Human neutrophil immunodeficiency syndrome is associated with an inhibitory Rac2 mutation

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A 5-week-old male infant presented with severe bacterial infections and poor wound healing, suggesting a neutrophil defect. Neutrophils from this patient exhibited decreased chemotaxis, polarization, azurophilic granule secretion, and superoxide anion (O_2^-) production but had normal expression and up-regulation of CD11b. Rac2, which constitutes >96% of the Rac in neutrophils, is a member of the Rho family of GTPases that regulates the actin cytoskeleton and O_2^- production. Western blot analysis of lysates from patient neutrophils demonstrated decreased levels of Rac2 protein. Addition of recombinant Rac to extracts of the patient neutrophils reconstituted O_2^- production in an *in vitro* assay system. Molecular analysis identified a point mutation in one allele of the Rac2 gene resulting in the substitution of Asp57 by an Asn ($Rac2^{D57N}$). Asp57 is invariant in all defined GTP-binding proteins. $Rac2^{D57N}$ binds GDP but not GTP and inhibits oxidase activation and O_2^- production *in vitro*. These data represent the description of an inhibitory mutation in a member of the Rho family of GTPases associated with a human immunodeficiency syndrome.

Neutrophils are the predominant phagocytes that protect the host against bacterial and fungal infections. Neutrophils are circulating leukocytes that are activated rapidly in response to inflammatory signals, causing them to adhere to the endothelial surface, diapedesis across the endothelial barrier, and migrate to the site of infection (1). Once in contact with opsonized microbes, neutrophils ingest the microorganisms. The contents of azurophilic, specific, and other granule classes are released into the developing phagolysosome along with toxic oxygen metabolites produced through the assembly and activation of the NADPH oxidase enzyme system in the plasma membrane. Death and dissolution of the microbe results from the variety of oxygen-dependent and oxygen-independent mechanisms generated in the phagolysosome. Thus, integration of stimulus-activated functions of neutrophils is required for efficient host defense.

The importance of neutrophils in host defense is emphasized by patients with disorders of neutrophil function who exhibit a predisposition to severe, life-threatening infections (2). Disorders of adhesion to endothelial surfaces such as leukocyte-adhesion deficiency (LAD) and deficient microbicidal activity such as chronic granulomatous disease (CGD) are examples of genetic defects in specific proteins resulting in abnormal neutrophil function and immunodeficiency (2). Activation of neutrophil function is regulated by cell surface receptors for chemoattractants and chemokines. Neutrophil receptors regulate specific signaling pathways that control cell shape change, motility, and the NADPH oxidase system. Rho family GTPases are critical in regulating neutrophil activation. One of these, Rac2, is involved in the control of the neutrophil actin cytoskeleton, cell migration, and the NADPH oxidase (3, 4). In this report, we describe a human Rac2 mutation that resulted in severe neutrophil dysfunction and a predisposition to bacterial infections.

Methods

Isolation of Neutrophils and Neutrophil Subcellular Fractions. Blood for these studies was obtained from the patient or controls with consent under a protocol approved by the Colorado Multiple Institutional Review Board at the University of Colorado Health Sciences Center. Neutrophil lysates from the patient and control were made by adding 1% Triton X-100/0.2 mM PMSF/1 μg/ml leupeptin to neutrophils (2×10^7/ml) and subcellular fractions by previously described techniques (5–7).

Neutrophil Function Assays. Chemotaxis was measured as the distance (microns) of the leading edge of neutrophils in a modified Boyden chamber with 10% zymosan-activated serum or buffer in the lower compartment (5). Expression of CD11b was determined after incubation of neutrophils with buffer, phorbol myristate acetate (PMA), formyl-methionyl, leucyl-phenylalanine (fMLP), or platelet-activating factor (PAF)/fMLP for 5 min at 37°C. Cell surface CD11b was detected with direct immunofluorescence by using flow cytometry (5).

For degranulation studies, neutrophils (10^6) were preincubated with buffer or 5 μg/ml cytochalasin b for 5 min at 37°C and then incubated with fMLP (1 μM) and PMA (200 ng/ml) for 60 min. Supernatants were removed from the cells after centrifugation and assayed for myeloperoxidase (MPO) and lactoferrin (LF) by using a colorimetric assay and ELISA, respectively (7, 8). Total cell content of alkaline phosphatase, lysozyme, MPO, and LF for 10^6 neutrophils was also measured (7–9). The amount of MPO and LF released into the supernatant was represented as a percentage of total cell content (7–9).

