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Regulation of NADPH Oxidase Activity by Rac GTPase Activating Protein(s)

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Activation of the NADPH oxidase of phagocytic cells requires the action of Rac2 or Rac1, members of the Ras superfamily of GTP-binding proteins. Rac proteins are active when in the GTP-bound form and can be regulated by a variety of proteins that modulate the exchange of GDP for GTP and/or GTP hydrolysis. The p190 Rac GTPase Activating Protein (GAP) inhibits human neutrophil NADPH oxidase activity in a cell-free assay system with a K_I of ~100 nM. Inhibition by p190 was prevented by GTPγS, a nonhydrolyzable analogue of GTP. Similar inhibition was seen with a second protein exhibiting Rac GAP activity, CDC42Hs GAP. The effect of p190 on superoxide (O_2^-) formation was reversed by the addition of a constitutively GTP-bound Rac2 mutant or Rac1-GTPγS but not by RhoA-GTPγS. Addition of p190 to an activated oxidase produced no inhibitory effect, suggesting either that p190 no longer has access to Rac in the assembled oxidase or that Rac-GTP is not required for activity once O_2^- generation has been initiated. These data confirm the role of Rac in NADPH oxidase regulation and support the view that it is the GTP form of Rac that is necessary for oxidase activation. Finally, they raise the possibility that NADPH oxidase may be regulated by the action of GAPs for Rac proteins.

INTRODUCTION

Phagocytic cells contain an NADPH oxidase that enables them to produce superoxide anion (O_2^-) for the purposes of microbial killing and tumoricidal activity (see Clark, 1990; Morel *et al.*, 1991 for reviews). This system consists of a membrane-bound cytochrome b_{558}, which serves to transmit electrons derived from NADPH to molecular oxygen, and two proteins of unknown function, p47*phox* and p67*phox*, that are found in the cytosolic fraction of disrupted, unstimulated neutrophils. It has recently been shown that the Rac GTP-binding protein is a third required cytosolic component of the system, with Rac2 identified in human neutrophils (Knaus *et al.*, 1991, Heyworth *et al.*, 1993) and Rac1 in guinea pig macrophages (Abo *et al.*, 1991, 1992.).

The NADPH oxidase can be activated in cell-free systems containing membranes and cytosol from unstimulated phagocytes by the addition of an anionic amphiphile, such as arachidonic acid or sodium dodecyl sulfate (SDS) (Bromberg and Pick, 1984; Heyneman and Vercauteren, 1984; Curnutte, 1985; McPhail *et al.*, 1985). Reconstitution of an active cell-free NADPH oxidase that produces O_2^- at rates sufficient to account for intact cell production minimally requires the intact cytochrome b_{558}, p47*phox*, p67*phox*, and Rac (Abo *et al.*, 1992; Rotrosen *et al.*, 1992). Upon activation of intact neutrophils, the three cytosolic components have been shown to become tightly associated with the membrane and/or cytochrome b_{558} to form a functional NADPH oxidase (Clark *et al.*, 1990; Heyworth *et al.*, 1991; Quinn *et al.*, 1993). The mechanisms that regulate this assembly remain undefined.

Rac1 and Rac2 are members of a large superfamily of proteins, of which Ras is the prototype, that regulate many types of cellular activity (Grand and Owen, 1991; Downward, 1992; Bokoch and Der, 1993). These proteins undergo regulated cycles of GTP binding and hydrolysis (Grand and Owen, 1991; Downward, 1992). The ability of members of the Ras superfamily to reg-

