Molecular Biology of the Cell
Vol. 4, 261–269, March 1993

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

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Submitted December 22, 1992; Accepted January 19, 1993

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^-)[1] [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-

[1] Abbreviations used: cell eq, cell equivalents; GDI, GDP dissociation inhibitor (also referred to as RhoGDI); GDS, GDP/GTP dissociation stimulator; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); O_2^-, superoxide; PIPES, 1,4-piperazinediethanesulfonic acid; SDS, sodium dodecyl sulfate.

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), (GTPγS)] cause a 2- to 4-fold enhancement in the rate of O_2^- 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 GTPγS) 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 GTPγS and stimulate O_2^- 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 σ1) 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 O_2^- 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 O_2^- 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 b_{558}, recombinant p47-*phox* and p67-*phox*, and either recombinant Rac1 or purified human neutrophil σ1, that these components are necessary and sufficient for O_2^- 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 O_2^- 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 $MgCl_2$, 1 mM ATP, 1.25 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 1,4-piperazinediethanesulfonic acid (PIPES), pH 7.3] (Borregaard *et al.*, 1983) by N_2 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 9×10^7 and 1.25×10^9 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 *Bam*HI 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 $(OD)_{600}$ 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 $MgCl_2$, 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 $MgCl_2$, 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-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 cell pellet was subjected to N_2 cavitation at 450 psi and 4°C for 20 min in a buffer consisting of 100 mM KCl, 3 mM NaCl, 3.5 mM $MgCl_2$, 10 mM PIPES (pH 7.3), 1 mM phenylmethylsulfonyl fluoride, 100 kallikrein inhibitory units of aprotinin/ml, 1 μM leupeptin, 1 mM 2-mercaptoethanol, and 0.2 mM dithiothreitol. The cavitated cells were collected into sufficient EGTA 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 $MgCl_2$, 50 mM NaCl, 0.1 mM dithiothreitol, 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 $MgCl_2$, 0.1 mM dithiothreitol, 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 isoprenylated by the incorporation of [^{3}H]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 cytosolic 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 [^{35}S]GTPγS

Recombinant GTP-binding proteins were quantified by their ability to bind [^{35}S]GTPγS, as determined by a rapid filtration technique

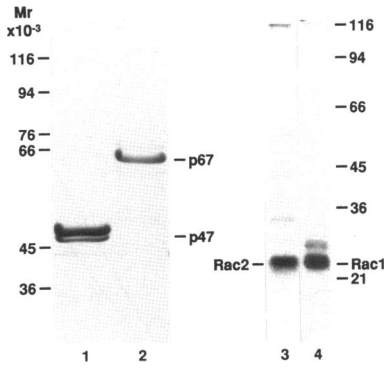

Figure 1. Purity of baculovirus recombinant proteins used in this study. Recombinant p47-*phox* (2 μg; lane 1), p67-*phox* (2 μg; lane 2), Rac2 (70 ng; lane 3), and Rac1 (100 ng; lane 4) were expressed in a baculovirus/Sf9 insect cell system and purified as described in MATERIALS AND METHODS. The proteins were subjected to SDS polyacrylamide gel electrophoresis and silver stained.

(Knaus *et al.*, 1992). For some experiments, Rac proteins were preloaded with [^{35}S]GTPγS by incubation with 40 mM HEPES (pH 7.5), 4 mM EDTA, 2 mM dithiothreitol and 2 μM [^{35}S]GTPγS ($1–2 \times 10^4$ cpm/pmol) at room temperature. The reaction was stopped by raising the concentration of free Mg^{2+} from the original 400 nM to 10 mM. After removal of free [^{35}S]GTPγS by ultrafiltration (Centricon 10, Amicon, Beverly, MA) the concentration of Rac-[^{35}S]GTPγS was determined as described (Knaus *et al.*, 1992).