O_2^- production by intact neutrophils was measured as superoxide dismutase (SOD)-inhibitable cytochrome *c* reduction. Neutrophils added to microtiter plates were stimulated with 200 ng/ml PMA, 1 μM fMLP, or 1 μM PAF/1 μM fMLP. The maximal rate of cytochrome *c* reduction over 5 min was measured as the OD_{550} in a microtiter plate reader for the first three stimuli (5). For opsonized zymosan (1 mg/ml), cells were incubated for 5 min, placed in a 4°C ice bath, and centrifuged at $1,000 \times g$ for 10 min. The supernatant was removed and the OD_{550} was measured (5, 6).

Neutrophil polarization was visualized by using Rhodamine-phalloidin to stain f-actin (red) and Hoechst 33342 to stain nuclei

Abbreviations: PMA, phorbol myristate acetate; PAF, platelet-activating factor; fMLP, formyl-methionyl, leucyl-phenylalanine; LAD, ; MPO, myeloperoxidase; SOD, superoxide dismutase; GTP[γS], guanosine 5′-[γ-thio]triphosphate; GST, glutathione *S*-transferase; EBV, Epstein–Barr virus.

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(blue) followed by digital fluorescence microscopy (10, 11). To determine f-actin content, 10^6 neutrophils were loaded with N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) phallacidin (NBD-phallacidin) and incubated with fMLP (100 nM), and fluorescence was measured by flow cytometry (5).

Oxidase SDS Cell-Free System. Guanosine 5′-[γ-thio]triphosphate (GTP[γS]) (10 μM), 1 μg of neutrophil plasma membrane, 50 μg of neutrophil cytosol, 112.5 μM SDS, and 75 μM cytochrome *c* in 10 mM Hepes, pH 7.4/0.5 mM EGTA/50 mM KCl/5 mM $MgCl_2$ were added to wells of a microtiter plate. The mixture in a final reaction volume of 150 μl was incubated for 3 min at 37° (5, 6). NADPH was added to give a final concentration of 200 μM. Assays were completed in triplicate with one of the wells containing 16.7 μg/ml SOD. The rate of SOD-inhibitable cytochrome *c* reduction was determined and O_2^- production was calculated by using the extinction coefficient of 8.4 mmol/liter^{-1}·cm^{-1} (5, 6).

Reconstitution of the patient's cytosol was done with 1 μg of membrane from control neutrophils, 5 μg of cytosol from the control or patient, and 600 ng of recombinant p47-phox, 600 ng of p67-phox, and/or 2.4 μg of Rac1 loaded with GTP[γS]. Recombinant p47-phox and p67-phox were expressed in a Sf/9-baculovirus expression system (12). Recombinant Rac1 expressed as a glutathione *S*-transferase (GST) fusion protein (13) was provided by Michael Kleinberg (VA Medical Center, Baltimore). The Rac1 protein was enzymatically cleaved from GST (14). Purified Rac1 was loaded with GTP[γS] by using a 5 molar excess of GTP[γS] in a 15-min incubation at room temperature to give an activated Rac1·GTP[γS] complex (15).

The inhibitory effect of the mutant Rac2 was assayed with 1 μg of plasma membrane, 5 μg of cytosol, or 173 ng of Rac1·GTP[γS], 600 ng each of p47/p67-phox, and 200 or 173 ng of WT Rac2 or $Rac2^{D57N}$ in the SDS cell-free system. Rac2 and $Rac2^{D57N}$ were preloaded with a 5 molar excess of GTP[γS] for 15 min at room temperature before being added to the assay (15).

Western Blot Analysis of Oxidase Components and Rac2. Cytosol (10 μg) or plasma membrane (25 μg) proteins from the patient and control were separated by SDS/PAGE, transferred, and probed with antibodies for p67-phox, p47-phox, p22-phox, gp91-phox, p38, or Rac2 (3, 5, 6).

Sequencing of Transcripts for Rac2. Genomic DNA isolated from the patient or control peripheral blood mononuclear cells and Epstein–Barr virus (EBV)-transformed B cell lines was amplified for 50 cycles of 5 sec at 95°C, 30 sec at 60°C, and 15 sec at 75°C. The gene structure of Rac2 was found by BLAST search, and PAC clones HS 151B14A (accession nos. Z8600 and Z82188) and HS 151B14B (accession no. Z85988) contained the genomic sequence of Rac2 in reverse orientation. All seven exons, along with intron–exon junctions, were amplified. Genomic PCR products were sequenced in both orientations.