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ulate cell function is determined by whether they are in the GTP-bound "active" state or the GDP-bound "inactive" state (Grand and Owen, 1991; Downward, 1992; Bokoch and Der, 1993). This is true for regulation of the NADPH oxidase as well; reduction of endogenous GTP levels in neutrophil cytosol by dialysis prevents the formation of O_2^- (Uhlinger *et al.*, 1991; Peveri *et al.*, 1992), and activity can be totally restored by supplementation of the system with 10 μM GTPγS. Our preliminary data indicates that this requirement for GTP can be met by addition of GTP-bound Rac (unpublished data). Rac must bind GTP to promote O_2^- formation in cell-free systems (Abo *et al.*, 1991; Mizuno *et al.*, 1992; Heyworth *et al.*, 1993; Kwong *et al.*, 1993), and the ability of Rac to interact with protein(s) able to stimulate the exchange of GDP for GTP is necessary for activation (Mizuno *et al.*, 1992; Heyworth *et al.*, 1993). Indeed, posttranslational isoprenylation of Rac appears to be necessary for this exchange reaction (Heyworth *et al.*, 1993), and inhibitors of protein isoprenylation block O_2^- formation in intact cells (Bokoch and Prossnitz, 1992). Rac translocates to the plasma membrane of human neutrophils upon cell activation (Quinn *et al.*, 1993), and it appears that conversion to the GTP-bound form may be necessary for this to occur (Phillips *et al.*, 1993; Bokoch *et al.*, unpublished data).

At present, three types of protein have been identified that can modulate the GTP/GDP state of Rac (Bokoch and Der, 1993). These include GTPase activating proteins (GAPs), which stimulate GTP hydrolysis; GDP/GTP dissociation stimulators (GDSs), which promote the exchange of GTP for GDP; and the so-called GDP dissociation inhibitors (GDIs), which inhibit GDP dissociation from Rac but that can also inhibit the intrinsic GTP hydrolytic activity of Rac as well as that stimulated by GAPs (Chuang *et al.*, 1993). The presence of [Rho]GDI[1] in human neutrophils and other phagocytes is well established (Abo *et al.*, 1991; Knaus *et al.*, 1992; Kwong *et al.*, 1993), and a Rac GDS activity has also been detected (Heyworth *et al.*, 1993). The addition of exogenous [Rho]GDI and smgGDS to cell-free oxidase systems has demonstrated that these proteins can regulate NADPH oxidase activity, probably through effects on Rac to prevent or stimulate GTP/GDP exchange, respectively (Mizuno *et al.*, 1992). Regulatory roles for Rac GAPs in the oxidase system have not been described. Because there is evidence that GAPs might act as downstream effectors as well as negative regulators, at least in certain systems, the activities of GAP proteins remain to be determined for each particular protein. A variety of molecules with GAP activity for Rac have been described (Bokoch and Der, 1993; Hall, 1992). Among these are the p190 protein, which tightly associates with p120 Ras GAP upon activation of growth factor receptor- and transforming tyrosine kinases (Ellis *et al.*, 1990; Settleman *et al.*, 1992a,b), and a distinct GAP, termed CDC42 GAP, that has activity for the Rac-related GTP-binding protein CDC42 (Hart *et al.*, 1991). This GAP appears to be identical to a previously identified Rho GAP (Garrett *et al.*, 1989). Although Rac GAP activity has been detected in human neutrophils (Bokoch, unpublished data) the proteins that mediate this activity in human phagocytes have not yet been identified.

In the present studies, we demonstrate that the p190 Rac GAP is present in human neutrophils and is able to inhibit NADPH oxidase activity in a cell-free O_2^- generating system. This inhibition appears to be the result of modulation of the GTP state of Rac and can be reversed by the addition of preactivated forms of Rac. Finally, we use the p190 Rac GAP to investigate Rac in the active NADPH oxidase complex.

[1] [Rho]GDI refers to a GDI originally described to be active on Rho (Ueda *et al.*, 1990) but that now is known to be active on other members of the Rho family.

