Requirement for Posttranslational Processing of Rac GTP-Binding Proteins for Activation of Human Neutrophil NADPH Oxidase

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Rac1 and Rac2 are closely related, low molecular weight GTP-binding proteins that have both been implicated in regulation of phagocyte NADPH oxidase. This enzyme system is composed of multiple membrane-bound and cytosolic subunits and when activated catalyzes the one-electron reduction of oxygen to superoxide. Superoxide and its highly reactive derivatives are essential for killing microorganisms. Rac proteins undergo posttranslational processing, primarily the addition of an isoprenyl group to a carboxyl-terminal cysteine residue. We directly compared recombinant Rac1 and Rac2 in a human neutrophil cell-free NADPH oxidase system in which cytosol was replaced by purified recombinant cytosolic components (p47-phox and p67-phox). Processed Rac1 and Rac2 were both highly active in this system and supported comparable rates of superoxide production. Under different cell-free conditions, however, in which suboptimal amounts of cytosol were present in the assay mixture, processed Rac2 worked much better than Rac1 at all but the lowest concentrations. This suggests that a factor in the cytosol may suppress the activity of Rac1 but not of Rac2. Unprocessed Rac proteins were only weakly able to support superoxide generation in either system, but preloading of Rac1 or Rac2 with guanosine 5'-O-(3-thiotriphosphate) (GTPγS) restored activity. These results indicate that processing is required for nucleotide exchange but not for interaction with oxidase components.

INTRODUCTION

In response to a variety of particulate and soluble stimuli, the phagocytic cells of the human immune system (neutrophils, eosinophils, monocytes, and macrophages) undergo a respiratory burst, catalyzed by NADPH oxidase, a multicomponent electron transport system that reduces oxygen to superoxide (O$_2^-$) [for review see Morel et al. (1991)]. Superoxide and its metabolites (hydrogen peroxide, hydroxyl radical, hypohalous acids) are essential for killing invading bacteria, fungi, and parasites. In unstimulated cells NADPH oxidase is dormant and consists of membrane-bound and cytosolic components, whereas in stimulated cells it is enzymatically active and confined to the plasma membrane (Morel et al., 1991). The two known membrane-bound components of NADPH oxidase, gp91-phox and p22-phox, are subunits of cytochrome b$_{558}$, the terminal electron carrier of the oxidase (Parkos et al., 1987; Segal, 1987). Recent evidence, including reconstitution data and sequence homologies, suggests that cytochrome b$_{558}$ is actually a flavocytochrome that not only binds heme, but also NADPH and the FAD cofactor required for transport of electrons from NADPH to the heme (Rotrosen et al., 1992; Segal et al., 1992). The two other well characterized oxidase components, p47-phox and p67-phox, are found in the cytosol fraction of disrupted, un-

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stimulated neutrophils (Nunoi et al., 1988; Volpp et al., 1988; Curnutte et al., 1989b). Upon activation they become tightly associated with the membrane-bound components (Clark et al., 1990; Heyworth et al., 1989, 1991). The cytoskeleton has also been implicated in this process, but the exact nature of its involvement is not clear (Quinn et al., 1989a; Nauseef et al., 1991; Woodman et al., 1991).

NADPH oxidase can be activated in cell-free systems containing cytosol and membranes from unstimulated phagocytes by the addition of an anionic amphiphile such as arachidonate or sodium dodecyl sulfate (SDS) (Bromberg and Pick, 1984; Curnutte, 1985; McPhail et al., 1985). Several reports have shown that GTP or one of its nonhydrolyzable analogs [e.g., guanosine 5'-O-(3-thiotriphosphate), (GTPyS)] cause a 2- to 4-fold enhancement in the rate of O2- generation by these systems (Seifert et al., 1986; Gabig et al., 1987). More recently, we demonstrated that there is an absolute requirement for GTP (or GTPyS) in the cell-free system (Uhlinger et al., 1991; Peveri et al., 1992). Moreover, the NADPH oxidase activity of differentiated HL-60 cells is dependent upon prenylation of a cytosolic component (Bokoch and Prossnitz, 1992). Taken together, this evidence was strongly indicative of a role for a GTP-binding protein in NADPH oxidase activation.