Full-length cDNA was obtained from RNA extracted (16) from peripheral blood mononuclear cells and EBV-transformed B cell lines by reverse transcription–PCR. Oligonucleotide probes used were 5′-ATGCAGGCCATCAAGTGTGT (sense) and 3′-TAGAGGAGGCTGCAGGCG (antisense). The reverse transcription reaction included 1 μg of RNA, 5 mM $MgCl_2$, 1 mM of each of the dNTPs, 20 units of RNase inhibitor, and 50 units of murine leukemia virus reverse transcriptase. After incubation for 10 min at room temperature, transcription was completed as follows: 15 min, 42°C; 5 min, 99°C; and 5 min, 5°C. After chloroform extraction, PCR amplification was completed with 2.5 units of *Taq* DNA polymerase, 2 mM $MgCl_2$, and primers as noted above. PCR was completed with 35 cycles as follows: 45 sec, 95°C; 45 sec, 63°C; 2 min, 72°C; and products were sequenced.

Preparation of Wild-Type Rac2 and Mutant $Rac2^{D57N}$. Wild-type human Rac2 was subcloned into pGEX-4T-2. Rac2 D57N was prepared by the PCR overlap extension method (17). Bacterial cultures expressing either the wild-type or mutant Rac2 were induced for 3 h with 1 mM isopropyl β-D-thiogalactoside, pelleted, and freeze-thawed. Pellets were lysed in buffer (1× PBS, pH 7.4/1% Triton X-100/1 mM PMSF), sonicated to disrupt cells, and pelleted. The supernatant was incubated with glutathione-Sepharose beads and GST-fusion proteins were eluted.

Rac2-Binding Studies. *[^{35}S-GTP[γS]-binding assay.* [^{35}S]GTP[γS] binding to GST-Rac proteins was measured in 50 mM Hepes, pH 8.0/1 mM DTT/2 mM EDTA. One microgram of wild-type GST-Rac2 or GST-$Rac2^{D57N}$ was incubated with 1 μM [^{35}S]GTP[γS] in the presence or absence of a 10-fold molar excess of unlabeled GTP[γS] at 30°C. Reactions were placed on ice and terminated by the addition of 2 ml of iced termination buffer (25 mM Tris, pH 8.0/100 mM NaCl/30 mM $MgCl_2$/2 mM DTT/1 mg/ml BSA). Samples were filtered over prewet nitrocellulose filters and washed four times with 2 ml of iced termination buffer, dried, and counted.

[^{3}H]GDP-binding assay. [^{3}H]GDP-binding assays were performed as described above for [^{35}S]GTP[γS] binding assays except 10 μM [^{3}H]GDP was used in the presence or absence of a 10-fold molar excess of unlabeled GDP.

Results

Patient History. A male infant of nonconsanguineous parents presented at 5 weeks of age with a perirectal abscess and failure of the umbilical stump to involute. In the subsequent 4 months, he exhibited recurrent perirectal abscesses, an infected urachal cyst, a failure to heal surgical wounds, and the absence of pus in infected areas. His older sibling was healthy, and there was no family history of an increased incidence of infections or poor wound healing. By all other criteria, the patient was normal for his age. Transfusions of neutrophils collected from healthy blood donors were required for the resolution of the abscesses and for complete healing of the surgical wounds. The patient exhibited leukocytosis and neutrophilia, normal levels of serum immunoglobulins, normal complement activity (CH50 and C3), and low-normal numbers of total T and B cells, as well as T cell subsets for his age.

Neutrophil Function Studies. Although quantitatively increased, the patient's neutrophils had a marked decrease in motility compared with neutrophils from healthy adult controls (Fig. 1*A*). These findings reflected the inability of the patient to mobilize neutrophils to sites of infection or inflammation and could be consistent with LAD in which the CD11b/CD18 complex is diminished or absent (18). However, both resting and up-regulated CD11b surface expression on the patient's neutrophils were identical to healthy adult control neutrophils (Fig. 1*B*), indicating that the neutrophil dysfunction was not due to LAD (18, 19).

The chemotactic defect exhibited by the patient's neutrophils was accompanied by a number of other functional deficiencies (Fig. 2). Although total cell myloperoxidase, lysozyme, lactoferrin, and alkaline phosphatase were similar in patient neutrophils compared with those of healthy adult controls (7, 8), the patient's neutrophils demonstrated a defect in azurophilic granule release in response to different stimuli. PMA- or fMLP-stimulated release of azurophilic granules (9), as measured by myloperoxidase, was completely absent (Fig. 2*A*). In contrast, the release of specific granules (7–9) as reflected by supernatant lactoferrin was similar in patient and control neutrophils (Fig. 2*B*). These data demonstrate a selective loss in azurophilic granule secretion.

