Regulation of the Human Neutrophil NADPH Oxidase by *rho*-Related G-Proteins

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ABSTRACT: Superoxide production by phagocytic white blood cells requires the assembly of an NADPH oxidase from membrane and cytosolic proteins. Recombinant cytosolic proteins p47^{phox} and p67^{phox} and neutrophil membranes were used to purify a third cytosolic component that is necessary and sufficient for cell-free reconstitution of NADPH oxidase. The component was isolated as a complex of *rho*-GDP dissociation inhibitor (*rho*-GDI) and two members of the *rho* subfamily of *ras*-related guanine nucleotide binding proteins, *rac*2 and CDC42Hs. Oxidase reconstitution with these pure cytosolic proteins was unaffected by GTPγS but was inhibited by GDPβS, suggesting that the active complex contained endogenous bound GTP. Direct binding of *rho*-GDI to the GTPγS-bound forms of these G-proteins was demonstrated by gel filtration following exchange with radiolabeled guanine nucleotide. *rho*-GDI was shown to be nonessential for cell-free oxidase reconstitution in experiments that compared the activities of pure recombinant forms of these G-proteins. Recombinant *rac* augmented superoxide production, while recombinant CDC42Hs, which shares 70% amino acid sequence identity with *rac*, did not. Three highly conserved regions of *rac*1 and *rac*2 were noted as markedly divergent in CDC42Hs. It is proposed that one or more of these regions of *rac* may be involved in the specific interaction of *rac* with the other NADPH oxidase protein(s).

Stimulation of neutrophils and other phagocytes activates an NADPH oxidase responsible for the production of superoxide anion and potent microbicidal oxidants. The identification of oxidase components has been facilitated by the development of a cell-free NADPH oxidase reconstitution system activated by anionic amphiphiles and requiring both phagocytic cell membranes and cytosol (Heyneman & Vercauteren, 1984; Bromberg & Pick, 1985, Curnutte, 1985; McPhail et al., 1985). Cytochrome b_{558} is the sole membrane component required for cell-free reconstitution of the oxidase (Rotrosen et al., 1992; Knoller et al., 1991; Segal et al., 1992). The cytochrome, which contains heme, NADPH, and flavin adenine dinucleotide (FAD) binding sites (Rotrosen et al., 1992; Segal et al., 1992), is a heterodimeric integral membrane protein composed of a 22-kDa peptide (p22^{phox}) and a 91-kDa glycopeptide (gp91^{phox}) (Parkos et al., 1987; Segal, 1987). Studies by Nunoi et al. (1988) demonstrated that cytosol could be separated by anion-exchange chromatography into three complementary fractions required for cell-free activation of the oxidase. These and related studies identified the active components of two of these three fractions as the proteins p47^{phox} and p67^{phox} (Nunoi et al., 1988; Volpp et al., 1988). Deficiencies of either cytochrome b_{558}, p47^{phox}, or p67^{phox} result in chronic granulomatous disease, a hereditary defect in superoxide production (Clark et al., 1989). Purified recombinant p47^{phox} and p67^{phox} were shown to restore the deficient oxidase preparations from patients lacking these factors (Leto et al., 1990; Lomax et al., 1989; Volpp et al., 1989), but the two recombinant proteins together did not replace whole cytosol in reconstituting the oxidase (Leto et al., 1991). Work of Bolscher et al. (1990) and Gabig et al. (1990) indicated that one of the cytosolic components of this oxidase is guanine nucleotide dependent. Subsequently, this component was identified as *rac*1 in guinea pig macrophages (Abo et al., 1991) and *rac*2 in human neutrophils (Knaus et al., 1991; Mizuno et al., 1992). *rac*1 and *rac*2 are 95% identical in their amino acid sequences (Didsbury et al., 1989). These *rac* proteins modulate superoxide production not only in cell-free systems of phagocytic cells but also in intact B lymphocytes, where antisense inhibition of *rac* synthesis resulted in diminished oxidase activity (Dorseuil et al., 1992).

In the present study, we report the purification from human neutrophils of the third cytosolic component of the oxidase, which we previously called NCF-3 (neutrophil cytosolic factor 3) (Nunoi et al., 1988). This purification is based on its ability to support NADPH oxidase activity reconstituted with neutrophil membranes and pure recombinant cytosolic proteins, p47^{phox} and p67^{phox}. This NCF-3 was purified as a complex of *rho*-GDP dissociation inhibitor (*rho*-GDI and the two *rho*-related G-proteins *rac*2 and CDC42Hs. The recombinant forms of CDC42Hs and *rac* were produced and their roles in superoxide production were compared to define G protein requirements for this system.

MATERIALS AND METHODS

Materials

Chromatographic materials, chemicals, and kits were obtained from the following sources: Superdex-75 and pGEX2T vector (Pharmacia, Piscataway, NJ); phenyl-5PW and DEAE-5PW (Thomson Instrument Co., Springfield, VA); ferricytochrome *c* (horse heart; Type VI), FAD, superoxide dismutase, phenylmethanesulfonyl fluoride (PMSF), leupeptin, glutathione–agarose, isopropyl β-D-thiogalactoside (IPTG), and a gel-filtration molecular weight marker kit consisting of bovine serum albumin, carbonic anhydrase, cytochrome *c*, and aprotinin (Sigma, St. Louis, MO); BA85 nitrocellulose filter discs (Schleicher and Schuell, Keene, NH); Ecoscint A (National Diagostics, Manville, NJ); NADPH and GTPγS (Boehringer Mannheim, Indianapolis, IN); dithiothreitol (DTT; Schwarz/Mann Biotech, Cleveland, OH); [γ-^{32}P]GTP and [^{35}S]GTPγS (guanosine 5′-*O*-(3-thiotriphosphate); Du Pont, Boston, MA); Bio-Rad protein assay kit (Bio-Rad, Richmond, CA); Novex precast polyacrylamide gels (Novel

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Experimental Technology, San Diego, CA); Daiichi silver staining kit (Integrated Separation Systems, Natick, MA); polymerase chain reaction (PCR) kit with Taq polymerase (Perkin Elmer Cetus, Norwalk, CT); subcloning efficiency DH5α *Escherichia coli* (Life Technologies Inc., Gaithersburg, MD). Recombinant proteins rp47^{phox} and rp67^{phox} were produced and purified from a baculovirus expression system as described by Leto et al. (1991).

