Characterization of membrane-localized and cytosolic Rac-GTPase-activating proteins in human neutrophil granulocytes: contribution to the regulation of NADPH oxidase

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We have investigated the intracellular localization and molecular identity of Rac-GTPase-activating proteins (Rac-GAPs) in human neutrophils. Immunoblot analysis detected the presence of both p190RhoGAP and Bcr mainly in the cytosol. An overlay assay performed with [γ-^{32}P]GTP-bound Rac revealed dominant GAP activity related to a 50 kDa protein both in the membrane and cytosol. This activity could be identified by Western blotting and immunoprecipitation with specific antibody directed against the GAP domain of p50RhoGAP. Using a semirecombinant or fully purified cell-free activation assay of the Rac-activated enzyme NADPH oxidase, we demonstrated the regulatory effect of both the membrane-localized and soluble GAPs. We suggest that in neutrophil granulocytes Rac-GAPs have redundant function and represent suitable targets for both the up-regulation and down-regulation of the NADPH oxidase.

Key words: overlay, phagocytes, prenylation.

INTRODUCTION

The small GTP-binding protein Rac belongs to the Rho GTP-ase subgroup of the Ras superfamily [1]. All three members of the Rho subfamily (Rac, Rho and Cdc42) have a central role in controlling the cytoskeleton [2–4] but there is growing evidence that Rac is also involved in the regulation of other biological processes such as mitogenesis and cellular stress responses [5–8].

The first biological response in which the involvement of Rac was proved was the production of superoxide ($O_2^{-\bullet}$) by professional phagocytes [9]. $O_2^{-\bullet}$ is produced by the NADPH oxidase enzyme complex, which transfers electrons from NADPH to molecular oxygen. NADPH oxidase is composed of five subunits: the membrane-localized cytochrome b_{558}, which forms a heterodimer (gp91^{phox} and p22^{phox}), the cytosolic proteins p40^{phox}, p47^{phox}, p67^{phox} and the small GTP-ase Rac1 or Rac2 [10–12]. Activation of the enzyme occurs by translocation of the cytosolic components to the cytochrome b_{558} in the membrane. The exact role of Rac in the activation process is still unclear. Binding to the oxidase component p67^{phox} was demonstrated under cell-free conditions and also in the yeast two-hybrid system [13,14]. This protein–protein interaction seems to be essential for the oxidase activity because mutation of the Rac-binding domain of p67^{phox} results in the complete failure of $O_2^{-\bullet}$ production, causing chronic granulomatous disease (CGD) [15].

The role of Rac is probably not restricted to the final steps of the activation process. The GTP-bound form of Rac interacts with p21-activated kinases (PAKs) which become activated after stimulation of neutrophil granulocytes by the receptor agonist fMet-Leu-Phe [16–19]. PAKs are able to phosphorylate the cytosolic p47^{phox} in a recombinant system [18] but their exact role *in vivo* remains to be clarified. Rac was also shown to have a role in the activation of phosphoinositide 3-kinase [20,21], which is essential for chemoattractant-induced neutrophil responses including the production of $O_2^{-\bullet}$ [22–25]. However, it was also demonstrated that the phospholipid product of phosphoinositide 3-kinase, PtdIns(3,4,5)P_3, participates in the activation of Rac [26].

As with other members of the Ras superfamily, the activity of Rac is controlled by three groups of regulatory proteins [27]. Guanine nucleotide exchange factors (GEFs) stimulate the exchange of GDP for GTP, therefore promoting the activation of Rac. Guanine nucleotide dissociation inhibitors (GDIs) form a complex with the GDP-bound form of Rac and inhibit the GDP–GTP exchange reaction. The third group of proteins are GTPase-activating proteins (GAPs), which stimulate the intrinsic rate of hydrolysis and thus accelerate the inactivation of the protein. We have previously demonstrated that fluoride ions inhibit a membrane-bound Rac-GAP activity in the crude membrane fraction of neutrophil granulocytes [28]. We proposed that the stimulatory effect of fluoride on NADPH oxidase under cell-free conditions reported by several groups [29–32] might be explained by the down-regulation of Rac-GAP activity in the system. Exogenous Rac-GAP protein was able to reduce $O_2^{-\bullet}$ production in a cell-free system [33] but information on the involvement of endogenous Rac-GAP proteins is scarce. A definitive role for Bcr, a protein possessing both Rac-GAP and Rac-GEF (Dbl homology) domains, was shown by experiments in which targeted disruption of the *bcr* gene resulted in enhanced $O_2^{-\bullet}$ production in neutrophil granulocytes [34]. However, knock-out experiments do not provide information on the site of interaction and the possible molecular partners of the investigated protein. Furthermore, the diversity of Rac targets makes it unlikely that negative regulation of all these functions is mediated

Abbreviations used: CGD, chronic granulomatous disease; DTT, dithiothreitol; GDI, guanine nucleotide dissociation inhibitor; GEF, guanine nucleotide exchange factor; GST, glutathione S-transferase; GTP[S], guanosine 5′-[γ-thio]triphosphate; PAK, p21-activated kinase; PMN, polymorphonuclear cells; Rac-GAP, Rac-GTPase-activating protein.