Two very closely related members of the Rho family of Ras-like low molecular weight GTP-binding proteins have recently been implicated in the regulation of NADPH oxidase. We purified Rac2 from human neutrophil cytosol on the basis of its ability both to bind GTPαS and stimulate O2- generation in the presence of a suboptimal amount of neutrophil cytosol (Knaus et al., 1991, 1992). In similar experiments by using guinea pig peritoneal macrophages, Rac1 was purified in an oxidase-enhancing complex (termed ρ1) with Rho GDP dissociation inhibitor (RhoGDI) (Abo et al., 1991). Subsequently, Mizuno et al. (1992) also purified Rac2 from differentiated HL-60 cells, a human myeloid cell line, and confirmed that it enhances NADPH oxidase activity in a cell-free assay. A different approach was taken by Dorseuil and colleagues (1992), who used Epstein-Barr virus-transformed B lymphocytes that produce O2- by an NADPH oxidase system similar (and perhaps identical) to the one in phagocytic cells. They showed that rac antisense (but not sense) oligonucleotides decreased the Rac protein content of the cells and inhibited O2- generation in a dose-dependent manner, thus confirming the physiological role of Rac proteins in the regulation of NADPH oxidase activity. Rac1 and Rac2 are 92% identical and both undergo posttranslational modification by the addition of a 20-carbon geranylgeranyl group to the cysteine of the carboxy-terminal CAAX box (Xu and Bokoch, unpublished observations; Kinsella et al., 1991). Rac1 is expressed in a wide variety of cell types, whereas Rac2 is apparently restricted to cells of myeloid and lymphoid origin (Didsbury et al., 1989; Reibel et al., 1991).

Two groups have shown, by using a combination of highly purified cytochrome b558, recombinant p47-phox and p67-phox, and either recombinant Rac1 or purified human neutrophil ρ1, that these components are necessary and sufficient for O2- production in the NADPH oxidase cell-free system (Abo et al., 1992; Rotrosen et al., 1992). In this study, we have used a similar system, containing neutrophil membranes and either recombinant cytosolic oxidase components (p47-phox and p67-phox) or suboptimal levels of whole cytosol, to directly compare the ability of Rac1 and Rac2 to support O2- generation. In addition, by using unprocessed (Escherichia coli) or processed (baculovirus) recombinant Rac1 and Rac2 in either their GDP- or GTP-bound forms, we have studied the effect of posttranslational modification on the ability of these proteins to undergo nucleotide exchange and interact with NADPH oxidase components.

**MATERIALS AND METHODS**

**Preparation of Neutrophil Subcellular Fractions**

Neutrophils were obtained from normal, healthy donors by leukapheresis after the oral administration of dexamethasone (12 and 2 h before the procedure) to increase neutrophil yields (Curnutte et al., 1989a). After hypotonic lysis of erythrocytes, neutrophils were purified by density-gradient centrifugation through Ficoll-Paque (Pharmacia LKB, Piscataway, NJ) as previously described (Badwey et al., 1982). Neutrophils were treated with 2.5 mM diisopropyl fluorophosphate for 10 min at 4°C, disrupted in relaxation buffer [100 mM KCl, 3 mM NaCl, 3.5 mM MgCl2, 1 mM ATP, 1.25 mM ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 1.4-piperazine-dithanesulfonic acid (PIPES), pH 7.3] (Borregaard et al., 1983) by N2 cavitation, and fractionated on discontinuous Percoll gradients. These methods, described in detail elsewhere (Curnutte et al., 1987; Babior et al., 1988), produce cytosol and plasma membrane fractions whose final concentrations were adjusted to 9-108 and 1.25-109 cell equivalents (cell eq)/ml, respectively. Fractions were stored at −80°C for up to 1 y without loss of activity.