Methods

Isolation of Neutrophil Membranes and Cytosol. Neutrophils were isolated from peripheral blood of normal donors (Metcalf et al., 1986). Neutrophil membranes and cytosol were separated as described by Nunoi et al. (1988). Briefly, neutrophils were treated for 30 min with 5 mM diisopropyl fluorophosphate in Dulbecco's phosphate-buffered saline without Ca^{2+} or Mg^{2+} and then resuspended at (1–2) × 10^8 cells/mL in relaxation buffer [0.1 M KCl, 3 mM NaCl, 1 mM ATP, 3.5 mM $MgCl_2$, and 10 mM 1,4-piperazinediethanesulfonic acid (PIPES) at pH 7.3] with PMSF (1 mM) and leupeptin (0.1 mM) prior to disruption by nitrogen cavitation at 400 psi for 30 min at 4 °C. The nuclei were removed from the cavitate by centrifugation (1000*g* for 10 min) and the postnuclear supernatant was centrifuged (12000*g* for 20 min) to remove granules. The resulting supernatant was centrifuged (100000*g*, 1 h) to separate membranes from cytosol. The resulting membrane pellet was washed in a high-salt buffer by resuspending in 0.4 M KCl, 10 mM PIPES (pH 7.3), 3.5 mM $MgCl_2$, and 1 mM DTT and then centrifuged for 1 h at 100000*g*. The pellet was homogenized in relaxation buffer at 1 × 10^9 cell equiv/mL and stored at −70 °C in the presence of 1 mM DTT. The cytosol was stored at −70 °C at a concentration of (1–2) × 10^8 cell equiv/mL.

Cell-Free NADPH Oxidase Assays. All cell-free assays were conducted in 96-well microplates using a Molecular Devices Thermomax microplate reader (Menlo Park, CA) equipped with a Softmax program for kinetic analysis. Human neutrophil membranes were solubilized by mixing equal volumes of thawed membrane suspension and extraction buffer (20 mM glycine, 2.33% deoxycholate, 1.7 μM $CaCl_2$, 50% glycerol, and 1 mM NaN_3). Each well contained 0.5 × 10^6 cell equiv of solubilized membranes, an aliquot from each column fraction being analyzed for NCF-3, 0.5 μg each of rp47^{phox} and rp67^{phox}, acetylated ferricytochrome *c* (200 μM) (Azzi et al., 1975), $MgCl_2$ (4 mM), NADPH (200 μM), GTPγS (5 μM), FAD (1 μM), and ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) (1 mM). The reaction mixture was incubated at room temperature for 5 min prior to activation of the oxidase by addition of 20 μL of arachidonic acid to give a final concentration of 40 μM and a final volume of 100 μL. Control wells contained 2.5 μg of superoxide dismutase. Superoxide generation was calculated from the superoxide dismutase-inhibitable changes in cytochrome *c* absorbance observed at 550 nm with a 1-nm bandwidth filter using an extinction coefficient of 21.1 mM^{-1} cm^{-1}. Absorbance readings were taken for 10 min at 30-s intervals following the addition of arachidonic acid. Maximum rates of superoxide generation were calculated from the linear region of the absorbance changes by least-squares fit and converted to micromoles of superoxide generated per minute per milligram of membrane protein.

Purification of NCF-3. NCF-3 isolation procedures were based on methods described by Abo and Pick (1991) with modifications. However, our assay for NCF-3 activity used purified recombinant cytosolic proteins, rp47^{phox} and rp67^{phox}, while the assay system of Abo and Pick used a crude ammonium sulfate-precipitated fraction of macrophage cytosol called σ2 to provide p47^{phox} and p67^{phox}. A saturated ammonium sulfate solution in water (4 °C) was added slowly over 5 min to 10 × 10^9 cell equiv of human neutrophil cytosol [(1–2) × 10^8 cell equiv/mL] to yield a final saturation of 40% (1.7 M). The solution was stirred gently at 4 °C for 1 h and then centrifuged at 10000*g* for 10 min. Solid ammonium sulfate was added to the supernatant to give a final saturation of 70%. After gentle stirring 4 °C for 1 h, the solution was centrifuged at 10000*g* for 10 min. This 40–70% ammonium sulfate precipitate was resuspended in buffer A (0.1 M KH_2PO_4, 1 mM $MgCl_2$, 1 mM DTT, 1 mM EGTA, and 1 mM NaN_3) at pH 7.4 containing 1.7 M ammonium sulfate and then centrifuged at 10000*g* for 10 min. The supernatant was applied at a flow rate of 1 mL/min onto a phenyl-5PW column (0.8 × 7.5 cm) equilibrated in buffer A containing 1.7 M ammonium sulfate at pH 7.4. After the column was washed with 20 mL of the starting buffer, NCF-3 was eluted by a 60-mL descending linear gradient of 1.7–0 M ammonium sulfate in buffer A at a rate of 1 mL/min. Fractions of 2 mL were collected and the NADPH oxidase-reconstituting activity of each fraction was determined in the presence of neutrophil membranes and rp47^{phox} and rp67^{phox} using the cell-free assay described above. The peak fractions containing the highest activity were pooled and dialyzed overnight against 4 L of buffer B (20 mM KH_2PO_4, 1 mM $MgCl_2$, 1 mM DTT, and 1 mM EGTA) at pH 7.4 with 0.5 mM PMSF and 2 μM leupeptin. The dialyzed protein solution was applied onto a DEAE-5PW column (0.8 × 7.5 cm) equilibrated in buffer B (pH 7.4) with 10% betaine. After the column was washed with 20 mL of equilibration buffer, NCF-3 was eluted by a 40-mL ascending gradient of 0–0.2 M KCl in buffer B containing 10% betaine at a flow rate of 0.5 mL/min. Fractions of 2 mL were collected.