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by a single protein. We therefore performed a detailed characterization of Rac-GAP activities and their possible function in human neutrophil granulocytes.

We show the following: (1) p50RhoGAP (also called Cdc42GAP) represents the dominant Rac-GAP activity in both the membrane and cytosol fractions, (2) Bcr and p190RhoGAP are present mainly in the cytosol, and (3) both membrane-localized and soluble GAPs are involved in the regulation of NADPH oxidase.

EXPERIMENTAL

Materials

Recombinant $p47^{phox}$ and $p67^{phox}$ were produced and purified as described [35]. In brief, both proteins were expressed with an N-terminal His-tag. $p67^{phox}$ was expressed in Sf9 insect cells; $p47^{phox}$ was expressed in *Escherichia coli* BL21(DE3) strain with the T7 expression system (Novagen). Cytochrome *b* was purified and relipidated as described in [36].

Prenylated Rac was isolated from the membrane fraction of Sf9 cells by extraction with 1% (v/v) CHAPS and purified as described in [37]. Non-prenylated Rac1, Rho-GDI and the GAP domain of p50Rho-GAP (residues 198–439) and Bcr (residues 871–1271) were produced in the form of GST fusion proteins and purified as described in [38]. The *E. coli* clones producing Rac1, Rho-GDI, Bcr and p50Rho-GAP were a gift from A. Hall. Antibody against p50Rho-GAP was raised in rabbits against glutathione S-transferase (GST) fusion proteins containing residues 198–439 of the protein. Rabbit serum was collected 12 weeks after four injections with the fusion protein. The antibody was affinity-purified and tested in immunoblot experiments. Mouse monoclonal antibody against p190Rho-GAP was purchased from Transduction Laboratories and mouse monoclonal antibody against Bcr was obtained from Santa Cruz Biotechnology. Horseradish-peroxidase-labelled anti-rabbit IgG, anti-mouse IgG, and enhanced chemiluminescence (ECL®) reagent was purchased from Amersham; Percoll, Protein G–Sepharose and nitrocellulose were from Pharmacia; glutathione agarose, arachidonic acid and ferricytochrome *c* (horse heart, type VI) were from Sigma; NADPH, superoxide dismutase, guanosine 5′-[γ-thio]triphosphate (GTP[S]) and GTP were from Boehringer Mannheim; [^{3}H]GDP was from Amersham and [γ-^{32}P]GTP was from Izotóp Intézet, Hungary. All other reagents were of the highest available quality. Incubations were performed in PBS [137 mM NaCl/2.7 mM KCl/8.1 mM Na_2HPO_4/1.5 mM KH_2PO_4 (pH 7.4)]. Arachidonic acid was dissolved in ethanol at 20 mM and added to the reaction mixture in small volumes (less than 3% of the final volume).

Preparation of neutrophils and subcellular fractions

Bovine neutrophils were isolated from bovine blood as described [31]. Neutrophils from 10 litres of blood were resuspended in 50 ml of PBS and disrupted by sonication on ice four times for 15 s each at 60 W output. Non-broken cells and nuclei were eliminated by centrifugation for 10 min at 10000 ***g***. The homogenate was subjected to ultracentrifugation for 45 min at 300000 ***g***. The membrane pellet was washed and resuspended in PBS. This crude membrane fraction was stored at −80 °C at a concentration of 3 mg/ml.

Human neutrophils were prepared from buffy coats of healthy volunteers as described [39], suspended in PBS and treated with 1 mM di-isopropyl fluorophosphate for 10 min at room temperature. After being washed in PBS, cells were suspended in PBS supplemented with 1 mM EGTA/10 μg/ml aprotinin/2 μM pepstatin/10 μM leupeptin/0.1 mM PMSF. Cells were broken by ultrasonic treatment and membrane and cytosolic fractions were prepared by the method described above [31].

Measurement of the GTP-ase activity of Rac

This was performed by the nitrocellulose filter binding assay as described [40]. Loading of Rac1 (1–4 μg of *E. coli* or Sf9-cell protein) was performed for 10 min at room temperature with a high specific radioactivity of [γ-^{32}P]GTP (more than 5000 Ci/mmol) in 16 mM Tris/HCl (pH 7.5)/20 mM NaCl/0.1 mM dithiothreitol (DTT)/5 mM EDTA/100 nM [γ-^{32}P]GTP (5 μCi). Thereafter, $MgCl_2$ was added at 20 mM to diminish further nucleotide exchange; the solution was kept on ice. The GTPase reaction was initiated by the addition of 3 μl of Rac loaded with [γ-^{32}P]GTP to 27 μl of a warmed (20 °C) buffer containing 16 mM Tris/HCl, pH 7.5, 0.1 mM DTT, 1 mM unlabelled GTP and, where indicated, membrane or cytosolic protein. BSA (1 mg/ml) was included as a carrier protein. Aliquots (5 μl) were taken at regular intervals and filtered through nitrocellulose filters (0.45 μm pore size), followed by washing three times with 2 ml of cold buffer consisting of 50 mM Tris/HCl and 5 mM $MgCl_2$, pH 7.7. The filters were dried and radioactivity was measured by the Cerenkov effect in a Beckman LS 5000TD liquid-scintillation spectrometer. GAP activity is either presented as the decrease in protein-bound radioactivity in time or expressed as the ratio of protein-bound [γ-^{32}P]GTP hydrolysed in 5 min to available protein-bound [γ-^{32}P]GTP.

