produced by transfection of the ψ-CRIP amphotropic packaging line were used to infect myoblasts. Supernatants from MFG and α-SGC producer cells were filtered (0.45 μm), mixed, and added with polybrene at 8 μg/ml to C2C12 mouse myoblasts for 12 hours, then replaced with fresh viral supernatant for an additional 12 hours. Transduced cells were tested for production of helper virus by L. Cohen (Somatix Therapy Corporation), who used the *his* mobilization assay [O. Danos and R. C. Mulligan, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 6460 (1988)]. Using ecotropic Moloney murine leukemia virus as the control for replication competent virus, we determined the sensitivity of the assay to be 1 particle per 2.0 ml of culture supernatant. All of the test samples registered negative.
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Regulation of Phagocyte Oxygen Radical Production by the GTP-Binding Protein Rac 2

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A major action of the microbicidal system of human neutrophils is the formation of superoxide anion (O_2^-) by a multicomponent oxidase that transfers electrons from the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) to molecular oxygen. The mechanism of assembly and activation of the oxidase from its cytosolic and membrane-bound components is unknown, but may require the activity of a guanosine 5′-triphosphate (GTP)-binding component. A cytosolic GTP-binding protein (G_{ox}) that regulates the NADPH oxidase of neutrophils was identified. G_{ox} was purified and shown to augment the rate of O_2^- production in a cell-free oxidase activation system. Sequence analysis of peptide fragments from G_{ox} identified it as Rac 2, a member of the Ras superfamily of GTP-binding proteins. Antibody to a peptide derived from the COOH-terminus of Rac 2 inhibited O_2^- generation in a concentration-dependent manner. These results suggest that Rac 2 is a regulatory component of the human neutrophil NADPH oxidase, and provide new insights into the mechanism by which this oxygen radical–generating system is regulated.

HUMAN NEUTROPHILS AND OTHER phagocytic cells undergo a respiratory burst in which superoxide anion (O_2^-) and its derivatives (H_2O_2, HOCl, HO·) are produced as a means of destroying ingested microorganisms. The significance of the respiratory burst in host defense is made evident by the recurrent and life-threatening infections that occur in patients with chronic granulomatous disease (CGD) in whose phagocytes the burst does not occur. It is known that CGD results from genetic defects in any one of the four known protein components of the NADPH oxidase enzyme responsible for generating O_2^- (*1*). Studies with various cell-free oxidase activation systems (*2*) and cellular material from CGD patients have helped to determine the identity and function of the components of the NADPH oxidase. The oxidase is composed of membrane-bound proteins that include cytochrome b_{558} and possibly a 45-kD flavoprotein, as well as cytosolic components, of which two have been well characterized: $p47_{[phox]}$ and $p67_{[phox]}$ (*3*). The NADPH oxidase is activated, at least in part, by the association of these components into a membrane-bound complex that can transfer electrons from NADPH to molecular oxygen, generating O_2^- (*3*).

A GTP-binding protein (*5*) appears to regulate NADPH oxidase activity. GTP is required for oxidase activation in cell-free systems and GDP analogs inhibit this activation (*4*). Also, inhibition of protein isoprenylation decreases the rate of O_2^- generation (*5*). Post-translational modifications that attach 15- or 20-carbon isoprenoid moieties are a common feature of GTP-binding proteins of the Ras superfamily, suggesting that a member of this family may be involved in oxidase activation. Members