The DEAE peak fraction containing the highest complementary activity was concentrated to 125 μL with a Centricon 10 microconcentrator (Amicon Corp., Beverly, CA). This concentrate (100 μL) was applied to a Pharmacia Superdex-75 gel-filtration column (1 × 30 cm) equilibrated in buffer A and eluted with the same buffer at a flow rate of 0.25 mL/min. Fractions (0.25 mL) were collected, tested for NCF-3 activity, and analyzed further as described below. In some experiments NCF-3 was labeled with radioactive GTPγS prior to the final gel-filtration step. In such experiments the DEAE peak fraction concentrate (100 μL) was preincubated at 4 °C with 4 pmol of [^{35}S]GTPγS (1.25 Ci/μmol) in the presence of 5 mM ethylenediaminetetraacetic acid (EDTA) to facilitate release of endogenous guanine nucleotide. At 10 min, 20 μL of 0.1 M $MgCl_2$ was added and incubated for another 20 min to facilitate binding of nucleotide prior to Superdex-75 gel-filtration chromatography.

Protein Quantitation, Electrophoresis, and Internal Sequencing of Proteins. Protein concentrations were determined with the Bio-Rad protein assay kit using bovine serum albumin as a standard. Electrophoresis was performed with SDS–Tris–glycine 8–16% polyacrylamide gels (SDS–PAGE) according to Laemmli et al. (1970). Staining was performed with a Daiichi silver staining kit. Electroblotting onto nitrocellulose was performed in 48 mM Tris, 39 mM glycine, and 10% methanol in a Hoeffer TE 50 Transphor unit (Hoeffer Scientific Instruments, San Francisco, CA) at 20 V for 10 h in the presence of 1 mM sodium thioglycolate. Nitrocellulose sheets were stained with Ponceau S for 90 s and rinsed with 1% acetic acid. Protein bands were excised and subjected to trypsin digestion *in situ*, reverse-phase high-pressure liquid chromatography (HPLC) fractionation, and amino acid

sequence analysis by the Harvard Microchemistry Laboratory (Cambridge, MA) according to procedures described by Aebersold et al. (1987).

Recombinant rac and CDC42Hs. The cDNAs of *rac*1 and *rac*2 and of human brain G25K are generous gifts of Dr. Richard F. Weber and Dr. Paul Polakis, respectively. The cDNA of the placental form of CDC42Hs (Shinjo et al., 1990) was produced by the polymerase chain reaction using human fetal brain CDC42Hs (G25K) cDNA (Munemitsu et al., 1990) as a template and an antisense primer that substituted the last 30 nucleotides of brain CDC42Hs with those of the placental CDC42Hs sequence (Shinjo et al., 1990). These cDNAs were subcloned using oligonucleotide linkers containing *Bam*HI (5′) and *Eco*RI (3′) sites, enabling a forced orientation into the bacterial expression vector pGEX2T. The pGEX constructs were used to transform *E. coli* strain DH5α. Expression and purification of bacterial fusion proteins, as well as thrombin-mediated release of G-proteins from the resin-immobilized fusion proteins, were performed essentially as described (Smith & Corcoran, 1991). The pGEX transformants were cultured in LB broth at 37 °C until the OD_{600} reached 0.6, at which time expression of the glutathione S-transferase–G protein fusion proteins was induced by 0.1 mM IPTG. Four hours later the induced *E. coli* were collected by centrifugation and resuspended into 3% of the original culture volume of buffer C [20 mM Tris (pH 7.4), 50 mM NaCl, 1 mM DTT, and 4 mM $MgCl_2$]. These cells were sonicated for 20 s (sonicator from Heat Systems-Ultrasonics, Inc.; Model W-220F equipped with standard tapered microtip; output control set at 3) and centrifuged for 5 min at 12000*g*. A 100-μL portion of a 50% slurry of glutathione–agarose in buffer C was added to 1.5 mL of supernatant and tumbled at 4 °C for 1 h to adsorb the fusion proteins. The glutathione–agarose-bound protein was washed extensively with buffer C and resuspended in 0.5 mL of buffer C containing 2.5 mM $CaCl_2$. Digestion conditions were explored by varying the thrombin concentration and digestion duration. *Rac*1 and CDC42Hs were cleaved from the respective resin-immobilized fusion proteins by digestion with thrombin (0.1–2 units/mL) for 1 h at room temperature or for 16 h at 4 °C. Release of *rac*1 from its fusion protein by thrombin was variable and a range of concentrations of thrombin (0.1–0.5 unit/mL) was used to avoid overdigestion. The cleavage of *rac*2 fusion protein with thrombin was inefficient in buffer C unless octyl glucoside was present. Efficient digestion of *rac*2 was attained in the presence of 25 mM octyl glucoside added to buffer C at a thrombin concentration of 20 units/mL. Octyl glucoside was removed by gel filtration prior to use in the superoxide production assay. Removal of thrombin by benzamidine–Sepharose had no apparent effect on the activity of these G proteins after their storage for 1–2 days at −70 °C. In all experiments reported, freshly prepared recombinant G proteins were used and thrombin was not removed prior to cell-free assay of superoxide production.