Measurement of Rac binding to Rho-GDI

Rac1 (1 μg, produced by *E. coli* or Sf9 cells) was loaded for 10 min with [^{3}H]GDP (15.3 Ci/mmol) in 16 mM Tris/HCl (pH 7.5)/20 mM NaCl/0.1 mM DTT/5 mM EDTA/0.015 μCi of [^{3}H]GDP. Thereafter, $MgCl_2$ was added to 20 mM so as to diminish further nucleotide exchange; the solution was kept on ice. Labelled Rac1 was incubated for 1 h with GST-conjugated GDI (5 μg) immobilized on glutathione–agarose beads, at 4 °C on a rotating wheel. The separated beads were washed twice in cold PBS and the radioactivity bound to the beads was determined in a toluene/Triton-based scintillation cocktail in a Beckman LS 5000TD liquid-scintillation spectrometer.

Immunoblotting

SDS/PAGE was perfomed by the method of Laemmli [41] with 7.5% or 12% (w/v) polyacrylamide in the separating gel. Proteins were then transferred to nitrocellulose sheets. After being blocked in PBS containing 5% (w/v) defatted milk powder, blots were exposed to anti-(p190Rho-GAP) (1:500 dilution), anti-Bcr (1:200 dilution) or anti-(p50Rho-GAP) (1:200 dilution). Bound antibodies were detected with horseradish-peroxidase-labelled anti-mouse IgG or anti-rabbit IgG and the reaction was detected with enhanced chemiluminescence Western blotting reagent as a substrate for peroxidase.

Overlay assay

The method described by Manser et al. [42,43] was used in these experiments. Proteins were separated by SDS/polyacrylamide gels containing 10% (w/v) glycerol, 5 mM DTT and 0.5 mM $MgCl_2$. Proteins were transferred to nitrocellulose membranes overnight at 4 °C in a semi-dry apparatus with a buffer containing 50 mM Tris base, 40 mM glycine, 10% (v/v) methanol, 0.5 mM $MgCl_2$, 0.2% SDS, 0.2% (v/v) Triton X-100. Transferred

proteins were renatured for 7–10 h in PBS containing 1 % (w/v) bovine albumin, 5 mM DTT, 0.5 mM $MgCl_2$ and 0.1 % (v/v) Triton X-100. Rac1 or Rac1–GST (1–5 μg) was labelled with 1–3 μl of [γ-^{32}P]GTP (specific radioactivity more than 5000 Ci/mmol) for 4 min at room temperature in 50 μl of an exchange buffer [50 mM NaCl/25 mM Mes/NaOH (pH 6.3)/2.5 mM EDTA/0.05 % (v/v) Triton X-100]. The [γ-^{32}P]GTP-bound protein was diluted in 2 ml of GAP buffer [50 mM NaCl/25 mM Mes/NaOH (pH 6.5)/2.5 mM DTT/1.25 mM $MgCl_2$/0.05 % Triton X-100] supplemented with 0.5 mM unlabelled GTP. Nitrocellulose filters with the separated proteins were soaked in this buffer and placed on 1 % (w/v) agarose gels containing 50 mM NaCl, 25 mM Mes/NaOH, pH 6.5, 5 mM DTT and 5 mM $MgCl_2$. After incubation for 5 min at room temperature and for 10 min at 4 °C, a second, wet nitrocellulose filter was overlaid on the original filter. After incubation for 5 min at 4 °C the second filter was carefully removed and washed three times in PBS containing 25 mM Mes/NaOH, pH 6.5, 5 mM $MgCl_2$ and 0.05 % (v/v) Triton X-100 and laid on X-ray film for 12–48 h at −70 °C.

Immunoprecipitation experiments

Protein G–Sepharose beads were incubated with the antibody against p50RhoGAP and washed three times in PBS to remove traces of unbound immunoglobulins. Membranes from human polymorphonuclear cells (PMN) corresponding to approx. 2 mg of protein were solubilized for 5 min in PBS containing 1 % (v/v) Triton X-100 at room temperature. The Triton-insoluble material was separated by centrifugation at 16000 ***g*** for 30 min. Immunodepletion of the clear supernatant was performed in three successive steps, incubating the solubilized material each time for 45 min with approx. 30 μl of antibody-loaded Sepharose beads. The separated beads were washed three times in PBS supplemented with 0.1 % (v/v) Triton X-100. The immunodepleted supernatants were analysed for total Rac-GAP activity and loaded on a gel for immunodetection and the overlay assay. Except for the solubilization, all steps of immunodepletion were performed at 4 °C.