**Production and Purification of Recombinant Proteins**

Recombinant Rac2 was expressed in E. coli and purified exactly as reported elsewhere (Knaus et al., 1992). The full-length cDNA encoding Rac1 was subcloned into the BamHI site on the expression vector pET3a. E. coli strain BL21-DE3 was used as the host cell for the recombinant plasmid, termed pER1, and was grown in LB medium at 37°C until an optical density (OD) of 0.7-0.9 was reached. Isopropyl β-D-thiogalactopyranoside was added to a final concentration of 1 mM to induce protein expression. The cells were grown for an additional 2 h at 37°C, harvested by centrifugation at 5 000 g and 4°C for 15 min, resuspended in 25 mM tris(hydroxymethyl)aminomethane [Tris]HCl (pH 8.0), 1 mM EDTA, 5 mM dithiothreitol, 5 mM MgCl2, 0.5 mM phenylmethylsulfonyl fluoride, and incubated for 1 h at 25°C in this buffer supplemented with lysozyme (0.5 mg/ml) and deoxyribonuclease I (10 μg/ml). The suspension was then subjected to two 10-s bursts of sonication at 4°C and to one cycle of freezing and thawing. After centrifugation for 25 min at 15 000 g, the supernatant was dialyzed against equilibration buffer [25 mM Tris-HCl (pH 8.0), 1 mM EDTA, 5 mM MgCl2, 5 mM dithiothreitol, 1 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride] and...
applied to a Mono Q HR 5/5 column (preequilibrated with the same buffer) connected to an FPLC system (Pharmacia LKB). The column was washed extensively with equilibration buffer and eluted at a flow rate of 0.5 ml/min with a 30 ml linear gradient of 0-300 mM NaCl, followed by a steeper 15-ml gradient of 300-1000 mM NaCl, both in the same buffer. The E. coli recombinant Rac1 eluted at 100-120 mM NaCl. This material was 70-80% pure as estimated by silver staining. Recombinant proteins expressed in bacterial systems do not undergo the normal posttranslational processing of the native protein and are referred to here as unprocessed protein. This was confirmed by the inability of E. coli-expressed Rac1 and Rac2 to partition into the detergent phase upon Triton X-114 partitioning analysis ( Bordier, 1981).

To produce forms of Rac1 and Rac2 that had undergone posttranslational modification (referred to as processed protein), a baculovirus/insect cell expression system was also utilized. The full-length cDNAs encoding Rac1 and Rac2, each with an additional sequence coding for the N-terminal epitope tag Met-Glu-Glu-Glu-Tyr-Met-Pro-Met-Glu, were subcloned into the baculovirus transfer vector pAC13 so as to place these genes under the control of the polyhedrin promoter. Spodoptera frugiperda host cells (SF9) were co-transfected with the Rac-containing transfer vector and wild-type baculovirus (AcMNPV) DNA (Summers and Smith, 1987). Recombinant viruses were identified by using a standard plaque assay, and virus from a single plaque was amplified and used to infect SF9 cells during log phase growth. Cells were harvested at 72 h postinfection. To purify the recombinant Rac, the baculovirus infected SF9 cells were centrifuged at 450 ps and 4°C for 20 min in a buffer consisting of 100 mM KCl, 3 mM NaCl, 3.5 mM MgCl₂, 10 mM PIPES (pH 7.3), 1 mM phenylmethylsulfonyl fluoride, 100 kallikrein inhibitory units of apronin/ml, 1 µM leupeptin, 1 mM 2-mercaptoethanol, and 0.2 mM diithiothreitol. The infected cells were collected into sufficient ETA to give a final concentration of 1 mM and centrifuged at 1 000 g and 4°C for 10 min to remove unbroken cells. The supernatant was centrifuged at 100 000 g for 35 min and the cytosol was removed from the pellet. The pellet (containing cell membranes) was washed once (with a buffer containing 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 10 mM MgCl₂, 50 mM NaCl, 0.1 mM diithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 1 mM 2-mercaptoethanol) to insure removal of contaminating cytosol, solubilized in extraction buffer (25 mM Tris-HCl (pH 7.5), 1 mM EDTA, 5 mM MgCl₂, 0.1 mM diithiothreitol, 1 mM phenylmethylsulfonyl fluoride and 0.9% cholate) for 4 h on ice and centrifuged at 100 000 g for 35 min. The supernatant was applied to a column of Protein G Sepharose 4 Fast Flow (Pharmacia LKB) cross-linked with a monoclonal antibody to the peptide Glu-Glu-Glu-Glu-Tyr-Met-Pro-Met-Glu, previously preequilibrated with extraction buffer (Grussenmeyer et al., 1985). After application of the supernatant, the column was washed with 15 column volumes of extraction buffer followed by 10 column volumes of extraction buffer containing 100 mM NaCl. Bound protein was then eluted with a solution of peptide (Glu-Glu-Tyr-Met-Pro-Met-Glu) in extraction buffer, at a concentration of 100 µg/ml. These methods produced highly pure preparations of Rac2 and Rac1 as shown by silver staining (lanes 3 and 4, Fig. 1). These proteins were shown to be isopyenically by the incorporation of [3H]mevalonic acid (Bokoch, unpublished observations).