Cell-free NADPH Oxidase Assay

Production of O_2^- by NADPH oxidase was monitored in a cell-free system at 25°C by following the superoxide dismutase-inhibitable reduction of cytochrome *c* at 550 nm, as previously described (Curnutte *et al.*, 1989b). Reaction mixtures (in 96-well microtitration plates) contained 0.1 mM cytochrome *c*, 6.5 mM $MgCl_2$, 87 mM KCl, 2.6 mM NaCl, 8.7 mM PIPES (pH 7.3), 10 μM GTPγS, 0.16 mM NADPH, and 4×10^5 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 (7.2×10^5 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-O_2^--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 O_2^- generated/min per 10^7 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 O_2^--dependent cytochrome *c* reduction), by using an extinction coefficient of 20.5 per mM/cm for reduced minus oxidized cytochrome *c* (Curnutte *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 O_2^- assay were obtained from the sources previously reported (Curnutte *et al.*, 1987). [^{35}S]GTPγS

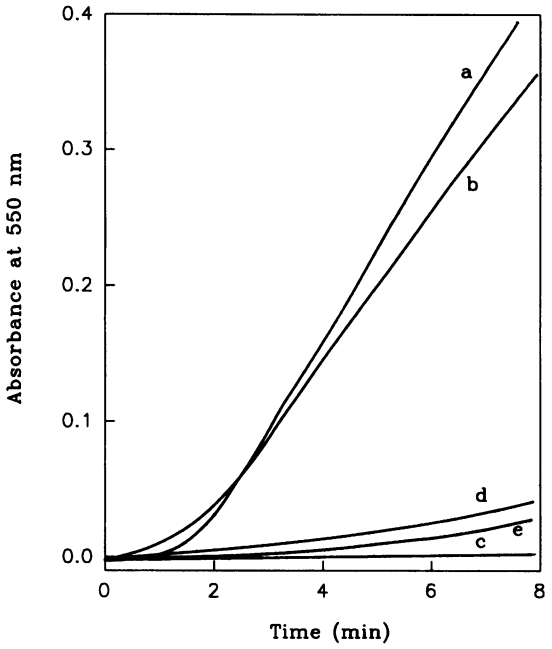

Figure 2. Comparison of time courses of O_2^- production in the presence of whole neutrophil cytosol or recombinant cytosolic NADPH oxidase components. Reaction mixtures contained either 20 μl neutrophil cytosol (1.8×10^6 cell eq) (trace a) or recombinant p47-*phox* (100 nM), p67-*phox* (100 nM), and Rac2 (27 nM) (trace b), in addition to neutrophil membranes (4×10^5 cell eq). Reaction mixtures for traces c-e were the same as for trace b, except that the following were omitted, p47-*phox* and p67-*phox* (trace c), Rac2 (trace d), and 10 μM GTPγS (trace e).

was from Du Pont-NEN Research Products (Boston, MA). Lysozyme (from egg white, 20 000 U/mg) was obtained from United States Biochemical Corp. (Cleveland, OH). Deoxyribonuclease I (from bovine pancreas) was purchased from Sigma Chemical Co. (St. Louis, MO).

RESULTS

In the cell-free NADPH oxidase system containing 4×10^5 cell eq of neutrophil membranes and 1.8×10^6 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 ± 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 ± 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 (Abo *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

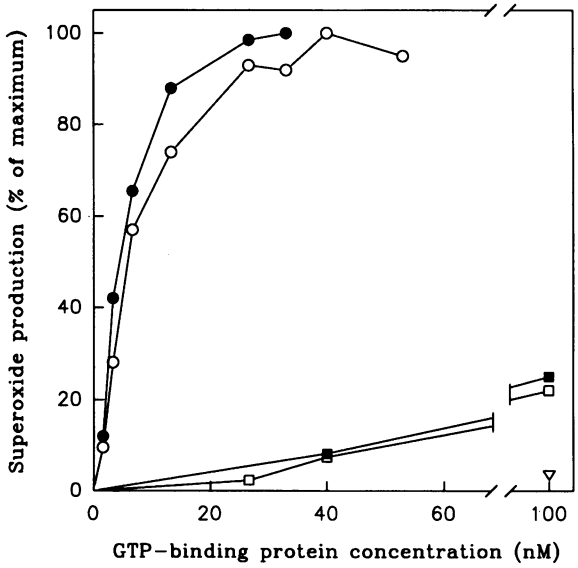

Figure 3. The importance of posttranslational processing on the ability of Rac1 and Rac2 to support O_2^- generation by NADPH oxidase in a cell-free system. Cell-free NADPH oxidase reaction systems containing neutrophil membranes (4×10^5 cell eq) and 100 nM each of baculovirus recombinant p47-*phox* and p67-*phox*, were supplemented with the indicated amounts of processed Rac1 (●), Rac2 (○), or CDC42Hs (▽), or unprocessed Rac1 (■) or Rac2 (□). The low rates of O_2^- production seen in the absence of recombinant Rac (e.g., see Figure 2) were subtracted from the rates obtained in the presence of GTP-binding protein. Results were then expressed as a percentage of the maximum rate of O_2^- production observed. The data shown are from a single representative experiment. Mean maximum rates were (in nmol/min per 10^7 cell eq membrane): 37.24 ± 5.79 (n = 6) for processed Rac1; 41.26 ± 10.73 (n = 8) for processed Rac2.