Removal of endogenous Rac by washing the crude membrane fraction with KCl

The crude membrane fraction of bovine neutrophils contains a substantial amount of Rac protein that masks the requirement for exogenous Rac in the cell-free activation assay. Freshly thawed membrane aliquots were therefore resuspended in an equal volume of 2 M KCl by gentle sonication with a microprobe. The membranes were then ultracentrifuged at 300000 ***g*** in a Beckman TL100 rotor for 20 min at 4 °C. They were rinsed with PBS and resuspended in half the initial volume of PBS to keep the protein concentration constant.

Measurement of $O_2^{-\bullet}$ generation in the semirecombinant and fully purified cell-free system

The rate of $O_2^{-\bullet}$ generation was determined as the superoxide-dismutase-sensitive portion of ferricytochrome *c* reduction measured at 550 nm [31] in a Labsystem IeMS microplate reader.

A two-step activation system was used. Washed membranes (3 μg of protein) or relipidated purified cytochrome b_{558} (0.3 pmol) was mixed with recombinant p47^{phox} (0.8 μg = 17 pmol), p67^{phox} (1.2 μg = 18 pmol) and Rac (0.9 pmol) preloaded with GTP[S] or GTP in 100 μl of PBS supplemented with 1 mM $MgCl_2$. This mixture was added to arachidonic acid that had been pipetted directly into the wells of the microplate. For each condition the optimal concentration of arachidonic acid was determined and used in the activation mixture. After 5 min of incubation, 0.1 ml of measuring solution (PBS supplemented with 200 μM cytochrome *c* and 500 μM NADPH) was added and the rate of change in absorbance was recorded between 60 and 120 s. Then 5 μg of superoxide dismutase was added and the rate of $O_2^{-\bullet}$ production was calculated from the difference in the rate of change in absorbance before and after the addition of superoxide dismutase, taking 21.1 $mM^{-1} \cdot cm^{-1}$ as the molar absorption coefficient for ferricytochrome *c*.

For loading of prenylated or non-prenylated Rac with guanine nucleotides, 1.8 μg of the small GTPase was incubated for 10 min in a final volume of 22 μl of PBS containing 4 mM EDTA and 20 μM GTP or GTP[S] at 25 °C. Subsequently 20 mM $MgCl_2$ was added and the protein was kept on ice [40].

Protein determination

The protein content was determined as described by Bradford [44], with BSA as standard.

Statistical analysis

Results are presented either as representative traces or pictures of the indicated number of experiments or as means ± S.E.M. for the indicated number of determinations (*n*).

RESULTS

Intracellular localization and interactions of Rac-GAP activity

We have demonstrated previously the presence of Rac-GAP activity in the membrane fraction of pig neutrophil granulocytes [28]. In the present study we first compared the GAP activity reacting with prenylated Rac1 in the membrane and cytosolic fractions of human PMN. The specific GAP activity was approx. 5-fold higher in the membrane fraction than in the cytosol. In typical preparations approx. 15 times more protein is obtained in the cytosol fraction than in the membrane fraction, so the total GAP activity in the cytosol might be threefold that in the membrane fraction. Strong Rac-GAP activity could also be demonstrated in the membrane fraction of PMN from patients with X-linked CGD, of non-differentiated and differentiated HL-60 cells, of peripheral lymphocytes and K 562 cells. Thus the appearance of the Rac-GAP activity is independent of the expression of the NADPH oxidase of phagocytic cells. Interestingly, no Rac-GAP activity could be detected in the membranes of mature human erythrocytes.

The Rac-GAP activity associated with the membrane fraction resisted washing in high (2 M) salt concentration but it was easily solubilized by 1 % (v/v) Triton.

The interaction of small GTPases with the GAP present in the neutrophil membranes was tested with Rac1 protein expressed either in Sf9 cells or in *E. coli*. As shown in Figure 1 (upper panels), addition of the PMN membrane fraction significantly increased the hydrolysis of [γ-^{32}P]GTP bound to Rac1 prepared from Sf9 cells, whereas it had very little effect on Rac1 from *E. coli*. In 12 measurements performed on five different PMN preparations, 74.4 ± 8.7 % and 16.5 ± 7.3 % of the [γ-^{32}P]GTP bound to prenylated and non-prenylated Rac respectively was hydrolysed in the first 5 min.

In mammalian cells Rac protein synthesized on the ribosomes is modified post-translationally by prenylating, hydrolytic and methylating enzymes localized mainly in the plasma membrane [27]. Sf9 insect cells are capable of performing this processing, whereas the protein purified from *E. coli* cells is not modified.