Recombinant full length Rap1a, H-Ras, and CDC42Hs were purified after expression in a baculovirus/SF9 insect cell system, using previously reported methods (Quilliam et al., 1990; Hart et al., 1991). Baculovirus/SF9-expressed CDC42Hs was generously provided for testing by D. Leonard and R. A. Cerione of Cornell University. Recombinant forms of the neutrophil NADPH oxidase catalytic components, p47- phox and p67-phox, were expressed in a similar system using the full-length cDNA clones and purified to near-homogeneity, as shown in lanes 1 and 2 of Figure 1, employing the methods described by Uhlinger et al. (1992).

Quantification of GTP-binding Proteins and Preloading with [35S]GTPγS

Recombbinant GTP-binding proteins were quantified by their ability to bind [35S]GTPγS, as determined by a rapid filtration technique (Knaus et al., 1992). For some experiments, Rac proteins were preloaded with [35S]GTPγS by incubation with 40 nM HEPS (pH 7.5), 4 mM EDTA, 2 mM diithiothreitol and 2 µM [35S]GTPγS (1–2 x 10⁵ cpm/ml) at room temperature. The reaction was stopped by raising the concentration of free Mg²⁺ from the original 400 mM to 10 mM. After removal of free [35S]GTPγS by ultracentrifugation (Centricon 10, Amicon, Beverly, MA) the concentration of Rac-[35S]GTPγS was determined as described (Knaus et al., 1992).

Cell-free NADPH Oxidase Assay

Production of O₂⁻ by NADPH oxidase was monitored in a cell-free system at 25°C by following the superoxide dismutase-inhibitable reduction of cytochrome c (350 nm) as previously described (Cumutte et al., 1989b). Reaction mixtures (in 96-well microtitration plates) contained: 0.1 mM cytochrome c, 6.5 mM MgCl₂, 87 mM KCl, 2.6 mM NaCl, 8.7 mM PIPES (pH 7.3), 10 µM GTPγS, 0.16 mM NADPH, and 4 x 10⁸ cell eq. of neutrophil membranes (1.6 µg protein), in a total volume of 150 µl. In addition, reactions contained varying amounts of recombinant low molecular weight GTP-binding protein, and either 1) 8 µl (7.2 x 10⁸ cell eq.; ~18 µg protein) of neutrophil cytosol or 2) 100 nM each of baculovirus recombinant p47-phox and p67-phox as specified in the text or in figure legends. Control wells (paired with each reaction well) contained 9 µg superoxide dismutase, to account for non-O₂⁻-dependent reduction of cytochrome c. Superoxide production was initiated by the addition of SDS to give a final concentration of either 90 µM (with recombinant p47-phox/p67-phox) or 110 µM (with neutrophil cytosol). Maximum rates of absorbance change, calculated from the first derivative of the time course using Softmax software (Molecular Devices, release 2.01), were converted to nmol O₂⁻ generated/min per 10⁸ cell eq of membrane (after subtracting the rate achieved in the presence of superoxide dismutase from that obtained in its absence, to give the rate of O₂⁻-dependent cytochrome c reduction), by using an extinction coefficient of 20.5 per mM/cm for reduced minus oxidized cytochrome c (Cumutte et al., 1989b). Standard deviations are given, with (n) representing the number of experiments.