MATERIALS AND METHODS

Protein Preparations

Recombinant Rac1 and Rac2 were prepared and purified from the membranes of baculovirus-infected Sf9 insect cells as described previously (Heyworth *et al.*, 1993). These proteins are posttranslationally isoprenylated and processed and are fully active in GTP binding and NADPH oxidase assays. The Rac2 (Q61L) mutant was prepared, expressed in Sf9 cells, purified, and characterized as described in Xu *et al.* (1993). p190 was prepared as previously described (Settleman *et al.*, 1992a). CDC42Hs GAP was purified as previously described (Hart *et al.*, 1991) and was the kind gift of Drs. Matthew Hart and Richard Cerione, Cornell University, Ithaca, NY. Recombinant Sf9 cell RhoA was provided by Larry Feig, Tufts University, Boston, MA.

GAP Activity Assay

The [γ^{32}P]GTP Rac1 or Rac2 complex was formed by incubation of purified recombinant Rac protein (150 nM) with 25 mM tris(hydroxymethyl)aminomethane-HCl pH 7.5, 1 mM dithiothreitol, 4.7 mM EDTA, 100 μg/ml bovine serum albumin, 10 μM GTP (10 000–15 000 cpm/pmol), and 35 nM free Mg^{2+} at 30°C for 4 min. GTP hydrolysis was initiated by addition of $MgCl_2$ to 19 mM and GTP to 200 μM in the presence of the indicated concentrations of p190 GAP plus or minus 110 μM SDS. The amount of [γ^{32}P]GTP that remained protein-bound was determined by filtration after 5 min incubation at room temperature (Knaus *et al.*, 1992; Chuang *et al.*, 1993). p190 does not stimulate dissociation of [α-^{32}P]GTP under the same conditions.

NADPH Oxidase Assay

Subcellular fractions from human neutrophils for use in cell-free NADPH oxidase assays were prepared as described previously (Curnutte *et al.*, 1989). Cell-free oxidase assays were conducted as described in Heyworth *et al.* (1993) and Curnutte *et al.* (1989). To deplete endogenous guanine nucleotides for some experiments, cytosol was dialyzed overnight against 3 2-L volumes of 100 mM KCl/3 mM NaCl/3.5 mM $MgCl_2$/10 mM piperazine-*N*,*N*'-bis(2-ethanesulfonic acid (PIPES) pH 7.3, as previously described (Peveri *et al.*, 1992). Reaction mixtures contained 4×10^5 cell equivalents of human neutrophil membranes and 1.8×10^6 cell equivalents of neutrophil cytosol. Reactions were performed in the absence of GTPγS, unless otherwise indicated (see Figure legends) and with the indicated concentrations

of p190 GAP. Rates of O_2^- production were calculated from maximum rates of absorbance change after initiation of the reaction with 110 μM SDS.

Miscellaneous Materials and Methods

SDS-polyacrylamide gel electrophoresis (PAGE) and Western blotting were performed as previously described (Settleman *et al.*, 1992a). Reagents used in these studies were of the highest available grade and were obtained from the sources indicated in the cited references. GTP and GTPγS were obtained from Sigma Chemical (St. Louis, MO). [^{35}S]GTPγS and [γ-^{32}P]GTP were from Du Pont-New England Nuclear Research Products (Boston, MA).

RESULTS

We used a standard GAP assay procedure to establish the activity of p190 to stimulate GTP hydrolysis by Rac under our NADPH oxidase assay conditions. The ability of p190 to produce a concentration-dependent stimulation of the rate of GTP hydrolysis by Rac1 and Rac2 is shown in Figure 1. The GAP activity of p190 was antagonized by the presence of 110 μM SDS (Figure 1) or 150 μM arachidonic acid. These are the concentrations of these anionic amphiphiles that are used in the cell-free NADPH oxidase assay. About 10-fold more p190 was required for a given level of GAP activity when SDS or arachidonic acid was present. In contrast, we found the activity of CDC42Hs GAP for Rac was essentially unchanged by the presence of SDS or arachidonic acid.