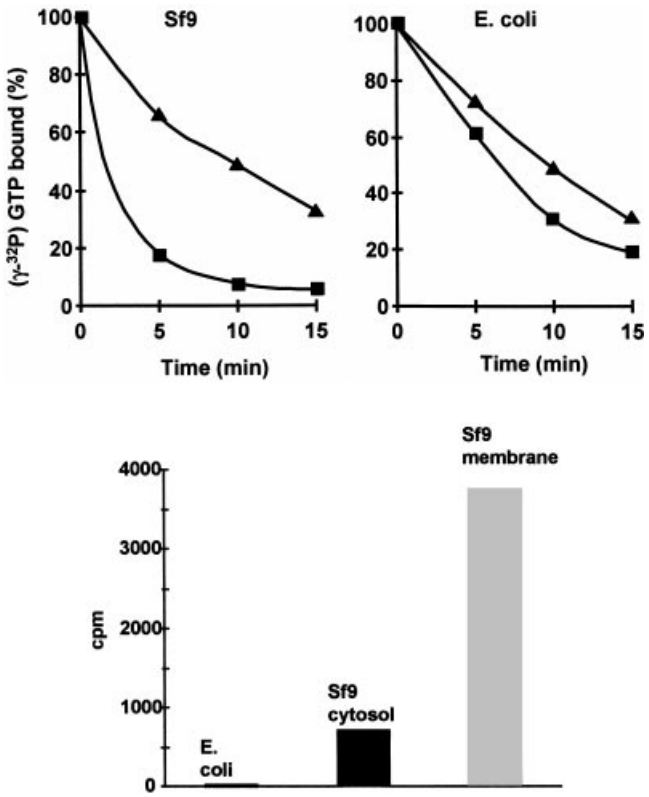

Figure 1 Reaction of membrane-associated Rac-GAP with Rac from different sources

Upper panels: change with time in the amount of Rac-bound [γ-^{32}P]GTP is shown in the absence of any Rac-GAP (▲) or in the presence of 15 μg of human neutrophil membrane protein (■). Data points are means for two separate determinations in one typical experiment of five similar ones. Lower panel: binding of Rac loaded with [^{3}H]GDP to Rho-GDI–GST fusion protein fixed to glutathione–Sepharose beads. Binding was assessed on the basis of radioactivity detected on the separated beads. Radioactivity attached to beads loaded only with GST protein has been subtracted.

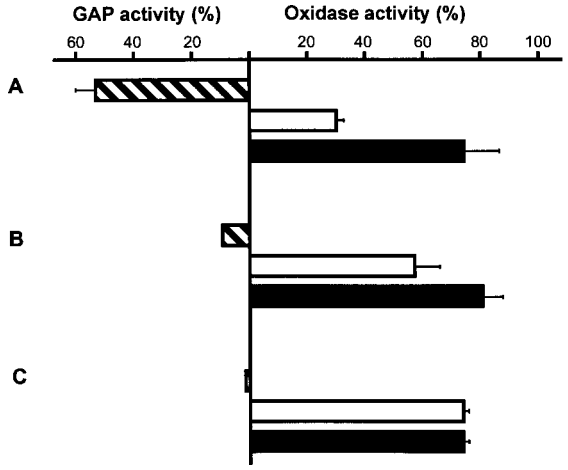

Figure 2 Relation of total Rac-GAP activity (hatched bars) to $O_2^{-\bullet}$ production elicited by Rac-GTP (open bars) or by combination of Rac-GTP plus fluoride (filled bars) in the semirecombinant (A and B) and in the fully purified (C) systems

Activation in the semirecombinant or the fully purified system was performed as described in the Experimental section. When applied, 30 mM NaF was added to the preincubation mixture; 100% of oxidase activity corresponds to the rate of $O_2^{-\bullet}$ production obtained with Rac-GTP[S]. (**A**) Washed membrane, prenylated Rac; (**B**) washed membrane, *E. coli* Rac–GST; (**C**) purified cytochrome b_{558}, prenylated Rac.

Prenylation has been described as being a crucial factor in the reaction of Rac with Rho-GDI [45]. We therefore measured the binding of the two Rac preparations used in our experiments to Rho-GDI–GST fusion protein. As shown in Figure 1 (lower panel), binding of *E. coli* Rac1 did not exceed the background level, whereas Rac1 purified from Sf9 cells exhibited significant binding to Rho-GDI. The difference detected in the binding properties of Rac1 protein purified from the cytosol or the membrane fraction of the insect cells probably reflects the abundance of prenylated Rac protein in the two preparations [37]. The similarity of the interaction of Rac1 from different sources with recombinant Rho-GDI and with GAP-containing biological preparations suggests that the differences observed can be ascribed to the differences in prenylation of the small GTPase produced in bacterial cells and in insect cells.

Involvement of the membrane-associated Rac-GAP activity in regulation of NADPH oxidase

In previous experiments, with the use of purified cytochrome b_{558} and crude cytosol, we obtained firm data on the participation of cytosolic Rac-GAPs in the regulation of NADPH oxidase [46]. In the following experiments we investigated the possible involvement of the strong membrane-associated Rac-GAP activity in modulating the enzymic function.

Our general approach was to compare $O_2^{-\bullet}$ production in the presence of Rac loaded with GTP (and therefore sensitive to the effect of GAP) or its non-hydrolysable analogue GTP[S] in different forms of the cell-free activation system containing various levels of Rac-GAP activity. We also investigated the enhancing effect of fluoride in these systems.