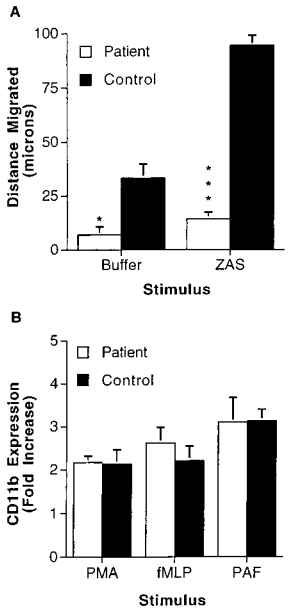

Fig. 1. (*A*) Impaired chemotaxis in the presence of normal CD11b up-regulation. Migration of the leading edge of neutrophils was measured in response to buffer only (random migration) or 10% zymosan activated serum (ZAS) (directed migration) in a modified Boyden chamber assay. Data are presented as the mean distance migrated in microns (±SEM) from three independent isolations. The *P* values were calculated by using Student's *t* test (*, $P < 0.05$; ***, $P < 0.001$). (*B*) Up-regulation of CD11b expression in response to 200 ng/ml PMA, 1 μM fMLP, or 1 μM PAF was determined by flow cytometry. The data represent the mean fold increase in CD11b surface expression (±SEM) from three to five experiments. The mean values of resting CD11b expression in mean channel fluorescence (MCF) units ± SEM were: control, 9.64 ± 1.52 ($n = 5$), and patient, 7.80 ± 3.09 ($n = 3$).

To further characterize the patient's neutrophils, we investigated two additional measurements of neutrophil function. First, we determined the activity of the NADPH oxidase (5, 20) by measurement of SOD-inhibitable cytochrome *c* reduction in response to several stimuli (Fig. 2*C*). O_2^- production stimulated by PMA was similar in patient and control neutrophils, indicating that the oxidase complex is functional in the patient's neutrophils. In contrast, O_2^- production in response to opsonized zymosan was partially, but significantly ($P < 0.005$), decreased in the patient's neutrophils compared with control neutrophils. Quite dramatically, the respiratory burst in response to fMLP with or without PAF priming was completely absent in patient neutrophils (Fig. 2*C*). Just as striking was the inability of patient neutrophils to polarize (10, 11) in response to 25 nM fMLP (Fig. 2*D*). This lack of polarization was characterized by a complete absence of lamellipodia and uropods in patient neutrophils. Additionally, there was a decrease in the amount of f-actin formed in response to fMLP (Fig. 2*D*) (5). Studies with neutrophils from the patient's mother, father, and brother showed normal chemotaxis and O_2^- production to all stimuli noted above (data not shown). Cumulatively, the phenotype of the patient's neutrophils is one of complete loss of chemotaxis, azurophilic granule secretion, superoxide generation, and polarization in response to a variety of receptor stimuli, especially fMLP. However, the defect does not reside in the receptors themselves because the patient's neutrophils did respond to PAF with CD11b up-regulation and to fMLP with CD11b up-regulation and specific granule secretion. These data suggest that the defect in the patient's neutrophils lies in a protein common to the regulation of O_2^- production and shape change/motility. A likely candidate is the low-molecular-weight GTP-binding protein, Rac2.

Investigation of Neutrophil Oxidase with a Cell-Free Activation System and Rac2. The striking loss of O_2^- production in response to fMLP prompted investigation of the NADPH oxidase in a cell-free system (6, 21) by using subcellular fractions from patient and control neutrophils. In this system, a low concentration of SDS is required for assembly of an active oxidase complex. In experiments with four different preparations of subcellular fractions, patient membrane and cytosol produce approximately 28% of the response generated by control fractions (Fig. 3*A*). Fig. 3*B* shows the results of membrane and cytosol fractions from patient and control neutrophils mixed in various combinations to reconstitute oxidase activity. The reconstitution experiments indicated that the defect in oxidase activity by the patient's subcellular fractions was due to a