GTPase Assay. Recombinant CDC42Hs (0.5 μg) was prebound with [γ-^{32}P]GTP by incubation with 7.5 μM radionucleotide (30 Ci/mmol) at room temperature in 0.8 mL of a buffer containing 50 mM Tris (pH 7.5), 0.1 mM DTT, 0.3 M NaCl, 1 mM $MgCl_2$, and 2 mM EDTA. This prebinding of radiolabeled GTP was stopped 10 min later by adding 320 μL of a buffer containing 50 mM Tris (pH 7.5), 0.3 M NaCl, 0.1 mM DTT, 5 mM $MgCl_2$, and 1 mM GTP. At time 0, 5, 10, 15, and 20 min, 30-μL aliquots of this reaction mix were applied directly to 0.45-μm nitrocellulose discs and washed 3 times (5 mL each) with an ice-cold buffer containing 50 mM Tris (pH 7.5), 0.3 M NaCl, 0.1 mM DTT, and 10 mM $MgCl_2$. The protein-bound radioactivity retained on these filters was then counted in 10 mL of Ecoscint A. GTPase activity was determined from the loss of radioactivity from CDC42Hs preloaded with [γ-^{32}P]GTP. Dissociation of GTP from CDC42Hs was assessed by using the nonhydrolyzable analogue [^{35}S]GTPγS (300 Ci/mM; 0.05 μM), in place of [γ-^{32}P]GTP in the same filter binding assay described above.

RESULTS

Purification of NCF-3. NCF-3 was purified from human neutrophil cytosol on the basis of the ability of individual column fractions to reconstitute oxidase activity in the presence of neutrophil membranes and recombinant p47^{phox} and p67^{phox}. A 40–70% ammonium sulfate fraction of human neutrophil cytosol depleted in p47^{phox} and p67^{phox} was prepared as described in *Methods*. This 40–70% ammonium sulfate precipitate was then fractionated by hydrophobic interaction chromatography on a phenyl-5PW column using a descending ammonium sulfate gradient (Figure 1A). NCF-3 was eluted at 0.91 ± 0.14 M ammonium sulfate (mean ±SE; $n = 6$). The two fractions containing the highest NCF-3 activity were pooled, dialyzed, and fractionated by DEAE-5PW as per *Methods* (Figure 1B). An ascending KCl gradient was applied and NCF-3 was eluted at 0.09 ± 0.003 M KCl (mean ± SE; $n = 6$). The DEAE fraction containing the highest NCF-3 activity was fractionated further by gel filtration on Superdex-75 (Figure 1C). The concentration dependence of the purified NCF-3 preparation in the presence of excess rp47^{phox} and rp67^{phox} showed saturable reconstituion levels apparently limited only by the amount of membranes used in the assays (Figure 2). The maximum oxidase activity reconstituted was comparable to that observed with crude unfractionated cytosol with an equivalent amount of membranes.

The NCF-3 preparations were analyzed by SDS–PAGE at each stage of purification. Electrophoresis of the purified NCF-3 in six preparations revealed two bands with apparent molecular weights of 26 000, and 21 000, which correlated with NADPH oxidase-reconstituting activity (Figure 3). These two bands were first observed in active fractions eluted during DEAE chromatography. The active proteins eluted from the final Superdex-75 gel-filtration column (Figure 1C) exhibited a higher apparent molecular weight (46 000) than either of the two components revealed by SDS–PAGE. These electrophoresed protein bands were electroblotted onto nitrocellulose paper and excised for *in situ* tryptic digestion and high-pressure liquid chromatography of the released peptides. Sequencing of three tryptic peptides of the 26-kDa protein yielded sequences corresponding to bovine *rho*-GDI (GDP dissociation inhibitor) (Fukumoto et al., 1990). These sequences were SIQE (residues 34–37), GVKIDKT (residues 135–141), and FTDDDKTRHLSWEWNLTIK (residues 180–198). Residue 185 obtained from this study was lysine, while the equivalent residue reported for bovine brain *rho*-GDI is arginine. The seven most prominent and best resolved tryptic peptides from the 21-kDa protein were sequenced. Four of these yielded sequences identical to those of human placental CDC42Hs (G25K) (Figure 4), a close relative of *rac*1 and *rac*2 (Shinjo et al., 1990). Two of these four were overlapping peptides. One peptide corresponded to an autolytic fragment of trypsin. Two peptides yielded sequences that corresponded to *rac*2 from human differential HL-60 cells (Didsbury et al., 1989). Assuming equal efficiency of tryptic digestion and electroblotting, these two G-proteins were present in approximately equal quantities, as judged from the length of the tryptic peptides and the area of the corresponding HPLC-resolved peaks.