In the semirecombinant cell-free activation system, the crude membrane fraction from bovine neutrophils was washed with KCl to remove endogenous Rac. Activation was performed in the presence of recombinant p47^{phox} and p67^{phox} proteins and prenylated Rac1 purified from the membrane fraction of Sf9 cells. Only results obtained with the optimal arachidonic acid concentrations are shown.

The membrane fraction applied in the semi-recombinant activation system exhibited intense Rac-GAP activity (Figure 2A, hatched bar). In this system, $33 \pm 2.9\,\%$ of the maximal rate of $O_2^{-\bullet}$ production was attained by Rac-GTP (Figure 2A, open bar) but it could be significantly increased by fluoride (Figure 2A, filled bar).

In accordance with the results shown in Figure 1, the membrane-associated GAP activity was less effective with non-prenylated Rac1 protein obtained from *E. coli* cells (Figure 2B, hatched bar). When non-prenylated *E. coli* Rac1 protein was applied in the semirecombinant activation system, Rac-GTP elicited $59.3 \pm 11\,\%$ of the maximal $O_2^{-\bullet}$ production (Figure 2B, open bar). Consistent with the poor GAP-action, the effect of fluoride on NADPH oxidase was less prominent with unprocessed than with prenylated Rac.

In the fully purified activation system relipidated purified cytochrome b_{558} was incubated with recombinant p47^{phox}, p67^{phox} and prenylated Rac1 loaded with GTP or GTP[S] in the presence of the optimal concentration of arachidonic acid. The cytochrome preparation did not have any detectable Rac-GAP activity (Figure 2C). $O_2^{-\bullet}$ production attained with Rac-GTP was

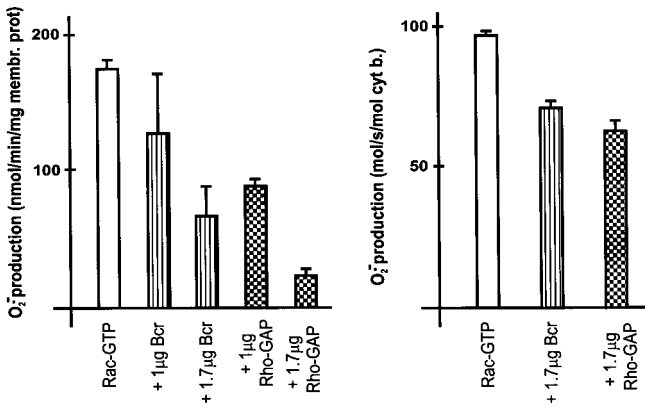

Figure 3 Effect of exogenous Rac-GAP proteins on Rac-GTP-elicited $O_2^{\cdot-}$ production

Activation in the semirecombinant (left panel) or fully purified (right panel) system was performed as described in the Experimental section. The indicated quantities of Bcr or Rho-GAP were added to the preincubation mixture.

$75 \pm 1.5\,\%$ of the maximal rate obtained with Rac-GTP[S] (Figure 2C, open bar) and was not increased further by fluoride (Figure 2C, filled bar).

Effect of exogenous Rac-GAP proteins on the rate of $O_2^{\cdot-}$ production

The effect of the GAP domains of Bcr and p50 Rho-GAP was investigated in both the semirecombinant and fully purified systems. The GAP activity of the two domains was tested on purified Rac1 and RhoA proteins and their effects corresponded to previous results [47]. The activity of the GAP domains was not influenced by arachidonic acid or the lipid mixture used for the relipidation of purified cytochrome b_{558} (results not shown). When added to the activation phase of the cell-free system, both Bcr and p50Rho-GAP decreased the rate of $O_2^{\cdot-}$ production elicited with Rac-GTP in a dose-dependent manner (Figure 3). Both GAPs seemed to be more effective in the semi-recombinant system. In control experiments, when Rac-GTP[S] was present in the preincubation phase, addition of the GAP domains was without effect (results not shown).

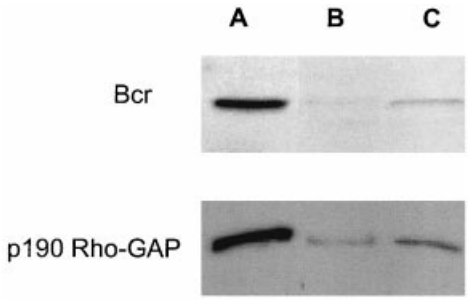

Figure 4 Detection of Bcr and p190Rho-GAP in the crude membrane and cytosol fractions of human neutrophil granulocytes and rat brain tissue extract

Proteins (200 μg) of rat brain tissue extract (lane A), the crude membrane fraction (lane B) and the cytosol fraction (lane C) of human neutrophil granulocytes were subjected to electrophoresis and immunoblotted with specific monoclonal antibodies against Bcr and p190Rho-GAP. A typical result from three similar ones is shown.

Detection of Bcr and p190RhoGAP in human neutrophil granulocytes

Our investigation of the presence of specific Rac-GAP proteins in human neutrophil granulocytes concentrated first on Bcr and p190RhoGAP. The involvement of Bcr protein was suggested by the observation of an increase in $O_2^{\cdot-}$ production in the neutrophil granulocytes of Bcr-deficient mice [34]. In contrast, the presence of p190RhoGAP was demonstrated in human neutrophils [33] and its translocation was described to occur in parallel with the translocation of $p47^{phox}$ and $p67^{phox}$ in the course of NADPH oxidase activation [48].