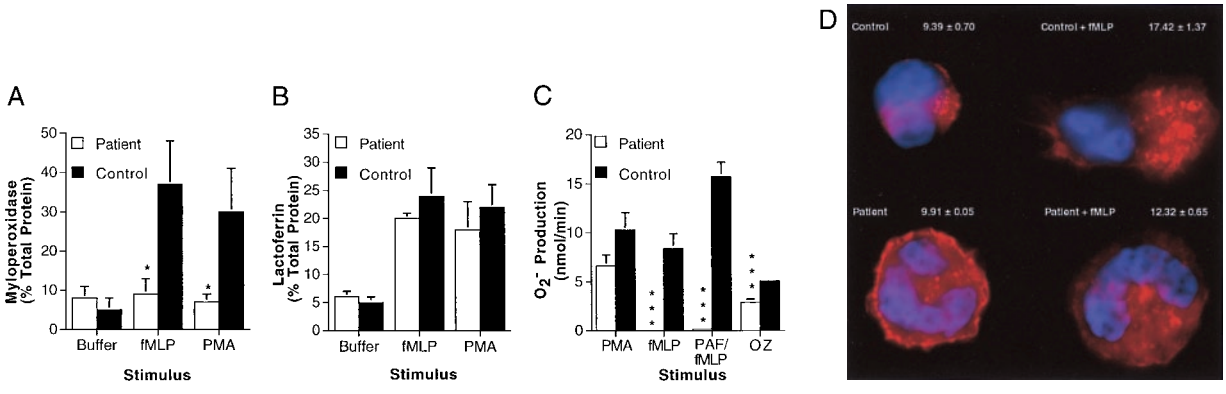

Fig. 2. Functional activities of patient and control neutrophils. The release of azurophilic granules and specific granules in response to buffer only, 1 μM fMLP, or 200 ng/ml PMA in the presence of 5 μg/ml cytochalasin b was measured by the presence of either myloperoxidase (*A*) or lactoferrin (*B*) in the supernatant. The data represent the mean supernatant myloperoxidase or lactoferrin as a percentage of total cellular marker protein (±SEM) from three independent experiments. The *P* values were calculated by using Student's *t* test (*, $P < 0.05$). (*C*) The production of O_2^- by intact neutrophils in response to 200 ng/ml PMA, 1 μM fMLP, 1 μM PAF plus 1 μM fMLP, or 1 mg/ml opsonized zymosan (OZ) was measured as SOD-inhibitable cytochrome *c* reduction. The data represent the mean O_2^- production in nmol/min (±SEM) from 5–11 independent experiments. The *P* values were calculated by using Student's *t* test (***, $P < 0.005$). (*D*) Neutrophil polarization in response to buffer only or 25 nM fMLP. The images shown are representative of three independent experiments. Numerical values represent the mean of f-actin staining with NBD-phallacidin in MCF units ± SEM in neutrophils stimulated with 100 nM fMLP from three to six independent experiments.

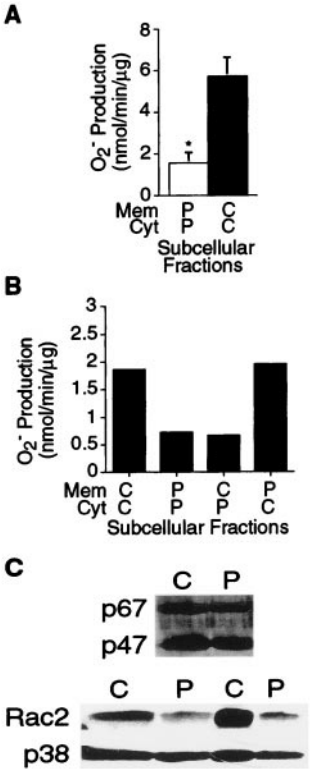

Fig. 3. Patient neutrophil cytosol is defective in reconstituting a cell-free NADPH oxidase system. Control (C) or patient (P) neutrophils were fractionated into plasma membranes (Mem) and cytosol (Cyt) by nitrogen cavitation and ultracentrifugation. The production of O_2^- was measured as SOD-inhibitable cytochrome *c* reduction in the SDS-dependent cell-free system. (*A*) The data shown are the mean values of O_2^- production as nmol/min·μg protein (±SEM) from four independent fractionations of patient and control neutrophils. The *P* value was calculated by using Student's *t* test (*, $P < 0.05$). (*B*) A representative experiment of four independent membrane and cytosol mixing experiments with patient and control fractions is shown. (*C*) Western blot analysis of the oxidase components p47-phox and p67-phox was performed on cytosol fractions from patient and control neutrophils. The blots shown are representative of three separate fractionations. Western blot analysis of Rac2 and p38 MAP kinase was performed on whole-cell lysates from patient and control neutrophils. The blot shown contains two separate lysates representative of five separate, whole-cell lysates. The amount of immunoreactive Rac2 protein from patient whole neutrophil lysate was 41 ± 18% of control.

deficiency in the patient's cytosol and not the plasma membrane fraction.