Miscellaneous Methods and Materials

SDS-polyacrylamide gel electrophoresis and silver staining were performed by using previously described methods (Knaus et al., 1992). Reagents used in the purification of neutrophils, the preparation of neutrophil fractions and the cell-free O₂⁻ assay were obtained from the sources previously reported (Cumutte et al., 1987). [35S]GTPγS
RESULTS

In the cell-free NADPH oxidase system containing $4 \times 10^8$ cell eq of neutrophil membranes and $1.8 \times 10^6$ cell eq of neutrophil cytosol [the normal amount of cytosol used in the assay system (Peveri et al., 1992)], the mean rate of $O_2^-$ generation was $41.70 \pm 7.36$ (n = 15) nmol/min per $10^7$ cell eq membranes. Figure 2 (trace a) depicts a typical time course of $O_2^-$ generation achieved under these conditions. When cytosol was omitted and replaced with 100 nM each of recombinant p47-phox and p67-phox as well as 27 nM recombinant processed Rac2, the rate of $O_2^-$ production (Figure 2, trace b) was $41.26 \pm 10.73$ (n = 8) nmol/min per $10^7$ cell eq membranes, closely matching that observed with whole cytosol. In the recombinant system, oxidase activity was entirely dependent on the presence of p47-phox and p67-phox (Figure 2, trace c) and almost totally dependent on the addition of Rac2 (trace d) and GTPγS (trace e). The low level of activity obtained in the absence of recombinant Rac2, which was dependent on GTPγS, varied slightly from one batch of neutrophil membranes to another and was probably due to the presence in membrane vesicles of small amounts of entrapped cytosol. (Protein immunoblots using antibodies with high affinities for p47-phox and p67-phox revealed very low levels of these components in some of our membrane preparations.)

Both Rac1 (Åbo et al., 1991) and Rac2 (Knaus et al., 1991) have been purified from phagocytic cells and implicated in the regulation of NADPH oxidase. We directly compared the abilities of the two proteins to support $O_2^-$ generation by NADPH oxidase of human neutrophils. Reaction mixtures containing neutrophil membranes and 100 nM recombinant p47-phox and p67-phox (levels giving near-maximal activities) were supplemented with increasing amounts of Rac1 and Rac2, in both their processed and unprocessed forms. As shown in Fig. 3, concentration-response curves for the processed forms of the two proteins were very similar, with maximum rates of $O_2^-$ generation [37.24 ± 5.79 (n = 6) and 41.26 ± 10.73 (n = 8) nmol/min per $10^7$ cell eq membrane with Rac1 and Rac2, respectively] achieved at final GTP-binding protein concentrations of 30-40 nM. Concentrations of Rac1 and Rac2 giving...
half-maximal activity were \( \sim 4 \) and 6 nM, respectively. Activity was totally dependent on the presence of guanine nucleotide, with maximal rates observed with either 10 \( \mu \)M GTP\( \gamma \)S or 10 \( \mu \)M GTP. In marked contrast to the activities obtained with processed recombinant Rac proteins purified from Sf9 cell membranes, the corresponding recombinant proteins expressed in \( E. \) coli, which undergo no isoprenylation, showed only a very weak ability, even at high concentrations, to promote \( O_2^- \) generation in the cell-free oxidase system (Figure 3).

In addition to Rac1 and Rac2, we also tested three other posttranslationally modified recombinant low molecular weight GTP-binding proteins, Rap1A, H-Ras, and CDC42Hs. None of these proteins was able to activate \( O_2^- \) production in the cell-free system at concentrations up to 115 nM for Rap1A, 72 nM for H-Ras, and 133 nM for CDC42Hs (see Figure 3 for CDC42Hs data).