By means of specific antibodies we revealed Bcr protein in the cytosolic fraction of human neutrophil granulocytes, whereas this protein was absent from the membrane fraction (Figure 4). Nervous tissue was shown to be the richest source of several GAP proteins, so an equal amount of protein of rat brain extract was loaded on the same gel as a positive control.

The antibody reacting with p190RhoGAP recognized a definitive band in the neutrophil cytosol; occasionally a much weaker signal was seen in the membrane fraction. It should be noted that both Bcr and p190RhoGAP are significantly less abundant in neutrophil granulocytes than in nervous tissue.

Investigation of the Rac-GAP proteins present in PMN membranes

To obtain information on the possible Rac-GAP proteins residing in the membrane fraction of human neutrophils, we used the Rac-[γ-^{32}P]GTP overlay technique described by Manser et al. [42,43]. In Figure 5 the image of the X-ray film is shown as a negative: proteins with GAP activity (inducing a decrease of bound radioactivity) appear as dark bands, whereas proteins that inhibit the endogenous GTP-hydrolysis of Rac are visible as light bands on a uniformly grey background. As shown in Figure 5, the most intense band with Rac-GAP activity appeared at approx. 50 kDa in both the membrane and cytosol fractions. In addition, two weaker bands are visible at approx. 35–40 kDa in the membrane fraction. Both the 50 kDa and the 35 and 40 kDa proteins also reacted with Rho and Cdc42 when these members of the Rho-family GTPases were used in the overlay technique (results not shown). In our experiments, no GAP activity was revealed in the higher-molecular-mass range in either the membrane or the cytosolic fractions. In agreement with the results of Manser et al. [42], in the rat brain extract also under our experimental conditions a clear GAP activity was visible around 180 kDa. The lack of the appearance of this band in the neutrophil preparations was probably due to the considerably smaller amount of Bcr and p190RhoGAP in PMN (Figure 4).

In the cytosolic fraction, three bands with strong GTPase inhibitory action were revealed. The bands at 66–70 kDa probably corresponded to the two PAKs [16,18,19], whereas the protein at approx. 180 kDa was, to our knowledge, a hitherto unidentified GTPase inhibitory protein.

The GAP activity at 50 kDa reacting with Rac, Rho and Cdc42 could correspond to p50RhoGAP, which has been described in many tissues. To verify this hypothesis we produced antibodies against a 29 kDa fragment of the protein, containing the GAP domain [49]. The purified polyclonal antibody specifically recognized the 29 kDa GAP domain of human p50RhoGAP that was used for immunization (results not shown) but did not react with rat proteins. It detected a single band in both human granulocyte membrane and cytosol at 50 kDa

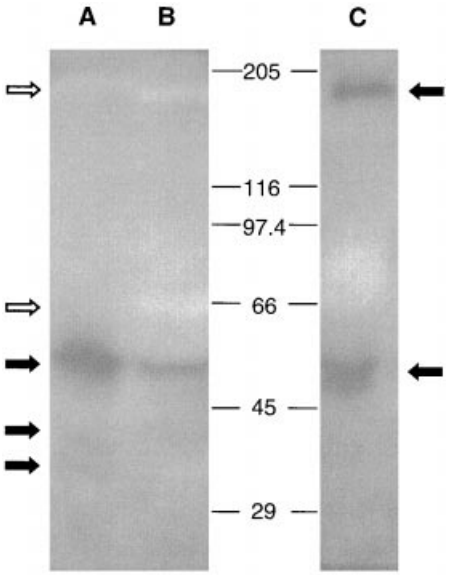

Figure 5 Detection of Rac-GAP activities by overlay assay in the crude membrane and cytosol fractions of human neutrophil granulocytes and rat brain tissue extract

Proteins (200 μg) of the crude membrane fraction (lane A) and the cytosol fraction (lane B) of human neutrophil granulocytes and of rat brain tissue extract (lane C) were subjected to electrophoresis and transferred to nitrocellulose membranes. The membrane-bound proteins were renatured and analysed for Rac-GAP activity as described in the Experimental section. Proteins with Rac-GAP activity appear as dark bands and are indicated by dark arrows. Proteins that have the opposite, inhibitory effect on the GTPase reaction appear as light bands and are indicated by white arrows. A typical result from five similar ones is shown. The positions of molecular mass markers are indicated (in kDa) between the panels.

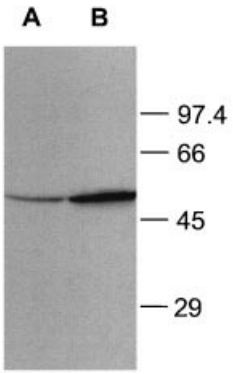

Figure 6 Detection of p50Rho-GAP in the cytosol and crude membrane fractions of human neutrophil granulocytes

Proteins (200 μg) from the cytosol fraction (lane A) and the crude membrane fraction (lane B) of human neutrophil granulocytes were separated by SDS/PAGE and immunoblotted with affinity-purified polyclonal antibody raised against a GST fusion protein containing residues 198–439 of p50Rho-GAP. A typical result from three similar ones is shown. The positions of molecular mass markers are indicated (in kDa) at the right.