Expression of oxidase activity requires the p47/p67-phox proteins and the GTP-binding protein Rac2 contributed by the cytosolic fraction of neutrophil lysates (22). Fig. 3*C* shows that the oxidase components p47-phox and p67-phox were expressed at similar levels in cytosol from patient and control neutrophils. Additionally, the membrane-associated p22-phox and gp91-phox proteins also were detected at similar levels in patient and control neutrophils (data not shown). Strikingly, however, the expression in patient neutrophils of Rac2, which represents >96% of the Rac protein expressed in neutrophils (23, 24), was markedly diminished relative to control neutrophils (Fig. 3*C*). The amount of Rac2 protein in patient neutrophils was only 41% of that expressed in control neutrophils. As a comparative control, the expression of the p38 MAP kinase protein from the same lysates was similar in control and patient neutrophils. These data, therefore, suggest that the defect in the patient's neutrophils could be due to decreased Rac2 protein expression and/or function.

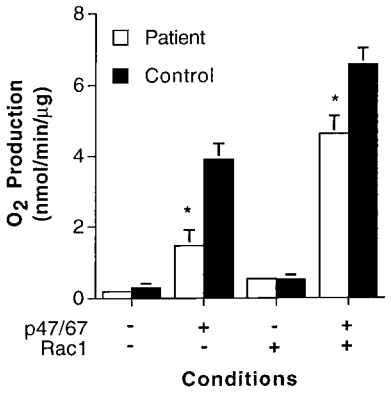

Fig. 4. Rac1·GTP[γS] restores O_2^- generation. Subcellular fractions were prepared as described in the legend to Fig. 3. The data represent the mean value of O_2^- production as nmol/min·μg protein (±SEM) from four independent experiments. The *P* value was calculated by using Student's *t* test (*, $P < 0.05$).

We tested this hypothesis by reconstituting the patient's cytosol with the recombinant cytosolic oxidase components p47/p67-phox and Rac1·GTP[γS]. For these reconstitution experiments, a decreased amount of cytosol was used. By diminishing the amount of cytosol added to the cell-free system, p47/p67-phox become rate-limiting, allowing the evaluation of the effect of adding recombinant p47/p67-phox and/or Rac·GTP[γS] to the patient's cytosol (25). Addition of p47/p67-phox proteins to the reconstitution system in the absence of recombinant Rac·GTP[γS] failed to restore the patient's O_2^- production to control values (Fig. 4). Previous studies have shown that both Rac1 and Rac2 similarly reconstitute the oxidase response (4, 26). An activated recombinant Rac1 protein complexed with GTP[γS] was added to the reconstitution system for measure of its effect on oxidase activity (14). The addition of Rac1·GTP[γS] to the system in the presence of p47/p67-phox reconstituted the patient's superoxide response to levels approaching that of cytosolic fractions from control neutrophils (Fig. 4). This result indicated that Rac activity was deficient in the patient's neutrophils, consistent with the diminished Rac2 expression shown in Fig. 3*C*.

Analysis of Genetic and Molecular Defects of Rac2. The diminished expression of Rac2 protein in the patient's neutrophils suggested a potential molecular defect in the gene encoding Rac2. To address this question, both alleles encoding Rac2 were sequenced from DNA isolated from patient and control mononuclear cells as well as from EBV-transformed B cell lines. Fig. 5*A* shows the mutation defined from the genomic analysis. The mutation is a transition of G → A in exon 3 at codon 57 (GAC → AAC) that results in the mutation of Asp57 to Asn. Asp57 in Rac2 is within the DX_2G motif that is conserved in all GTPases (27). The aspartate residue in the DX_2G motif binds the catalytic Mg^{2+} through an intervening water molecule (28). This Mg^{2+} binds additional residues in the GTP-binding pocket and oxygens of the β- and γ-phosphates of GTP and is required for GTP hydrolysis. In Ras, mutation of Asp57 to other residues inhibits the functional activity of Ras signaling (29, 30). Thus, the mutation identified would be expected to result in defective Rac2 signaling. The D57N mutation was also detected in cDNA prepared from reverse transcriptase reactions by using RNA prepared from mononuclear leukocytes or EBV-transformed B cells. In this analysis, both the mutant and wild-type Rac2 sequences were detected, demonstrating that the $Rac2^{D57N}$ mRNA was expressed. The patient's parents and sibling expressed only the normal allele. The diminished levels of Rac2