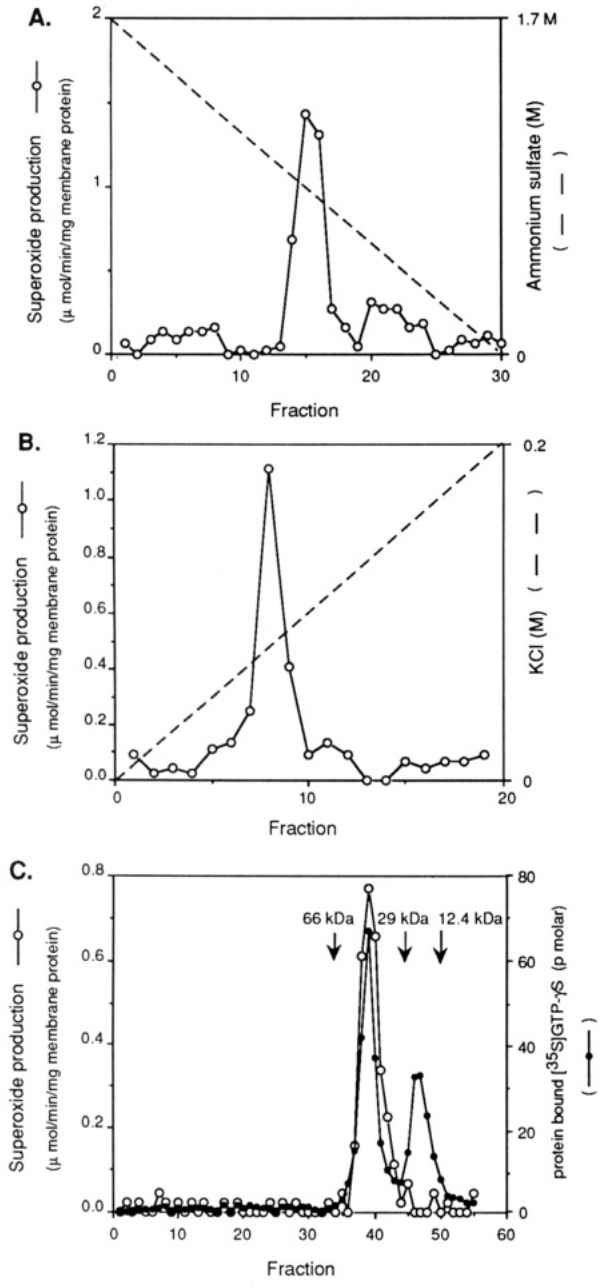

FIGURE 1: Chromatographic purification of NCF-3. (A) Phenyl-5PW chromatography of the 40–70% ammonium sulfate fraction of neutrophil cytosol. A 2-μL aliquot of each fraction was assayed for oxidase reconstituting activity (open circles). (B) DEAE-5PW chromatography. Fractions 15 and 16 of the phenyl-5PW column were pooled, equilibrated in buffer B, and applied to the column. A 6-μL aliquot of each fraction was tested for activity in the cell-free assay. (C) Superdex-75 gel filtration. The DEAE-5PW peak fraction was concentrated to 125 μL, and 100 μL was applied to the column. A 3-μL aliquot of each fraction was assayed for activity, and radioactivity of protein-bound [^{35}S]GTPγS was determined in 10-μL samples (filled circle). Molecular weight markers used to calibrate the Superdex-75 column were bovine serum albumin (66 000), carbonate anhydrase (29 000), and cytochrome *c* (12 400). The cell-free assay of superoxide production of fractions from each chromatographic step was performed in the presence of pure recombinant proteins p47-phox and p67-phox and neutrophil membranes as described in Methods. Binding of [^{35}S]GTPγS to NCF-3 was described in Methods. Results were representative of eight experiments, two of which involved nucleotide exchange prior to gel filtration.

Guinea pig macrophage *rac*1 (Abo et al., 1991b) and human *rac*2 (Knaus et al., 1991; Mizuno et al., 1992) have been shown to serve as guanine nucleotide binding proteins capable of augmenting NADPH oxidase activity in the presence of membranes and crude cytosolic fractions. In the present study,

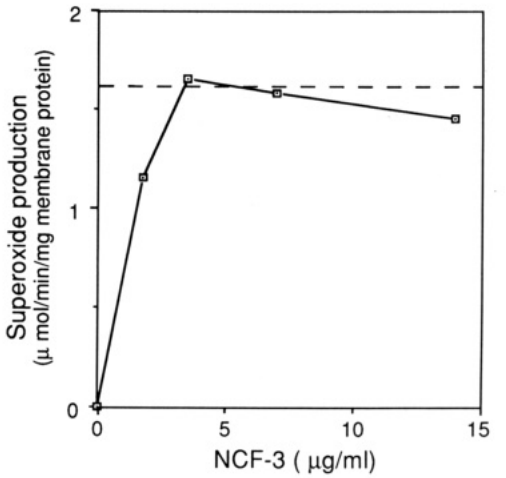

FIGURE 2: Concentration dependence of purified NCF-3 for reconstitution of NADPH oxidase. The data shown are means of duplicate determinations. The solid line represents activity of purified NCF-3 in the presence of recombinant p47^{phox} and p67^{phox} (0.5 μg/well each) and solubilized cell membranes (0.5 million cell equiv/well). The dotted line represents the activity of 1 million cell equiv of crude cytosol and the same amount of membranes in the absence of recombinant proteins. Similar results were obtained in two other experiments.

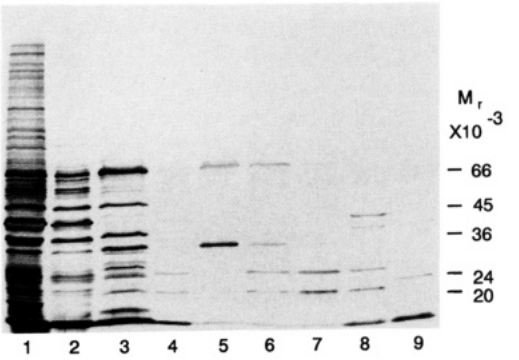

FIGURE 3: Silver-stained polyacrylamide (8–16%) gel electrophoresis (SDS–Tris–glycine) of cytosolic fractions at various stages of NCF-3 purification. Lane 1, crude cytosol; lane 2, 40–70% ammonium sulfate fraction; lane 3, phenyl-5PW peak fraction; lane 4, DEAE-5PW peak fraction; lanes 5–9, Superdex-75 fractions 35, 37, 39, 41, and 43, respectively. The molecular weights of protein standards are indicated.