The inability of unprocessed Rac proteins to support \( O_2^- \) generation prompted us to define more clearly the step(s) in oxidase activation at which it is essential for Rac1 and Rac2 to be in their processed forms. By converting \( E. \) coli recombinant Rac1 to its GTP\( \gamma \)S-bound form (Rac1-GTP\( \gamma \)S) before adding it to the other constituents of the reaction mixture, the requirement for isoprenylation of the protein was circumvented (Fig. 4). The maximum rate of \( O_2^- \) production by unprocessed Rac1-GTP\( \gamma \)S (28.79 \( \pm \) 4.97 [\( n = 3 \)] nmol/min per 10\(^7\) cell eq membranes) compared favorably with the rate obtained using processed Rac1-GTP\( \gamma \)S (40.43 \( \pm \) 12.69 [\( n = 3 \)] nmol/min per 10\(^7\) cell eq membranes) (Figure 4). The concentration at which this maximum rate was achieved (20-30 nM) was similar to the concentration of processed Rac1 required for maximal activity (Figure 3). In two experiments to confirm that preloading \( E. \) coli recombinant Rac2 with GTP\( \gamma \)S also circumvented the requirement for posttranslational processing, mean \( O_2^- \) generation rates of 18.78 and 24.87 nmol/min per 10\(^7\) cell eq were achieved with 36 and 90 nM unprocessed Rac2-GTP\( \gamma \)S, respectively.

Preloading processed Rac1 with GTP\( \gamma \)S did not significantly change its ability to support NADPH oxidase activity; maximum rates with the GDP-bound (no preloading) and GTP\( \gamma \)S-bound forms were 37.24 \( \pm \) 5.79 (\( n = 6 \)) and 40.43 \( \pm \) 12.69 (\( n = 3 \)) nmol/min per 10\(^7\) cell eq membranes, respectively. However, in contrast to the situation observed with Rac protein that was not preloaded, Rac1-GTP\( \gamma \)S (whether processed or unprocessed) no longer required guanine nucleotide to be added to the system. We have previously demonstrated that binding of GTP\( \gamma \)S to Rac2 is very slow at the level of Mg\(^2+\) (6.5 mM) present in the oxidase assay (Knaus et al., 1991). It is also evident that the GDP-bound form of Rac1 or Rac2 will not support \( O_2^- \) production (see Figure 2). We conclude, therefore, that there is a stimulatory guanine nucleotide exchange protein (GDP/GTP dissociation stimulator; GDS) operative in our assay system (presumably derived from the membrane fraction) that allows processed but not unprocessed Rac to exchange GDP for GTP\( \gamma \)S (or GTP).

Our initial purification of Rac2 from human neutrophil cytosol was based on the ability of the protein to both bind \( ^{35} \)S\( \)GTP\( \gamma \)S and to augment the \( O_2^- \)-generating ability of a suboptimal amount of cytosol (6.3 \( \times \) 10\(^5\) cell eq) in the cell-free NADPH oxidase system (Knaus et al., 1991, 1992). This amount of cytosol is sufficient to provide at least a small amount of each of the cytosolic components necessary for NADPH oxidase activation and provides a means of detecting components that are below saturating concentrations in the aliquot of whole cytosol (Cumutte et al., 1989b). Having ascertained that processed Rac1 and Rac2 are very similar in their ability to activate NADPH oxidase in the presence of highly purified recombinant p47-\( \)phox and p67-\( \)phox, we tested them in the presence of a suboptimal amount of cytosol, which also contains RhogDI (Knaus et al., 1992) and probably other proteins that can modulate Rac activity. As demonstrated for native protein purified from human neutrophil cytosol (Knaus et al., 1991), baculovirus recombinant Rac2 was able to augment the rate of \( O_2^- \) production in this system (Figure 4). Maximum activity was reached at 50 nM Rac2, resulting in more than a fourfold increase over the basal rate, a result comparable to that seen with the endogenous neutrophil protein. In contrast to Rac2 in this basal cytosol system, and to Rac1 and Rac2 in the re-
DISCUSSION