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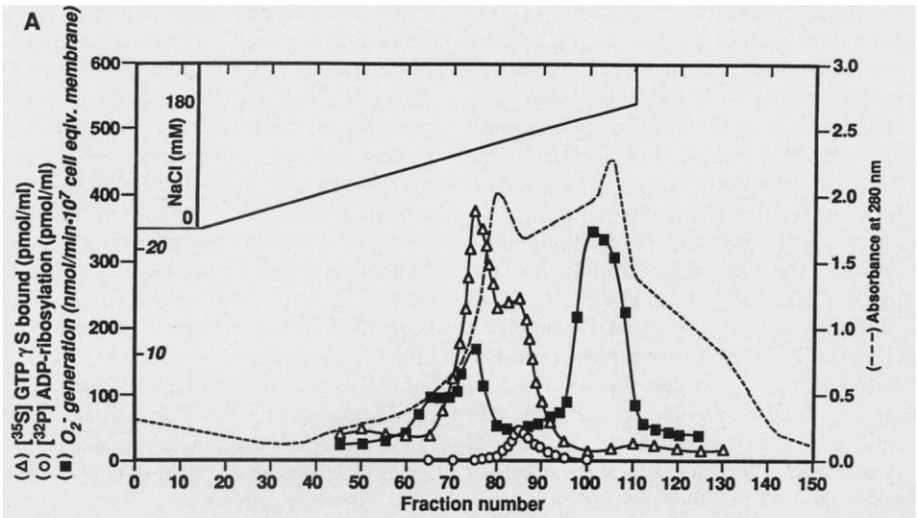

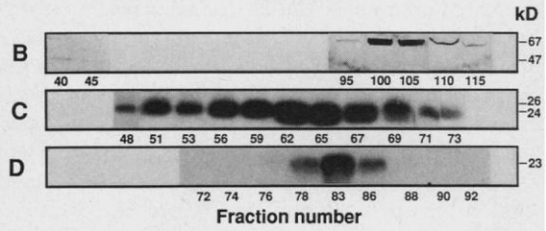

Fig. 1. Ion exchange chromatography of G_{ox}. (**A**) Human neutrophil cytosol was prepared (7), concentrated, and applied as a 1:10 dilution in a buffer containing 25 mM tris-HCl (pH 7.5), 1 mM EDTA, 5 mM MgCl, 0.1 mM DTT, 1 mM 2-mercaptoethanol, 0.5 mM phenylmethylsulfonyl fluoride, 1.5 mM ATP onto a DEAE-Sephacel column (Pharmacia, 2.5 × 25 cm). After a 300-ml wash, the bound protein was eluted by a 280-ml linear gradient of 0 to 180 mM NaCl in the above buffer containing 0.1 mM ATP followed by 200 ml of the same buffer containing 1M NaCl. Fractions (3.0 ml) were collected and portions of the indicated fractions were assayed (*8*) for O_2^- generation (■) in a cell-free system (containing a threshold amount of neutrophil cytosol), for guanine nucleotide binding (△) with [^{35}S]GTPγS in a filtration assay (*23*), and for ADP-ribosylation (○) with purified C3 ADP-ribosyltransferase of botulinum toxin (*24*). Further characterization of the fractions 40 to 115 is shown in panels B to D. Fractions not shown did not contain detectable amounts of the component being probed. (**B**) Immunoblot of DEAE-Sephacel fractions with antibodies to peptides from $p47_{[phox]}$ and $p67_{[phox]}$ (*25*). (**C**) Autoradiogram of [α-^{32}P]GTP-labeled nitrocellulose blots of DEAE-Sephacel fractions (*10*). (**D**) Immunoblot of DEAE-Sephacel fractions with antibody to a peptide from CDC42Hs (*11*). Protein immunoblotting was performed as described (*12*). Data shown are from a single purification, but are representative of the results obtained in at least five experiments.