	97	107
CDC42Hs	WVPEITHHCPK	
Rac2	WFPEVRHHCPS	
	134	144
CDC42Hs	QKPITPETAEK	
Rac2	LAPITYPQGLA	
	167	191
CDC42Hs	NVFDEAILAALEPPEPK	
Rac2	TVFDEAIRAVLCPQPTR	

FIGURE 4: Alignment of the sequence of tryptic peptides from the 21-kDa protein of purified NCF-3 with cDNA predicted sequences of CDC42Hs and *rac*2. Internal sequences of the 21-kDa protein band obtained from the present study are underlined.

the copurification of the closest relative of *rac*, CDC42Hs, with oxidase-reconstituting activity raised the possibility that it might also play a role in oxidase activation. To assess this possibility, we produced and purified full-length recombinant *rac*1, *rac*2, and CDC42Hs using the pGEX2T expression vector (Smith & Corcoran, 1991). Recombinant proteins were

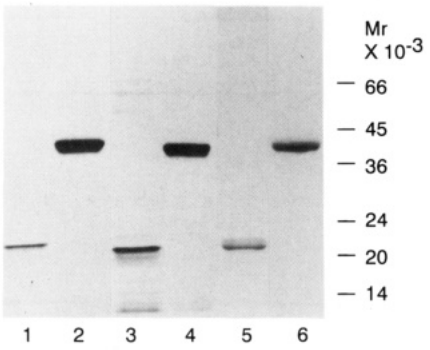

FIGURE 5: Coomassie blue stained electrophoretogram (8–16% Tris–glycine SDS–PAGE) of glutathione–agarose affinity-purified recombinant CDC42Hs, *rac*1, and *rac*2. Lane 1, CDC42Hs generated by cleavage of fusion protein with 2 units/mL thrombin; lane 2, uncleaved CDC42Hs fusion protein; lane 3, *rac*1 generated by cleavage of fusion protein with 0.2 unit/mL thrombin; lane 4, uncleaved *rac*1 fusion protein; lane 5, *rac*2 generated by cleavage of fusion protein with 20 units/mL thrombin; lane 6, uncleaved *rac*2 fusion protein. Purification and cleavage of fusion proteins were described in Methods.

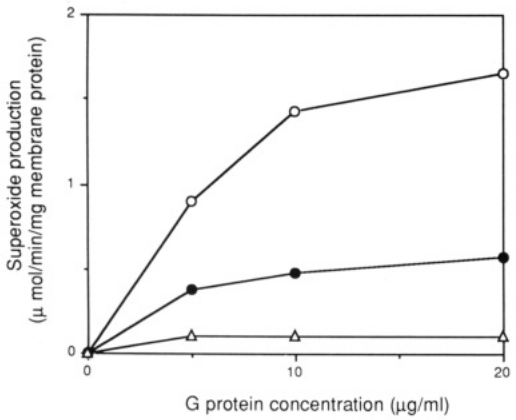

FIGURE 6: Concentration dependency of *rac*1, *rac*2, and CDC42Hs. Superoxide production was determined as described in Methods. Open circles, *rac*1; solid circles, *rac*2; open triangles, CDC42Hs. Result was representative of three experiments.

induced with IPTG and harvested in parallel, using the same buffers. Fusion proteins were purified with glutathione–agarose and digested with thrombin to release the G proteins. The three fusion proteins exhibited significant differences in thrombin susceptibility. *rac*1 and CDC42Hs were readily cleaved, while very little *rac*2 was released from its fusion protein unless octyl glucoside was included. *rac*1, *rac*2, and CDC42Hs were cleaved from the corresponding fusion proteins, yielding products which appeared to be the full-length proteins, as judged by polyacrylamide gel electrophoresis (Figure 5). The activity of *rac*1, *rac*2, and CDC42Hs in supporting superoxide production in a cell-free assay consisting of rp47^{phox}, rp67^{phox}, and neutrophil membranes was determined. Figure 6 shows that recombinant *rac*1 and *rac*2 were active while CDC42Hs prepared in the same manner was not. To show that the recombinant CDC42Hs had the proper sequence and retained function, we verified the nucleic acid sequence of the cloned gene in the pGEX2T vector and demonstrated that the purified recombinant protein cleaved from the fusion protein possessed high intrinsic GTPase activity (Figure 7) as reported previously for platelet CDC42Hs by Hart et al. (1991). The amount of superoxide produced with saturating amounts of recombinant *rac* was comparable to the activity observed with saturating amounts of NCF-3 (Figure 2). However, exact comparison of relative potency of NCF-3 and recombinant *rac* cannot be made because the lack of isoprenylation of G protein by *E. coli* could potentially affect activity of these proteins.

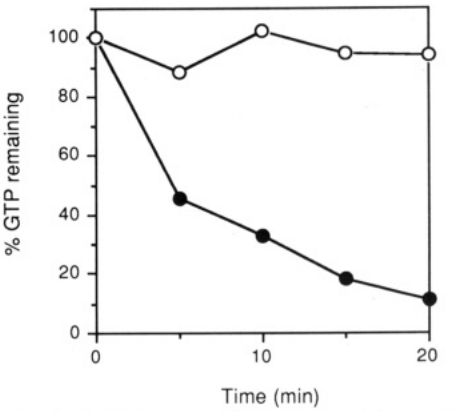

FIGURE 7: Intrinsic GTPase activity of recombinant CDC42Hs. Recombinant CDC42Hs was preincubated with [γ-^{32}P]GTP or [^{35}S]-GTPγS in low Mg^{2+} as described in Methods. GTP loading was stopped by excess free Mg^{2+} and the intrinsic GTPase activity (solid circles) was determined at room temperature by measuring the loss of protein-bound [γ-^{32}P]GTP using a nitrocellulose filter-binding assay. Dissociation of guanine nucleotide from CDC42Hs (open circles) in high Mg^{2+} was determined in a parallel experiment in which [^{35}S]GTPγS was used in place of [γ-^{32}P]GTP.