In this study we provide the first direct comparison of the abilities of Rac1 and Rac2, in both their unprocessed and processed forms, to activate neutrophil NADPH oxidase. Recently, Rotrosen et al. (1992) were able to reconstitute oxidase activity in a system containing purified, relipidated cytochrome b$_{558}$, recombinant p47-phox and p67-phox, and a σ1-like complex of RhOGRD1 and an undefined Rac protein, isolated from human neutrophil cytosol. Abo and colleagues (1992) had similar results with a system in which σ1 was replaced by unprocessed (E. coli) Rac1 preloaded with GTP. Neither of these studies provided data for either recombinant or native, purified Rac2. We find in the current study that in the presence of neutrophil membranes and recombinant p47-phox and p67-phox, processed forms of the two Rac proteins are equally able to support O$_2^-$ generation. This is perhaps not surprising as Rac1 and Rac2 differ in only 15 of their 192 residues, the largest region of dissimilarity being at the carboxyl terminus (Figure 6). The ability of these two closely related proteins to support NADPH oxidase activity is clearly quite specific, as three other low molecular weight GTP-binding proteins (Rap1A, H-Ras, and CDC42Hs), were unable to substitute for Rac. It is of particular interest

![Figure 5](image-url)  
**Figure 5.** The effect of processed low molecular weight GTP-binding proteins on O$_2^-$ production in the cell-free system containing a suboptimal amount of neutrophil cytosol. Baculovirus recombinant Rac1 ( ), Rac2 ( ), and Rap1A ( ) were added to NADPH oxidase reaction mixtures containing 4 × 10$^9$ cell eq membranes and 7.2 × 10$^6$ cell eq cytosol to give the final concentrations indicated. Where n > 2 error bars representing the standard deviation are shown; other data points represent mean values. The basal rate of O$_2^-$ production obtained in the absence of added GTP-binding protein (6.68 ± 1.31 [n = 7] nmol/min per 10$^9$ cell eq membrane) was not subtracted from the rates obtained in the presence of GTP-binding proteins.

![Figure 6](image-url)  
**Figure 6.** The predicted amino acid sequences of Rac1, Rac2, and CDC42Hs. For Rac1 and CDC42Hs only residues that are different from Rac2 are indicated. The 4 regions of CDC42Hs that differ most widely from the Rac proteins are boxed.
that CDC42Hs was unable to support NADPH oxidase activity because, of these three proteins, CDC42Hs is most closely related structurally to the Rac proteins, exhibiting ~70% overall identity with Rac1 and Rac2, and differing from Rac largely in four distinct regions, as outlined in Figure 6. Several of these domains appear to play critical roles in the activity of GTP-binding proteins and may account for the inability of CDC42Hs to function in NADPH oxidase activation. The variable region from amino acids 41-52 is immediately adjacent to the putative ‘effecter’ domain (amino acids 32-40), an area reported to be crucial for biological activities of Ras and Rap (Zhang et al., 1990; Marshall et al., 1991; Nur-E-Kamal et al., 1992; Schaber et al., 1992). The variable region from amino acids 101-111 has been associated with binding of heterotrimeric G protein α subunits to adenyllylcyclase (Itoh and Gilman, 1991), as well as binding of Ras to downstream effectors (Willumsen et al., 1991), and forms an exposed loop in the three-dimensional structure of Ras. Finally, the sequence from amino acids 173-192 contains the ‘hypervariable’ region of these low molecular weight GTP-binding proteins. The functional significance of this region is unknown, but it has been suggested that it directs localization of the Rab proteins to various intracellular compartments (Chavrier et al., 1991). It is perhaps significant that an antibody directed against a peptide corresponding to this region of Rac2 inhibited NADPH oxidase activity (Knaus et al., 1991).

The neutrophil membranes used in our studies contain high levels of Rap1A, apparently in its posttranslationally processed form (Bokoch and Prossnitz, 1992; Quilliam et al., 1991; Quinn et al., 1992). In the presence of GTP or GTPγS, but in the absence of Rac, we observed only very low rates of O2− generation. This activity could be accounted for by the (variable) low level contamination of our membrane vesicles with Rac due to entrapped cytosol. Although we cannot rule it out absolutely, we see no evidence to suggest that this low level of activity is due to the presence of an additional GTP-binding protein, such as Rap1A. We have tested both posttranslationally processed and unprocessed neutrophil and recombinant Rap1A, in both native and GTPγS-loaded forms, in the cell-free oxidase assay and have observed no oxidase-stimulatory activity, even at concentrations as high as 115 nM [this report (e.g., Figure 5) and unpublished data]. Rap1A is physically (Quinn et al., 1989b) and perhaps functionally (Bokoch et al., 1991) associated with cytochrome b558, but it appears not to be necessary for the function of NADPH oxidase, at least in cell-free systems in which highly purified cytochrome b558 replaced membrane vesicles (Abo et al., 1992; Rotrosen et al., 1992).