half-maximal activity were ~4 and 6 nM, respectively. Activity was totally dependent on the presence of guanine nucleotide, with maximal rates observed with either 10 μM GTPγS or 10 μM 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 GTPγS-bound form (Rac1-GTPγS) 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-GTPγS (28.79 ± 4.97 [n = 3] nmol/min per 10^7 cell eq membranes) compared favorably with the rate obtained using processed Rac1-GTPγS (40.43 ± 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 GTPγS 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-GTPγS, respectively.

Preloading processed Rac1 with GTPγS did not significantly change its ability to support NADPH oxidase activity; maximum rates with the GDP-bound (no preloading) and GTPγS-bound forms were 37.24 ± 5.79 (n = 6) and 40.43 ± 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-GTPγS (whether processed or unprocessed) no longer required guanine nucleotide to be added to the system. We have previously demonstrated that binding of GTPγS to Rac2 is very slow at the level of Mg^{2+} (6.5 mM) present in the oxidase assay (Knaus *et al.*, 1992). 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 GTPγS (or GTP).

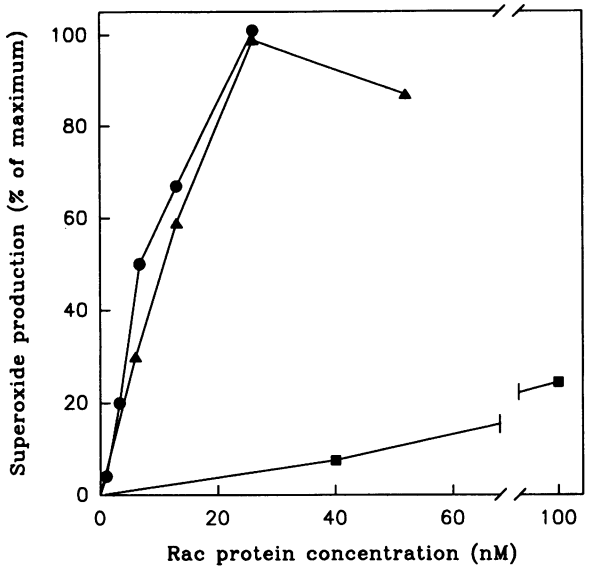

Figure 4. The effect of preloading unprocessed and processed Rac1 with GTPγS on their ability to support O_2^- generation. Processed (●) and unprocessed (▲) Rac1 were preloaded with [^{35}S]GTPγS as described in MATERIALS AND METHODS and added to NADPH oxidase reaction mixtures to give the final concentrations indicated. All other details are as described in the legend to Figure 3. For comparative purposes the data points from Figure 3 obtained with unprocessed Rac1 (without GTPγS) are also included here (■).

Our initial purification of Rac2 from human neutrophil cytosol was based on the ability of the protein to both bind [^{35}S]GTPγS and to augment the O_2^--generating ability of a suboptimal amount of cytosol (6.3 × 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 (Curnutte *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 5). 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-