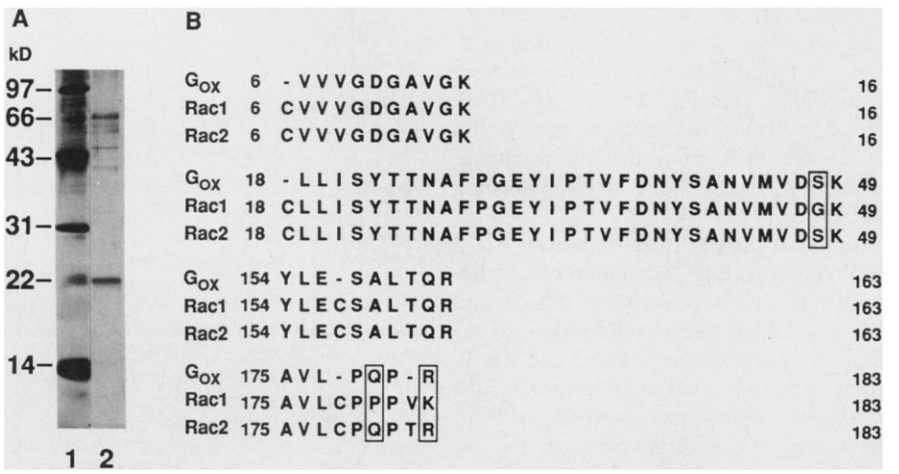

Fig. 2. Purified G_{ox} protein and its partial amino acid sequence. (**A**) SDS-PAGE (15% gel) of G_{ox} after five purification steps, visualized with silver stain in lane 2. The result shown is representative of more than five similar purifications. Lane 1 shows standard proteins and their relative molecular sizes in kD, with Rap1A as a 22-kD standard. (The faint bands at ~50 to 67 kD in lanes 1 and 2 are staining artifacts seen across the entire gel). (**B**) Comparison of the amino acid sequences of peptides derived by tryptic digestion of purified G_{ox} with those deduced from the cDNAs encoding Rac 1 and Rac 2 (*16*). Amino acids at which G_{ox} peptides and Rac 2 differ from Rac 1 are indicated by boxes. Unidentified residues of G_{ox} peptides are marked by hyphens. Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

of the Ras superfamily have molecular weights of ~20 kD and contain conserved GTP-binding and GTP-hydrolysis motifs. The GDP-bound forms of the proteins are generally considered to be inactive, and the GTP-bound forms are active. Although members of the Ras superfamily have been implicated in the regulation of a wide variety of cell functions, none of these proteins has yet been shown to exert direct effects in any cellular system. Here, we describe the purification and identification of a low molecular weight GTP-binding protein that stimulates the NADPH oxidase system of human phagocytes.

The protein mediating the GTP dependence of the NADPH oxidase appears to be cytosolic (*5, 6*). We therefore chromatographically fractionated human neutrophil cytosol in order to identify oxidase-stimulatory activities that might co-elute with GTP-binding activity. Cytosol prepared from unstimulated neutrophils (7) was concentrated, adjusted to 10 mM KCl by dilution, and applied to a DEAE-Sephacel column. The column was washed extensively, proteins were eluted with a linear salt gradient, and the column was washed with 1 M NaCl (Fig. 1A). A cell-free NADPH oxidase activation system containing a low concentration of cytosol at the threshold of being able to support activation (*8*) was used to assay oxidase-enhancing activity. Activity was detected in the column flow-through and in two peaks eluted in the salt gradient (Fig. 1A). Analysis of protein immunoblots showed that the majority of the $p47_{[phox]}$ component was in the unbound fraction (*9*), with low levels in fractions 40 to 45 (Fig. 1B). The second peak of oxidase-enhancing activity (fractions 95 to 115) contained the $p67_{[phox]}$ component (Fig. 1B), whereas neither one of these known neutrophil cytosolic oxidase factors was present in the remaining peak of oxidase-enhancing activity (fractions 71 to 77). GTP-γ-S–binding activity was resolved mainly into two overlapping peaks (Fig. 1A). The peak of maximal GTP-γ-S binding was associated with the oxidase stimulatory activity in fractions 71 to 77. We further characterized this stimulatory activity associated with GTP-γ-S binding (G_{ox}) by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and nitrocellulose blot analysis of the eluted fractions. Some small GTP-binding proteins bind [α-^{32}P]GTP after denaturation by SDS-PAGE and transfer to nitrocellulose (*10*). Although labeling with [α-^{32}P]GTP revealed binding of [α-^{32}P]GTP by 24- and 26-kD proteins that eluted in fractions 51 to 71, G_{ox} was not detectable by this technique (Fig. 1C). The labeled proteins may be identical to two previously described human