Rac1 and Rac2 were routinely added without prior preloading with GTPγS and were presumably in the GDP-bound form, as the addition of GTPγS (or GTP) to the system was required for activity. This suggests that the neutrophil membrane in the assay system contains sufficient guanine nucleotide exchange protein to stimulate conversion of Rac protein from its GDP-bound to its GTP-bound form in the reaction mixture. Alternatively, either p47- phox or p67-phox would have to fulfill this role, but there is no structural homology nor published evidence to indicate that they could be guanine nucleotide exchange factors.

The very weak ability of unprocessed Rac1 and Rac2 to support O2− generation implies that the posttranslational modifications that the native and baculovirus/Sf9-expressed recombinant Rac proteins undergo are essential for some aspect of the proteins’ function in regulating NADPH oxidase. Preloading unprocessed Rac1 (or Rac2) with GTPγS was sufficient to convert it to a fully active form [Abo et al. (1992) and this study] indicating that processing is not an absolute requirement for interaction with NADPH oxidase components or for membrane association, but is essential for efficient guanine nucleotide exchange. The addition of the geranylgeranyl group may promote interaction of Rac with a protein possessing GDP dissociation stimulatory (GDS) activity. Such a requirement for posttranslational processing has been reported for the interaction of Rap1 with its GDS (Hiroyoshi et al., 1991).

In the presence of a suboptimal amount of neutrophil cytosol, rather than recombinant p47-phox and p67-phox, processed Rac2 worked much better than Rac1 at all but the lowest concentrations of GTP-binding protein. The physiological significance of this observation is not clear, but it does suggest that a factor(s) in cytosol may suppress the activity of Rac1, but not of Rac2. However, we cannot exclude the possibility that the activity of Rac1 was inhibited by detergent tightly bound to the protein, although this seems unlikely as Rac2 was purified in the same manner, and the same preparations of Rac1 were highly active in the recombinant cell-free system.

The processes of molecular reorganization that regulate the assembly of NADPH oxidase on the plasma membrane are not fully understood, but the recent discovery that Rac1 and Rac2, members of the Rho family of Ras-like GTP-binding proteins, are able to regulate O2− generation, promises to help elucidate this mechanism. In fibroblasts, Rho and Rac1 regulate the cytoskeleton by organizing polymerized actin (Ridley and Hall, 1992; Ridley et al., 1992), and Rac proteins have the potential to associate with the plasma membrane by virtue of their carboxy-terminal isoprenoid groups (Didsbury et al., 1990). In addition, recent evidence from one of our laboratories (Chuang et al., 1993) shows that RhoGDI, with which Rac proteins appear to exist as a complex in neutrophil cytosol (Bokoch, unpublished observations) (Abo et al., 1991; Knaus et al., 1992), inhibits the very high intrinsic GTPase activity of Rac1. These results raise the possibility that Rac might exist in its GTP-bound form with RhoGDI, and that oxidase...
activation may involve, among other things, breaking this complex to release active GTP-bound protein. Active Rac may then mediate, at least in part, the changing affinities of the cytosolic oxidase components for cytoskeletal proteins and the membrane-bound flavocytochrome b558, which accompany activation.

Note added in proof. After submission of this paper, Takai’s group reported that processed Rac1 and Rac2 were equally able to support NADPH oxidase activity in a cell-free system containing recombinant p47-phox, and p67-phox, and solubilized membranes from differentiated HL-60 cells (Ando et al., 1992). In contrast to our results (and those of Abo et al., 1992), this group found that GTP-S-bound forms of unprocessed Rac1 and Rac2 had only very weak activity in their oxidase system. Their conclusion that posttranslational processing of Rac proteins is important not only for their interaction with guanine nucleotide exchange proteins but also for activation of NADPH oxidase per se, is therefore substantially different from ours.

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