein) were mixed with plasma membranes (40 μg). After activation with SDS and GTPγS and centrifugation 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^0 membranes (5.27 and 0.35); (lanes *2* and *4*) recombinant p47-*phox* and p67-*phoxΔ*^{58}Lys with normal or Xb^0 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).

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-*rac*1 bound to the wild-type GST–p67-*phox* but not to the ^{58}Lys-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-*rac*2 could not be detected in either the wild-type or mutated form of p67-*phox* (data not shown). These results suggest that ^{58}Lys of p67-*phox* is in a putative binding site for p21-*rac*1, or, alternatively, is important for maintaining the NH_2 terminus in a proper conformation for p21-*rac*1 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-*rac*1, whereas interaction with p21-*rac*2 (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-*rac*1, but not that of p21-*rac*2, is dependent on the presence of p47-*phox* and p67-*phox*. The interaction between p21-*rac*1 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.

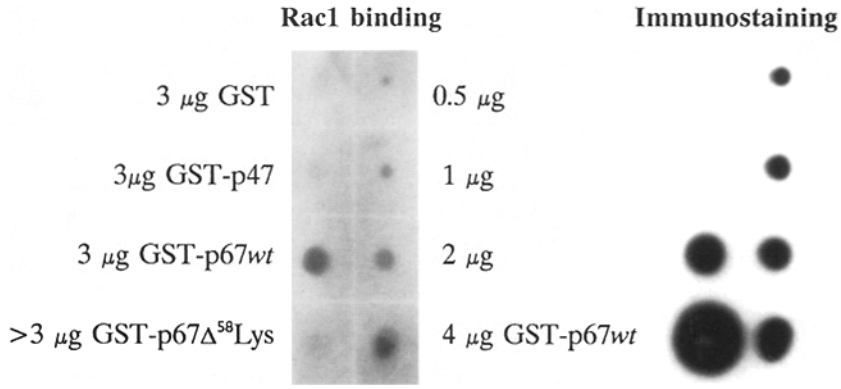

Figure 5. Analysis of p21-*rac*1 binding to GST fusion proteins. 3 μg of GST, GST–p47-*phox*, GST–p67-*phox*, or GST–p67-*phoxΔ*^{58}Lys were spotted onto nitrocellulose (*left lane*). For comparison, increasing amounts of GST–p67*wt* were spotted in the adjacent (*right*) lanes. The blot was then incubated with [^{32}P]αGTP-loaded p21-*rac*1 (*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-*rac*2.

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.

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

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