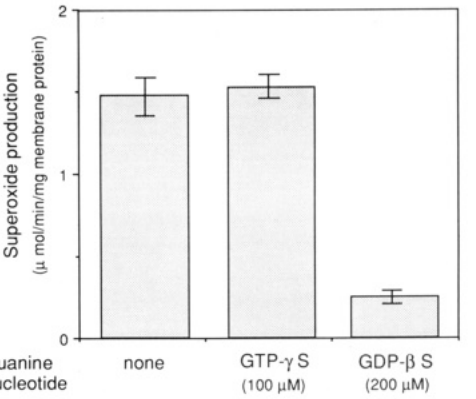

FIGURE 8: Effects of guanine nucleotides on cell-free reconstitution of NADPH oxidase. The cell-free assay for superoxide production consisted of neutrophil membranes, pure rp47^{phox} and rp67^{phox} (0.5 μg/well each), and purified NCF-3 (0.3 μg/well). The data shown are the means of triplicate determinations (±SE). Similar results were obtained in five other experiments, three of which were performed with a lower concentration of GTPγS (10 μM).

Purified NCF-3 Was in a GTP-Bound Form. Oxidase activity reconstituted with NCF-3 was modulated by guanine nucleotides as shown in Figure 8. The addition of GDPβS inhibited oxidase activity, while the addition of GTPγS had no stimulatory effect. These results suggest that part or all of the purified G-proteins were already in a GTP-bound form. The lack of a stimulatory effect by GTPγS was not attributed to trace amounts of GTP in the neutrophil membranes in the cell-free assay because substitution of membranes with purified functionally active cytochrome b_{558} (Rotrosen et al., 1992) also did not require exogenous GTP for activity (not shown). Direct evidence for GTPγS binding to the complexes of *rho*-GDI and the two *rho*-related G-proteins was provided in our experiment where the final gel-filtration chromatography step in NCF-3 purification was preceded by exchange of the endogenous guanine nucleotide with [^{35}S]GTPγS (Figure 1C). In this case, the G-protein-bound [^{35}S]GTPγS eluted in a pattern that closely resembled that of NCF-3 that was not subjected to this nucleotide exchange procedure.

DISCUSSION

The copurification of *rho*-GDI with *rac*2 and CDC42Hs by anion-exchange chromatography suggested that each of these proteins was isolated as a complex with *rho*-GDI, since their

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                            10                  20                  30                  40                  50
CDC42Hs   · · T · · · · · · · · · · · · · · · · · · · · · · · |K · · S · · V · · · · · · · A V T · · I G G E ·
rac2      M Q A I K C V V V G D G A V G K T C L L I S Y T T N|A F P G E Y I P T V F D N Y S A N V M V D S K P
rac1      · · · · · · · · · · · · · · · · · · · · · · · · · · |· · · · · · · · · · · · · · · · · · · · · G · ·

                            60                  70                  80                  90                  100
CDC42Hs   Y T|· · · F · · · · · · · · · · · · · · · · · · · · · · · V · · · V · · · S · F · · · K E · · V · ·
rac2      V N|L G L W D T A G Q E D Y D R L R P L S Y P Q T D V F L I C F S L V S P A S Y E N V R A K W F P E
rac1      · ·|· · · · · · · · · · · · · · · · · · · · · · · · · · · · · · · · · · · · · F · · · · · · · Y · ·

                            110                 120                 130                 140                 150
CDC42Hs   I T · · · · K · · F L · · · · Q I · · · · · P S · · · · · |A K N · Q K · · · P E T A E K · · R D L K|
rac2      V R H H C P S T P I I L V G T K L D L R D D K D T I E K L|K E K K L A P I T Y P Q G L A L A K E I D|
rac1      · · · · · · N · · · · · · · · · · · · · · · · · · · · · · |· · · · · · T · · · · · · · · M · · · · G|

                            160                 170                 180                 190
CDC42Hs   A · · · V · · · · · · · · K · · · N · · · · · |L · A · E · P E P K K S R · – · V|· ·
rac2      S V K Y L E C S A L T Q R G L K T V F D E A I|R A V L C P Q P T R Q Q K R A C S|L L
rac1      A · · · · · · · · · · · · · · · · · · · · · · |· · · · · · P · V K K R · · K · L|· ·
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FIGURE 9: Comparison of predicted amino acid sequence of *rac*2 with that of CDC42Hs and *rac*1. Amino acids of *rac*1 and CDC42Hs identical to those of *rac*2 are represented as solid dots. Boxed sequences represent conserved regions of *rac*1 and *rac*2 which are divergent from CDC42Hs. A gap which optimized the alignment of CDC42Hs is denoted by a dash.