Figure 2 shows a Western blot of isolated human neutrophil membranes and cytosol, as well as total lysates from HL60 promyelocytic cells that had been fully differentiated into a neutrophil-like cell by exposure to 1.25% dimethylsulfoxide (DMSO) for 6 d. The presence of p190 in human phagocytes could be seen after immunoblotting with a p190-specific antibody (Settleman *et al.*, 1992a,b). p190 was found to be primarily localized to the neutrophil cytosol, although a small amount could be detected in the purified plasma membrane fraction.

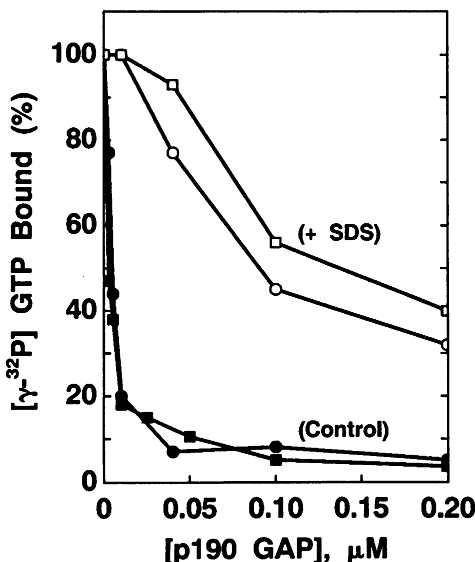

Figure 1. Activation of Rac GTPase activity by p190. Rac1 (squares) or Rac2 (circles) was loaded with [γ^{32}P]GTP, and GTP hydrolysis initiated in the presence of the indicated concentrations of p190 as described in MATERIALS AND METHODS. Closed symbols are in the absence (control) and open symbols are in the presence (+SDS) of 110 μM SDS.

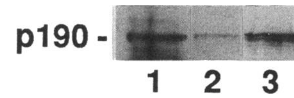

Figure 2. Presence of p190 in human phagocytic leukocytes. Human neutrophil cytosol (30 μg, lane 1) or membranes (50 μg, lane 2), and DMSO-differentiated HL60 cell lysates (2 × 10^5 cell eq, lane 3) were immunoblotted with a 1:1000 dilution of a p190-specific antibody as described in MATERIALS AND METHODS.

The ability of Rac to stimulate NADPH oxidase activity is dependent upon Rac being in the GTP-bound state (Abo *et al.*, 1991; Mizuno *et al.*, 1992; Heyworth *et al.*, 1993; Kwong *et al.*, 1993). To investigate whether GAP proteins that are able to modulate the GTP/GDP state of Rac could regulate NADPH oxidase activity, we examined the effect of adding p190 GAP to a cell-free NADPH oxidase assay (Figure 3). The addition of recombinant p190 to the system 1 min before initiation of the reaction with SDS resulted in a concentration-dependent inhibition of O_2^- formation with half-maximal inhibition occurring at ~100 nM p190. This correlates well with the amount of p190 required for half-maximal stimulation of Rac GTPase activity in the presence of SDS (see Figure 1). The inhibitory effect of p190 in the cell-free system was substantially reversed by the addition of 10 μM GTPγS (Figure 3). Because the free GTP concentration in the whole cytosol reaction is estimated to be 2.8 μM (Peveri *et al.*, 1992), it is likely that a portion of the endogenous Rac would still contain bound GTP even when 10 μM GTPγS is added. By adding GTPγS to dialyzed cytosol in which endogenous GTP levels have been depleted, nearly all of the Rac present will have bound GTPγS. Under these conditions, NADPH oxidase activity was almost totally resistant to inhibition by p190 (Figure 3).