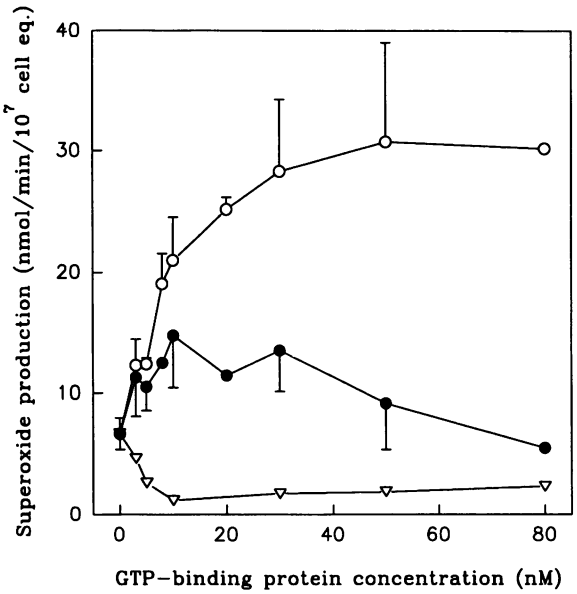

Figure 5. The effect of processed low molecular weight GTP-binding proteins on O_2^- production in the cell-free system containing a suboptimal amount of neutrophil cytosol. Baculovirus recombinant Rac1 (●), Rac2 (○), and Rap1A (▽) were added to NADPH oxidase reaction mixtures containing 4×10^5 cell eq membranes and 7.2×10^5 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 10^7 cell eq membrane) was not subtracted from the rates obtained in the presence of GTP-binding proteins.

combinant system (Figure 3), processed Rac1 caused only a modest augmentation (2-fold) of the rate of O_2^- production at low concentrations (≤10 nM). When the concentration was increased, (≥20 nM) this enhancing effect decreased until Rac1 actually caused a slight inhibition of the basal rate at concentrations ≥80 nM (Figure 5). Unprocessed Rac1 and Rac2 had no effect on the basal rate of O_2^- production in this system at concentrations ≤100 nM, providing further evidence that posttranslational modifications of Rac are essential for NADPH oxidase activity. Processed Rap1A was not only unable to stimulate oxidase activity but actually inhibited the basal rate of O_2^- production (Figure 5).

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 RhoGDI 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

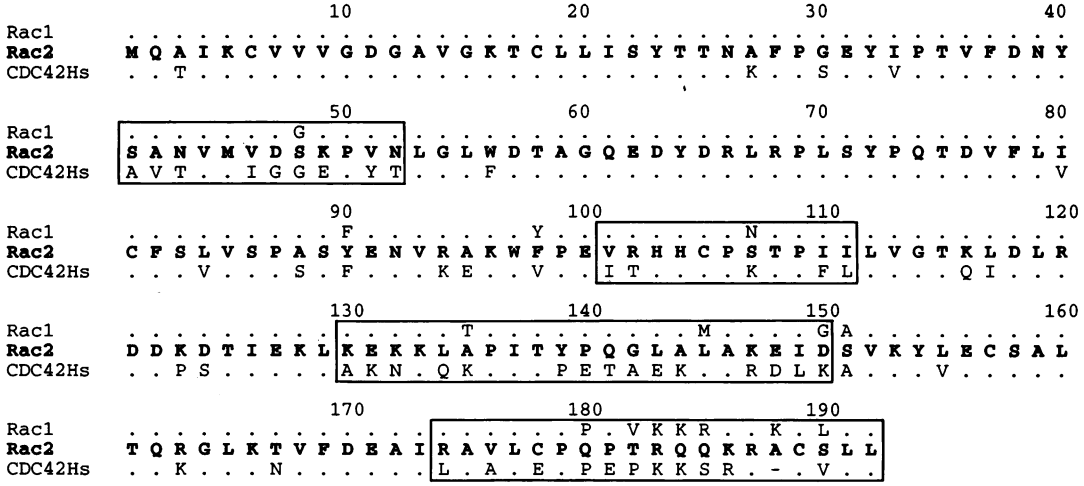

Figure 6. The predicted amino acid sequences of Rac1, Rac2, and CDC42Hs. For Rac1 and CDC42Hs only residues that are different from Rac2 are indicated. The 4 regions of CDC42Hs that differ most widely from the Rac proteins are boxed.

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 "effector" 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 adenylylcyclase (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 O_2^- 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 b_{558}, but it appears not to be necessary for the function of NADPH oxidase, at least in cell-free systems in which highly purified cytochrome b_{558} 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 O_2^- 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 O_2^- 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 b_{558}, 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 GTPγS-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.

ACKNOWLEDGMENTS

The authors thank Benjamin P. Bohl and David C. Barry for technical assistance, and J. David Lambeth for helpful advice. Ulla G. Knaus is the recipient of a Postdoctoral Fellowship from the Arthritis Foundation. This work was supported by grants AI-24838 and RR-00833 (to J.T.C.), HL-48008 (to G.M.B.), and AI-22809 and CA-46508 (to D.J.U.) from the National Institutes of Health. Support from the Sam and Rose Stein Charitable Trust Fund is also gratefully acknowledged.

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