isoelectric points differ significantly (p*I* 5.0, 7.6, and 6.5, respectively). Furthermore, these proteins eluted together in the final gel-filtration column with an apparent molecular weight of 46 000, though the calculated molecular weights of *rho*-GDI and *rac*2/CDC42Hs are 23 400 and 21 000, respectively. Copurification of *rho*-GDI with *rac*1 from guinea pig macrophages has also been described by Abo et al. (1991). In the present study, the apparent association of *rho*-GDI with the GTP-bound form of *rac*2 and CDC42Hs was directly demonstrated by radiolabeling studies, in which NCF-3 with [^{35}S]GTPγS still coeluted with *rho*-GDI from the final gel-filtration column (Figures 1C and 3). This suggested that *rho*-GDI was capable of binding to the GTP-bound form of *rac*. This observation is somewhat surprising in view of studies showing that only the GDP-bound form, but not the GTP-bound form, of *rho* purified from brain associates with *rho*-GDI (Ueda et al., 1990). The lack of stimulatory effect of GTPγS on NCF-3 activity suggests that most if not all of the neutrophil-derived active *rac*2 purified under the present experimental condition is in the GTP-bound form. This does not imply that *rac*2 is principally in this form in resting neutrophils; to the contrary, G proteins are expected to exist predominantly in a GDP-bound form in resting cells. One possible explanation for all the selective purification of this *rho*-GDI-associated form of *rac*2 is that noncomplexed *rac*2 was labile or its activity could not be assessed under the experimental conditions employed. This possibility is supported by later observations showing that recombinant *rac* had high activity in Tris buffer with 4 mM $MgCl_2$ but little activity in buffer A used in the NCF-3 purification protocol, which contained relatively high phosphate concentration (0.1 M) and low magnesium concentration (1 mM) (data not shown). Purification of *rac*2 to near homogeneity from differentiated HL-60 cells by Mizuno et al. (1992) was achieved in Tris buffer with 5 mM $MgCl_2$. Their *rac*2 appeared to be predominantly in either a nucleotide-free or GDP-bound form because a 5-fold stimulation of superoxide production was noted when GTPγS was added to their cell-free assay consisting of recombinant p47^{phox} and p67^{phox} and HL-60 cell membranes. When bovine brain *rho*-GDI was added to their assay, a 53% inhibition of superoxide production was noted. This finding was consistent with its known action in inhibiting exchange of G-protein-bound GDP with free GTP (Hiraoka et al., 1992). The copurification of *rho*-GDI and *rac*2 in a GTP-bound form in the present study would suggest that *rho*-GDI also binds and stabilizes the GTP-bound form of *rac* as well. Such a role for *rho*-GDI is further supported by recent observations that brain GDI, a protein structurally similar or identical to *rho*-GDI, inhibited the GTPase activity of CDC42Hs (Hart et al., 1992). Whether *rho*-GDI would similarly inhibit the GTPase activity of *rac*, thus stabilizing its activity, remains to be determined.

Although *rho*-GDI was copurified with *rac*2 in the present study, *rac*2 alone was capable of supporting superoxide production by neutrophil membranes in the presence of p47^{phox} and p67^{phox} (Figure 6; Mizuno et al., 1992). Similarly, Abo et al. (1991) reported copurification of *rho*-GDI with *rac*1 from guinea pig macrophage and showed that recombinant *rac*1 in the presence of $\sigma 2$ (cytosolic fraction containing p47^{phox} and p67^{phox}) was capable of supporting superoxide production by macrophage membranes. However, Mizuno et al. (1992) purified both *rac*1 and *rac*2 from differentiated HL-60 cells but found that only *rac*2 was capable of supporting superoxide production. The reason why *rac*1 was not active in their study is not clear. While *rac*2, but not *rac*1, was identified from human neutrophils in the present study, recombinant *rac*1 and *rac*2 were both shown to be active in supporting rp47^{phox}, rp67^{phox}, and neutrophil membranes for superoxide production (Figure 6). Although the amino acid sequence of CDC42Hs is 70% identical to those of *rac*1 and *rac*2, it is not active in augmenting the NADPH oxidase (Figure 6). This recombinant CDC42Hs preparation was otherwise active as a GTPase, as shown in Figure 7. In this assay, GTPase activity was determined from the loss of radioactivity of CDC42Hs prebound with [γ-^{32}P]GTP. This loss of radioactivity is due to hydrolysis of GTP rather than dissociation of GTP because little loss of radioactivity was noted if CDC42Hs was prebound with nonhydrolyzable [^{35}S]GTPγS instead. Consistent with the inability of CDC42Hs to activate the NADPH oxidase, Knaus et al. (1991) showed that DEAE column fractions of neutrophil cytosol containing CDC42Hs did not support superoxide generation. This lack of activity of CDC42Hs in supporting superoxide production indicates that certain protein domain(s) of *rac* essential for oxidase activation are absent in CDC42Hs. Alignment of the cDNA-predicted amino acid sequences of *rac*2 with *rac*1 and CDC42Hs revealed three regions in these *rho*-related G proteins (Figure 9) which are well conserved between the active *rac*1 and *rac*2 but markedly divergent from their oxidase-inactive relative, CDC42Hs. It appears likely that one or more of these regions of *rac* is

required for its interaction with the other oxidase component(s). It is interesting to note that among these three regions, residues 27–52 correspond closely to the region of *ras* (residues 21–54) reported to be essential for *ras* transforming activity (Zhang et al., 1990). Part of this region of *ras* (residues 26–36 and 44–51) exists as loop structures lying on the surface of the protein (Pai et al., 1989). Similarly, the functional determinant of K-*rev*-1 was described also in the N-terminal third of the protein. By analogy, the best candidate determinant specifying the function of *rac* is the region spanning residues 27–52 highlighted in Figure 9. The observation that CDC42Hs could not substitute for *rac* in the cell-free NADPH oxidase assay implies that it serves a different function involving different effector proteins in neutrophils. The observation that human CDC42Hs, but not human *rac*1 and *rac*2 (Shinjo et al., 1990), was capable of complementing a lethal mutation of CDC42Sc in *Saccharomyces cerevisiae* further supports this notion that CDC42 and *rac* have distinct effectors.

In conclusion, NCF-3 was purified as a complex consisting of *rho*-GDI and *rac*2, which appeared to be predominantly in a GTP-bound form. Prebinding of *rac*2 with [^{35}S]GTPγS showed that it still coeluted with *rho*-GDI in gel filtration as a 46-kDa complex, indicating that *rho*-GDI was capable of binding to the GTP-bound form of *rac*. Both *rac*1 and *rac*2 were active in supporting human neutrophil membranes for superoxide production, but their closest relative CDC42Hs was not. Three distinct regions of *rac*1 and *rac*2 were identified which appear likely to determine the functional specificity of these G proteins.

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