The ability of GTPγS to reverse the inhibition of O_2^- formation by p190 suggests that this effect of p190 was due to its ability to stimulate GTP hydrolysis. When we tested a second GAP (CDC42Hs GAP) active on Rac in the cell-free system, we also observed inhibition of NADPH oxidase activity in a GTPγS-sensitive fashion. Furthermore, there was an excellent correlation between the relative activity of these two GAPs for stimulating GTP hydrolysis by Rac in vitro and their ability to inhibit NADPH oxidase activity. Thus, we observed that it took ~10- to 20-fold more p190 to stimulate a level of Rac GTP hydrolysis equal to that produced by a given amount of CDC42Hs GAP, and it also took 10- to 20-

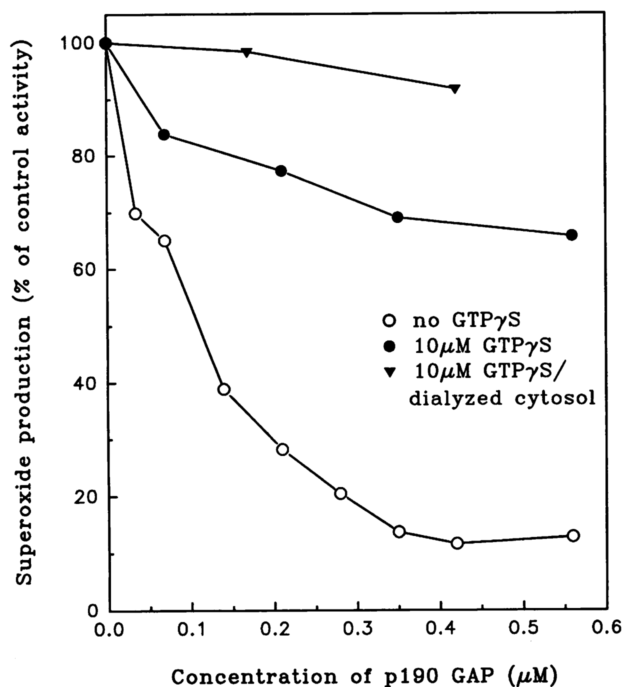

Figure 3. Effect of p190 GAP on NADPH oxidase activity in the presence and absence of GTPγS. Recombinant p190 GAP was added to reaction mixtures to give the final concentrations indicated 1 min before activation of O_2^- production with SDS. Reaction mixtures contained 4×10^5 cell eq membranes and 1.8×10^6 cell eq of either normal cytosol (○, ●) or dialyzed cytosol (▼). The final concentrations of endogenous GTP contributed by this amount of normal and dialyzed cytosol were 2.8 μM and <0.02 μM, respectively. Reactions were either performed at 2.8 μM GTP (○) or were supplemented with 10 μM GTPγS (●, ▼). The control rate of O_2^- production was 26.7 ± 3.2 (SD, n = 4) nmol/min/10^7 cell eq membranes. Data shown are representative of at least two experiments.

fold more p190 to inhibit O_2^- formation in the cell-free assay.

Rac2 and Rac1 have been shown to support NADPH oxidase activity, whereas the other members of the Rho family of GTP-binding proteins, Rho and CDC42Hs, are inactive in this system (Mizuno *et al.*, 1992; Heyworth *et al.*, 1993; Kwong *et al.*, 1993). Because p190 can stimulate GTP hydrolysis by all members of the Rho family, we wanted to be certain that its inhibitory effect on the NADPH oxidase was the result of an action on Rac. Figure 4 shows that the addition to the cell-free assay of a Rac2(Q61L) mutant protein that does not hydrolyze GTP and is unresponsive to p190 GAP activity (Xu *et al.*, 1993) to the cell-free assay was able to overcome totally the inhibitory effects of p190 GAP. Similarly, a GTPγS-loaded Rac1 protein also reversed the inhibitory activity of p190. In marked contrast, a GTPγS-bound form of RhoA did not reverse inhibition of NADPH oxidase activity by p190, even though this GTP-binding protein interacts even more strongly with p190 (Settleman *et al.*, 1992a). Both Rac-GTPγS and Rac2-GTP (i.e., the Q61L mutant) caused an increase in the rate of O_2^- production over the control cytosol, suggesting that the level of endogenous Rac protein present was insufficient for maximal stimulation of the NADPH oxidase, perhaps because of the action of endogenous GAPs and other regulatory factors.