Table 1. Augmentation of the rate of O_2^- generation in the cell-free NADPH oxidase assay system by purified G_{ox}. Purified G_{ox} (22 pmol in experiments 1 to 3; 16 pmol in experiment 4) or an equivalent volume of G_{ox} buffer (83 μl) were added to NADPH oxidase reaction mixtures containing a threshold amount of neutrophil cytosol, sufficient by itself to give approximately 10% of the maximal rate obtainable. Assays were performed in 96-well microtiter plates in a total volume of 150 μl, as described (*8*). All reaction mixtures contained cytosol from 6.3×10^5 cells and 10 μM GTP-γ-S. Experiment 1 was performed with membrane vesicles (4×10^5 cell equivalents) and the reactions were initiated with 110 μM SDS. In experiments 2 through 4, deoxycholate-solubilized membranes (1.25×10^6 cell equivalents) and 40 μM SDS were used. Superoxide generation was measured by following the rate of superoxide dismutase–inhibitable cytochrome c reduction at 550 nm in a kinetic microplate reader (*8*).

Addition to reaction mixture	O_2^- generation (nmol/min·10^7 cell equivalents of membrane)			
	Exp. 1	Exp. 2	Exp. 3	Exp. 4
None	6.68	4.24	3.27	4.02
G_{ox}	17.90	14.00	7.80	11.22
G_{ox} Buffer	8.29	7.80	2.50	6.06
Fold increase*	2.4	2.5	2.6	2.3

*This value represents the fold increase in rate caused by addition of G_{ox} after allowing for the effect of the buffer.

neutrophil GTP-binding proteins (*10*). Immunoblots with an affinity-purified antibody to a COOH-terminal peptide from the GTP-binding protein CDC42Hs (*11*) showed the presence of this abundant protein in fractions 78 to 86 (Fig. 1D). We also detected a substrate for adenosine diphosphate (ADP)–ribosylation (fractions 80 to 92) with the C3 ADP-ribosyltransferase of botulinum toxin, which presumably represents the GTP-binding protein Rho (Fig. 1A). The GTP-γ-S–binding peaks were further probed with antiserum to Rap1A (*12*) and with the monoclonal antibody 142-24E05 that recognizes a common consensus region of GTP binding in Ras-related proteins (*13*). Only the 142-24E05 antibody weakly recognized a 22-kD protein present in the G_{ox} peak. Thus, the GTP-γ-S–binding protein that co-elutes with the oxidase-enhancing activity does not appear to be any of these previously described GTP-binding proteins.

The peak of NADPH oxidase-enhancing and GTP-γ-S–binding activity was subjected to five additional purification steps (*14*): gel filtration on Sephacryl-S200 HR, ion exchange chromatography on Fractogel TSK 650M, Mono Q fast protein liquid chromatography (FPLC), hydrophobic-interaction chromatography on heptylamine Sepharose, and phenyl-Superose FPLC. During all purification procedures, GTP-γ-S–binding and oxidase-enhancing activity co-eluted. The resulting G_{ox} protein was purified nearly to homogeneity (Fig. 2A) and migrated on SDS-PAGE with a molecular size of 21 to 22 kD, similar to the 22-kD Rap1A protein used as a standard (Fig. 2A). After transfer to nitrocellulose, the purified protein did not react with antibody to the CDC42Hs peptide or bind [α-^{32}P]GTP. The botulinum toxin C3 ADP-ribosyltransferase ADP-ribosylated G_{ox} poorly compared to the Rho protein. The purified G_{ox} protein was also assayed for its stimulatory effect on the NADPH oxidase (Table 1). When the amount of cytosol was sufficient to provide at least a small amount of each of the cytosol components required for NADPH oxidase activation, the basal rate of O_2^- generation was increased 2.5 times when 22 pmol of G_{ox} was added to the assay (150 μl). This augmentation could be shown either with deoxycholate-solubilized or intact neutrophil membranes (*8*). The stimulation of O_2^- generation by a partially purified G_{ox} preparation was concentration-dependent (*9*).