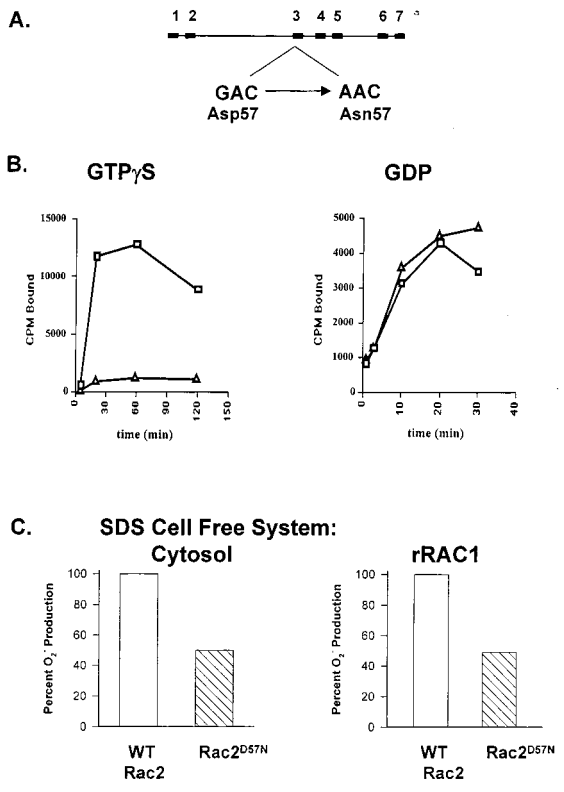

Fig. 5. (*A*) Molecular analysis of the Rac2 gene. Genomic DNA was prepared from both patient and control mononuclear cells and EBV-transformed B cells. Using specific primers, all six exons and exon–intron boundaries were sequenced. The genomic structure of the Rac2 locus is shown, with black boxes representing exons at their approximate position within the locus. One allele has a single-nucleotide transition, G → A, in exon 3 in the first nucleotide position of codon 57. (*B*) Binding of [^{35}S]GTP[γS] and [^{3}H]GDP to wild-type GST-Rac2 and GST-Rac2^{D57N}. Equal amounts of wild-type and mutant Rac2 (1 μg/tube) were used in [^{35}S]GTP[γS]- and [^{3}H]GDP-binding experiments. These data are representative of three such experiments performed in duplicate, where the difference between replicates varied less than 5%. □, Wild-type GST-Rac2; △, GST-Rac2^{D57N}. (*C*) Inhibition of O_2^- production by Rac2^{D57N}. The production of O_2^- was measured as SOD-inhibitable cytochrome *c* reduction with the addition of 1 μg of membrane and 5 μg of cytosol from control neutrophils, 600 ng of recombinant p47-phox, 600 ng of p67-phox, and 200 ng of wild-type (WT) or D57N GST-Rac2^{D57N} that had been preincubated with GTP[γS]. The data are presented as the percentage of O_2^- production in the presence of WT Rac2. A representative result from three such experiments is shown. Inhibition of Rac1·GTP[γS] stimulated O_2^- production by Rac2^{D57N}. The production of O_2^- was measured as noted above except for the addition of Rac1 (173 ng) preincubated with GTP[γS] instead of cytosol in the presence of 173 ng of either WT or D57N GST-Rac2 preincubated with GTP[γS]. The data are presented as the percentage of O_2^- production in the presence of added recombinant WT Rac2. A representative experiment of three such experiments is shown.

protein expression in the patient neutrophils suggested that not only is the mutation of Asp57 to Asn potentially inhibitory for Rac2 function but may also influence the stability of the Rac2 protein. The stability of other mutant GTP-binding proteins has been shown to be diminished significantly (31).

Comparison of Recombinant Wild-Type Rac2 and Rac2^{D57N}. The finding that Asp57 is mutated in one Rac2 allele in the patient has significant implications for defining Rac2-signaling functions in neutrophils. In the Ras protein, mutation of Asp57 induces a strong dominant-negative interfering mutant that inhibits the action of activators of Ras (29, 30). The mutant Ras preferentially binds GDP relative to GTP and is able to sequester Ras exchange factors. A mutation of the Asp in the DX_2G motif in Rac2 is predicted to have the same inhibitory property as mutation of this Asp in Ras. To test this possibility, recombinant wild-type Rac2 and Rac2^{D57N} were prepared as GST fusion proteins (17).

Recombinant wild-type Rac2 and Rac2^{D57N} were assayed for their ability to bind [^{35}S]GTP[γS] and [^{3}H]GDP (Fig. 5*B*). Wild-type Rac2 binds both [^{35}S]GTP[γS] and [^{3}H]GDP. In contrast, Rac2^{D57N} binds GDP similar to wild-type Rac2 but does not bind GTP[γS]. Rac2^{D57N} is unable to assume a conformation allowing GTP[γS] binding. When added to the oxidase reconstitution system, GST-Rac2^{D57N} protein inhibited the ability of cytosol to activate O_2^- production relative to wild-type GST-Rac2 (Fig. 5*C*). Similarly, the GST-Rac2^{D57N} protein inhibited the ability of Rac1·GTP[γS] to stimulate O_2^- production (Fig. 5*C*). Heterologous expression of Rac2^{D57N} in CD34$^+$ bone marrow-derived cells also was shown to inhibit cell motility and the O_2^- system (Williams, D. A., Tao, W., Yang, F., Kim, C., Gu, Y., Mansfield, P., Levine, J. E., Petryniak, B., Derrow, C. W., Harris, C., *et al.*, unpublished results). Thus, Rac2^{D57N} is an inhibitory Rac2 mutant protein that is unable to bind GTP.