The experiments described above suggest that p190 GAP effectively inhibits NADPH oxidase activity through an effect on the levels of active Rac-GTP. We examined the susceptibility of Rac to the action of p190 GAP in the activated oxidase complex by determining the ability of GAP to inhibit O_2^- formation in the cell-free assay once the reaction had been initiated with SDS. As shown in Figure 5, after the addition of stimulus to form active oxidase, p190 GAP was no longer able to inhibit O_2^- formation even when added within 100 s of the addition of SDS and before the maximum rate of O_2^- generation was achieved. However, the NADPH oxidase could be inhibited at this stage by the addition of diphenylene iodonium, a potent NADPH oxidase inhibitor (Cross and Jones, 1986). This loss of the inhibitory effect of the Rac GAP when added after oxidase activation suggests either that the GAP no longer has access to Rac in the active, multiprotein complex formed, or that the action of Rac-GTP is no longer necessary once an assembled, active enzyme has been established.

DISCUSSION

In this study we provide evidence that the human neutrophil NADPH oxidase is susceptible to regulation by

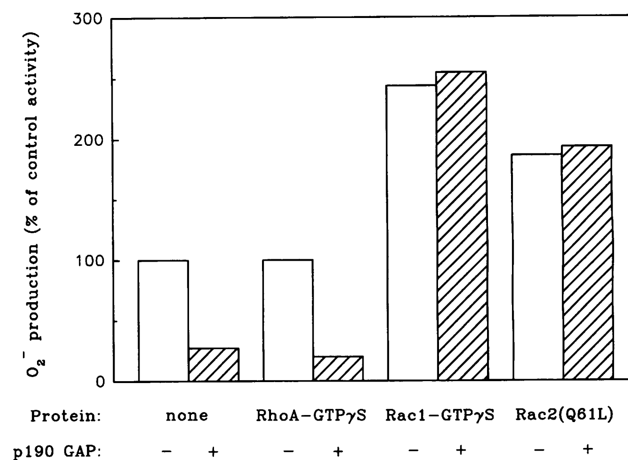

Figure 4. The addition of Rac GTP-binding proteins overcomes p190 GAP inhibition of O_2^- production by NADPH oxidase. Reaction wells were supplemented with either control buffer (none), RhoA preloaded with GTPγS (100 nM), Rac1 preloaded with GTPγS (30 nM), or Rac2(Q61L) (10 nM) and contained either zero (−)(□) or 280 nM (+)(▨) p190 GAP, as indicated in the figure. Reaction mixtures contained 4×10^5 cell eq membranes and 1.8×10^6 cell eq cytosol. The control rate of O_2^- generation was 30.1 ± 4.3 nmol/min/10^7 cell eq membrane. The data shown are representative of at least two experiments.