To obtain further structural information on G_{ox}, the protein was isolated by preparative SDS-PAGE. After electroelution of the protein and precipitation with a chloroform-methanol mixture, we found that the amino terminus of G_{ox} was blocked. The protein was therefore transferred to nitrocellulose and cleaved with trypsin, and the resulting peptides were separated by reverse-phase HPLC (*15*). The amino acid sequences of four peptide fragments were determined (Fig. 2B) and were compared with the sequences of other GTP-binding proteins. The peptide sequences are identical with regions of the amino acid sequence deduced from cDNA encoding Rac 2 (*16*). These peptide sequences also contain three amino acids that differ from the corresponding residues predicted from cDNA encoding Rac 1 (*16*).

Rabbit antiserum to recombinant Rac 2 (*17*) reacted with G_{ox} on immunoblots. Because this antibody recognizes Rac 1 and Rac 2, we then utilized affinity-purified antibodies to peptides from Rac 1 and Rac 2. These antibodies were generated against COOH-terminal sequences upstream from the CAAX box of both proteins that are specific in each type of Rac (*18*). Only the antibody to Rac 2 bound to G_{ox} on protein immunoblots (*9*).

Confirmatory evidence that Rac 2 accounted for the oxidase regulatory activity in the G_{ox} preparation was obtained with the antibody to the COOH-terminus of Rac 2 (Fig. 3). In the cell-free activation system with intact membranes, this antibody caused a concentration-dependent inhibition of O_2^- generation that, at the highest concentration tested, resulted in 70% inhibition (Fig. 3). This effect was partially reversed by preincubation of the Rac 2 antibody with G_{ox} protein, although experiments were limited by the small amount of the GTP-binding protein available. In contrast, antibodies to the COOH-terminal peptides of Rac 1 and CDC42Hs showed much less inhibitory activity (Fig. 3). An antibody to an undefined neutrophil surface antigen also did not inhibit O_2^- generation.

Our results indicate that Rac 2 stimulates the O_2^--generating NADPH oxidase of human neutrophils and is likely to account for the GTP dependence of the activation process. However, the involvement of other neutrophil GTP-binding proteins is not ruled out. Rap1A interacts with the oxidase-associated cytochrome b (*19*), and a truncated form of recombinant Rap1A has been reported to restore oxidase activity to immunodepleted cytosol (*20*). We have been unable to detect any stimulatory activity, however, of full-length baculovirus-expressed

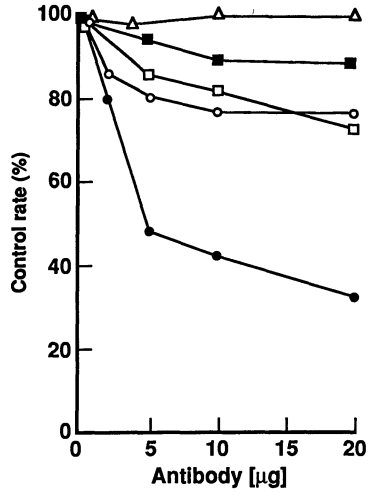

Fig. 3. Inhibition of the NADPH oxidase by a Rac 2 peptide antibody. Human neutrophil cytosol was incubated for 16 hours at 4°C with the indicated amount of affinity-purified antibody and assayed in the cell-free oxidase assay (150 μl) as described (*8*). The amount of cytosol used in the assay (2×10^6 cell equivalents) was sufficient to support about 95% of the maximal rate obtainable. Results are expressed as percent of the rate of O_2^- formation in the absence of antibody or antibody buffer and are indicative of at least three experiments. Control values of O_2^- generation varied from 30 to 50 nmol/min·10^7 cell equivalents of membrane in individual experiments. Antibodies specific to Rac 2, (●), and Rac 1, (○), were prepared with peptides representing amino acids 178 to 188 of each protein (*18*). Also tested were antibody to CDC42Hs Apl (□) (*11*), monoclonal antibody to an undefined neutrophil surface antigen (△) (*26*), and the buffer in which the affinity-purified antibodies were dissolved (■) (200 mM glycine neutralized to pH 7.5 with phosphate buffer).