Discussion

The neutrophil uses a variety of signaling proteins and pathways to integrate its function in protecting the host. Included in these are small GTPases of the Rho family, Rho, Rac, and Cdc42 (3, 32). These GTPases regulate the actin cytoskeleton and intracellular signaling pathways by cycling from the inactive, GDP-bound state to an active, GTP-bound state (3, 27, 32). The activated GTPase interacts with and regulates the assembly of specific proteins that control actin polymerization and the activation of signal cascades, ultimately regulating cell function. Guanine nucleotide exchange factors (GEFs) stimulate the release of GTPase-bound GDP, allowing the binding of GTP. GTPase-activating proteins (GAPs) that bind specific GTPases enhance the hydrolysis of bound GTP to GDP, returning the GTPase to an inactive state. GEFs and GAPs for Rho family GTPases are regulated in response to neutrophil activation (33–37).

In neutrophils, the GTPase Rac has been shown to play an important role in activation of the respiratory burst that initiates production of toxic oxygen metabolites (22, 26). Studies in a variety of systems have confirmed the role of Rac in the regulation and activity of the phagocyte NADPH oxidase (22, 26, 38, 39). Rac1 and 2, two highly homologous (92%) Rac proteins, are expressed in neutrophils. Rac2 accounts for >96% of the Rac protein expressed in neutrophils (23, 24). Rac2 is required for oxidase activity most probably through its direct interaction with p67-phox and cytochrome b_{558} (24, 40, 41). Other Rho GTPases, in addition to Rac2, have been shown to play a role in actin cytoskeletal dynamics, leukocyte migration, and ingestion (42–44).

Congenital defects of neutrophil dysfunction traditionally have been categorized according to the functional characteristic(s) most significantly involved as well as the clinical and clinical laboratory profile (2). Our patient exhibited a profile of multiple functional defects that was distinct from those of other neutrophil dysfunction diseases, including LADI and II, neutrophil actin dysfunction, and CGD. The patient's neutrophils were found to be deficient in polarization, chemotaxis, azurophilic granule release, and superoxide anion production, but not CD11b expression or specific granule release. These abnormalities suggested a defect in a signaling pathway controlling shape change/motility as well as assembly and activation of the NADPH oxidase. Normal expression of p47-phox and p67-phox, decreased expression of Rac2 in patient's neutrophils, and reconstitution of patient cytosol with Rac1·GTP[γS] confirmed the existence of Rac2 deficiency as the cause of the patient's

neutrophil dysfunction. Expression of $Rac2^{D57N}$ in cells causes a similar loss in motility and oxidase regulation as described for this patient's neutrophils (Williams, D. A., Tao, W., Yang, F., Kim, C., Gu, Y., Mansfield, P., Levine, J. E., Petryniak, B., Derrow, C. W., Harris, C., *et al.*, unpublished results), confirming that the Rac2 mutation is responsible for the neutrophil defects we have characterized.

Interestingly, the findings with our patient's neutrophils are similar to that recently described for Rac2 gene disruption in the mouse (38), consistent with the patient's neutrophil defects being a result of the mutation of Rac2. However, $Rac2^{-/-}$ mouse neutrophils have one important difference relative to the $Rac2^{D57N}$ expressed in human neutrophils. The $Rac2^{-/-}$ neutrophils had normal fMLP-stimulated oxidase activity, indicating Rac2 deficiency can be compensated for normal oxidase regulation. Low-level expression of Rac1 in neutrophils may rescue this response in $Rac2^{-/-}$ mice. In contrast, oxidase activation was inhibited in the patient neutrophils harboring the $Rac2^{D57N}$ mutation. This difference is consistent with the $Rac2^{D57N}$ protein being unable to bind GTP and functioning as an inhibitory mutant in the oxidase activation pathway. The changes in Rac2 expression and function would explain the phenotype observed in the patient's neutrophils. Thus, Rac2 mutations can contribute to nonclassical LAD syndrome with patients that have normal neutrophil CD11b/CD18 expression.

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