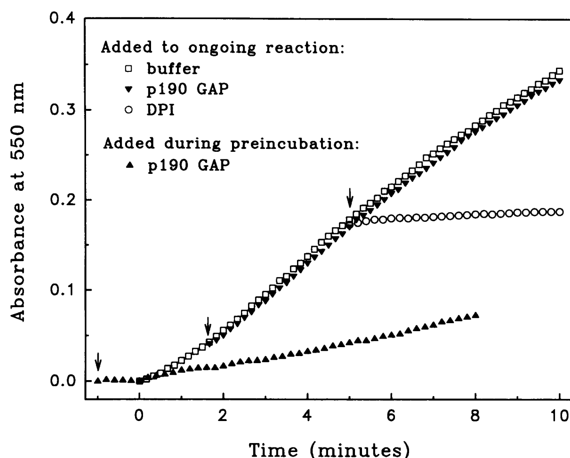

Figure 5. Comparison of the effects of adding p190 GAP before and after initiation of NADPH oxidase activity. Reaction mixtures were as in Figure 3 with normal cytosol and no GTPγS. Recombinant p190 GAP was added to reaction mixtures (to give a final concentration of 350 nM) either 1 min before (▲) or 1.7 min or 5 min after (▼) (see arrows) the initiation of the reaction at 0 min with SDS (110 μM). In control reactions buffer (□) or diphenylene iodonium (DPI, final concentration 1 μM) (○) was added to ongoing reactions 1.7 (not shown for DPI) or 5 min after initiation. Microplate reader data points at every 10 or 12 s are shown. The maximum rate of O_2^- generation in the control was 29.8 nmol/min/10^7 cell eq membrane. The data shown are representative of at least two experiments.

GAP proteins able to stimulate GTP hydrolysis by Rac. Previous studies have demonstrated that the oxidase system can be modulated by other proteins known to interact with and regulate the nucleotide state of Rac, including smgGDS and [Rho]GDI (Abo *et al.*, 1991; Knaus *et al.*, 1992; Mizuno *et al.*, 1992; Kwong *et al.*, 1993). Because GAPs act by stimulating the conversion of GTP to GDP, forming the inactive state of the low molecular weight GTP-binding protein substrate, these data indicate that the GTP-bound form of a small GTP-binding protein is essential for NADPH oxidase activation to occur. This is consistent with previous data that showed that GTP was an absolute requirement of the NADPH oxidase (Uhlinger *et al.*, 1991; Peveri *et al.*, 1992), and cell-free data that indicated that only the GTP-bound form of Rac was active in supporting NADPH oxidase activation (Abo *et al.*, 1991; Mizuno *et al.*, 1992; Heyworth *et al.*, 1993; Kwong *et al.*, 1993).

The GAPs used in these studies were the p190 GAP and CDC42Hs GAP. The cDNAs encoding p190 protein have been cloned, and the predicted protein product has been found to contain a region of homology with the breakpoint cluster region (Bcr) gene product associated with chronic myelogenous leukemia (Settleman *et al.*, 1992b; Groffen *et al.*, 1984). This region of Bcr exhibits GAP activity for the Rac1 protein (Diekmann *et al.*, 1991), and a number of proteins containing this domain have the ability to stimulate GTP hydrolysis by GTP-binding proteins of the Rho family (Hall, 1992; Bokoch and Der, 1993). It has previously been shown that p190 is active on members of the Rho family of GTP-binding proteins (Settleman *et al.*, 1992a), and we also found it to be active on both Rac1 and Rac2 (Figure 1). Similarly, we found the CDC42Hs GAP to be nearly as active with Rac1 and Rac2 as with CDC42Hs. The inhibitory action of p190 on NADPH oxidase was almost certainly because of its ability to stimulate GTP hydrolysis by Rac because 1) inhibition was prevented in the presence of the nonhydrolyzable GTP analogue GTPγS (Figure 3), 2) the degree of inhibition caused by p190 GAP versus CDC42 GAP was directly correlated with their relative GAP activity toward Rac, and 3) the inhibitory effect of p190 could be reversed by addition of GTP-bound forms of Rac1 and Rac2 but not by the GTPγS-bound form of RhoA (Figure 4). These data confirm the critical role of Rac in regulating the human phagocyte NADPH oxidase.