Rap1A under similar conditions (*21*). Rac 1, a protein closely related to Rac 2 (92% identity) may also have oxidase regulatory activity. Although Rac 1 mRNA is expressed in a large number of different cell types, Rac 2 mRNA is expressed only in cells of myeloid origin (*16*). In HL-60 cells induced to differentiate with dibutyryl cyclic adenosine monophosphate, expression of Rac 2 mRNA, but not Rac 1 mRNA, increased by seven- to ninefold. This may indicate a specialized action of Rac 2 in neutrophil function or differentiation. Also of interest in this regard are the isoelectric points of the Rac proteins. On the basis of their amino acid sequences, the isoelectric point (pI) of Rac 2 is calculated to be 7.56 and that for Rac 1 is 8.53 (*16*). The four cytosolic oxidase components have been resolved by preparative isoelectric focusing and have approximate pIs of 3.1, 6.0, 7.1, and 9.5. Two of these components (pI 6.0 and 9.5) are $p67_{[phox]}$ and $p47_{[phox]}$, respectively. The pI of 7.1 reported for one of the unidentified factors is similar to that predicted for Rac 2.

The mechanism by which Rac 2 acts to enhance activity of the NADPH oxidase and its exact function in the assembly and translocation of the other cytosolic components to form an active complex at the membrane should be amenable to investigation. Additionally, the availability of a cell-free assay for the activity of this member of the Ras superfamily will enhance our ability to determine regulatory features of the low molecular weight GTP-binding proteins in general.

Note added in proof: It has recently been reported that Rac 1 also has oxidase regulatory capability (*22*).

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25. SDS-PAGE was performed on 4 to 20% polyacrylamide gradient gels (Mini Protean II, Bio-Rad). After electrophoretic transfer to Zeta-Probe blotting membrane (Bio-Rad) the blots were blocked with BSA (3%) and goat serum (10%) in Hepes-buffered saline. The blots were probed with a cocktail of antibodies to peptides from $p47_{[phox]}$ (amino acids 340 to 355) and $p67_{[phox]}$ (amino acids 437 to 450) and developed after exposure to goat antibody to rabbit IgG that was conjugated to alkaline phosphatase.
26. J. T. Curnutte, unpublished data.

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NMDA Antagonist Neurotoxicity: Mechanism and Prevention

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Antagonists of the *N*-methyl-D-aspartate (NMDA) subtype of glutamate receptor, including phencyclidine (PCP) and ketamine, protect against brain damage in neurological disorders such as stroke. However, these agents have psychotomimetic properties in humans and morphologically damage neurons in the cerebral cortex of rats. It is now shown that the morphological damage can be prevented by certain anticholinergic drugs or by diazepam and barbiturates, which act at the γ-aminobutyric acid (GABA) receptor-channel complex and are known to suppress the psychotomimetic symptoms caused by ketamine. Thus, it may be possible to prevent the unwanted side effects of NMDA antagonists, thereby enhancing their utility as neuroprotective drugs.

ANTAGONISTS OF THE NMDA SUBtype of glutamate receptor are potentially useful for preventing neuronal degeneration in neurological disorders such as stroke (*1*). However, treatment of adult rats with noncompetitive (phencyclidine, MK-801, tiletamine, ketamine) or competitive [D-2-amino-5-phosphonopentanoate (D-AP5)] NMDA antagonists causes neurotoxic side effects consisting of pathomorphological changes in neurons of the cingulate and retrosplenial cerebral cortices (*2, 3*). After low doses these changes may be reversible, but higher doses can cause irreversible neuronal necrosis (*4*). Therefore, it has been questioned whether NMDA antagonist therapy can be applied without incurring serious side effects. However, we now report that certain anticholinergic or GABAergic agents protect cerebrocortical neurons against the adverse side effects of NMDA antagonists.

The neurotoxic action of MK-801 in the adult rat cingulate cortex is potentiated by pretreatment with the cholinergic agonist pilocarpine (*5*). This potentiating effect was abolished by coadministration of scopola-

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