p190 is a phosphoprotein that becomes tightly associated with p120 Ras GAP in mitogenically stimulated and tyrosine kinase-transformed cells (Ellis *et al.*, 1990). p190 may therefore provide a link between the Rho/Rac signaling pathways and signal transduction pathways regulated by Ras. We have shown by immunoblotting that p190 is present in mature human neutrophils and fully DMSO-differentiated HL60 cells (Figure 2). Whether p190 serves as a normal physiological regulator of the NADPH oxidase is not known, and our studies allow no conclusions to be made in this regard other than establishing that p190 is present and that it can regulate the activity of the NADPH oxidase. The cell-free assay of O_2^- generation is conducted in the presence of 110 μM SDS as an activating stimulus. The levels of p190 required to cause inhibition under these conditions are in the range of 10–400 nM, which correlates well with the concentrations of p190 required to stimulate GTP hydrolysis by Rac in the presence of identical concentrations of SDS (compare Figures 1 and 3). Whether p190 is active in intact stimulated neutrophils and whether its activity might be regulated by phosphorylation in these cells remains to be investigated. We have detected Rac GAP activity(s) in human neutrophils distinct from p190 (Bokoch *et al.*, unpublished data), and these proteins may also be important in determining NADPH oxidase activity, as well as in regulating other functions modulated by Rac, such as actin assembly (Ridley *et al.*, 1992). It will be important in future studies to begin to define the mechanisms by which individual Rac GAPs are regulated during phagocyte activation.

All of the three required cytosolic oxidase factors have been shown to translocate to the plasma membrane upon cell activation (Clark *et al.*, 1990; Heyworth *et al.*, 1991; Quinn *et al.*, 1993), and membrane association of at least the p47*phox* and p67*phox* components is dependent upon cytochrome b_{558} (Kleinberg *et al.*, 1990; Heyworth *et al.*, 1991). The nature and mechanism of

action of the active oxidase complex formed have not yet been defined however. The Rac protein would appear to be in the active, GTP-bound state at this stage, because Rac must be in a GTP-bound form for oxidase activation to occur (Mizuno *et al.*, 1992; Heyworth *et al.*, 1993; Kwong *et al.*, 1993) and GTP binding to Rac may preceed the translocation event (Bokoch *et al.*, unpublished data; Phillips *et al.*, 1993). Whether Rac participates as a component of the assembled NADPH oxidase along with p47*phox*, p67*phox*, and cytochrome b_{558} is not known. We observed that Rac was no longer responsive to p190 action once the NADPH oxidase was activated and assembled (Figure 5). This could indicate that p190 does not have access to the Rac protein when it is interacting with other oxidase components. Such an hypothesis would be consistent with what is known about Ras structure. In Ras both GAP and downstream effectors (i.e., Raf kinase) bind to a shared "effector" domain made up of amino acid residues 32–40 (Moodie *et al.*, 1993; Vojtek *et al.*, 1993). Whether the equivalent domain in Rac is important for NADPH oxidase activation has not yet been established. If Rac in the active oxidase is indeed protected from GAP action, then this would suggest that Rac GAP(s) do not serve as the immediate signal for termination of the respiratory burst response.

Alternatively, the inability of p190 to inhibit once the system has been stimulated might indicate that the GTP-bound Rac is no longer required to maintain activity of the enzyme once activation has been initiated. Although this possibility cannot be ruled out, the observation that Rac translocation is continuous during the time course of oxidase activation by chemoattractants and phorbol esters (Quinn, *et al.*, 1993) suggests that continuous activity of Rac is necessary to maintain an active enzyme. On the basis of current knowledge of GTP-binding protein action, this active Rac is likely to be the GTP-bound form.

In summary, we have demonstrated that the human neutrophil NADPH oxidase is subject to negative regulation by proteins, such as p190, that can stimulate GTP hydrolysis by Rac. Thus the NADPH oxidase appears subject to regulation by each of the three known classes of proteins able to modulate the GTP/GDP state of Ras-related GTP-binding proteins. It is of interest that the activity of certain GAPs may be modulated by the activation of cell surface hormone receptors (Li *et al.*, 1992; Marti and Lapetina, 1992), and the possibility that Rac GAP(s) in neutrophils is subject to chemoattractant-mediated regulation is a viable one. Rac-GTP appears not to be accessible to p190 after assembly of the active oxidase complex. Thus, the use of a GAP protein capable of modulating the activity of Rac in an intact system has enabled us to gain insight into the structure and function of the phagocyte NADPH oxidase system.

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