(Figure 6). The reaction was more intense in the membrane fraction than in the cytosol. Although the antibody is directed against the GAP domain, it did not react with proteins at lower molecular masses.

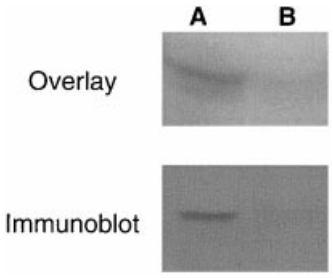

Figure 7 Immunodepletion of p50Rho-GAP content of the Triton-solubilized membrane fraction of human neutrophil granulocytes

Immunodepletion was performed as described the Experimental section. Proteins (50 μg) from solubilized control membrane fraction (lane A) or immunodepleted membrane fraction (lane B) were subjected to electrophoresis, transferred to nitrocellulose membranes and analysed by the overlay technique and by immunodetection. A typical result from three similar ones is shown.

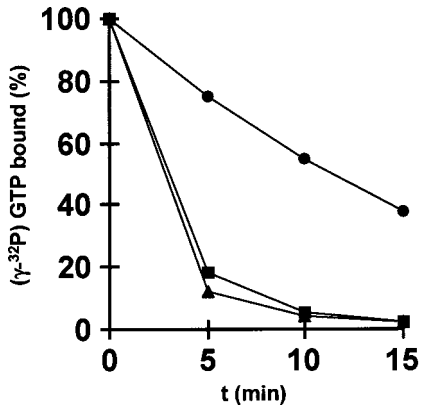

Figure 8 Detection of Rac-GAP activity in the supernatant after immunodepletion with anti-p50RhoGAP antibodies

Immunodepletion was performed as described in the Experimental section. The change with time in the amount of Rac-bound [γ-^{32}P]GTP is shown in the absence of any Rac-GAP (●) or in the presence of 7 μg of control (▲) or immunodepleted (■) solubilized membrane protein. Rac was prepared from Sf9 insect cells. Data points are means for three separate determinations in one typical experiment of three similar ones.

Immunoprecipitation of p50RhoGAP

To decide whether the dominant GAP activity at approx. 50 kDa revealed by the overlay technique could be ascribed to the massive presence of p50RhoGAP detected in the immunoblot, we performed the immunodepletion of this protein from solubilized membranes. Three successive treatments of the solubilized membrane fraction with anti-(p50Rho-GAP) antibodies immobilized on Protein G–Sepharose beads resulted in the removal of most of the immunoreactive 50 kDa protein and in the virtual disappearance of the GAP activity at approx. 50 kDa in the overlay (Figure 7). Thus the dominant renaturable GAP activity detected after the electrophoretic separation of solubilized human neutrophil granulocyte membrane proteins can be ascribed to p50RhoGAP.

Measurement of the total GAP activity of the immunodepleted solubilized membrane fraction yielded surprising results (Figure 8): immunodepletion of most of the p50RhoGAP protein caused only a moderate decrease in the total GAP activity of the preparation. In six experiments, a $12.8 \pm 4.6\,\%$ difference was detected between the amount of protein-bound [γ-^{32}P]GTP

hydrolysed in the first 5 min by immunodepleted solubilizates and that hydrolysed by control solubilizates.

DISCUSSION

Our overlay experiments revealed that in neutrophil granulocytes the dominant renaturable GAP activity reacting with Rho-family small GTPases corresponds to p50RhoGAP (also called Cdc42GAP). This protein was first purified as a 25–29 kDa fragment from human platelets [50] and human spleen [51]. Probably the same protein was purified as a 28 kDa fragment from bovine adrenal gland [52]. Cloning of the protein revealed its true molecular mass of 50 kDa and it was shown that the protein reacted with Cdc42, Rho and Rac [49,53]. p50RhoGAP seems to be a ubiquitous protein [43] and in bovine adrenal gland it was detected in both the membrane and the cytosol [53]. However, neither its expression in blood cells nor its participation in any cellular function has yet been demonstrated.

In neutrophil granulocytes the behaviour of p50RhoGAP was similar to that described previously in other cell types: it was present in both the cytosol and the membrane and it reacted with Rac, RhoA and Cdc42 in the overlay assay. However, immunodepletion of p50RhoGAP did not significantly impair the intense Rac-GAP activity of the solubilized membrane fraction. Different explanations are possible for this finding. (1) p50RhoGAP contains a proline-rich region that allows its interaction with various proteins containing SH3 domains [53]. It is therefore possible that in resting neutrophils p50RhoGAP is present as part of a supramolecular complex that is not interrupted by solubilization and in its cellular environment does not function as a GAP. The fact that only a proteolytic fragment of the protein could be purified might be the consequence of its participation in a complex; the formation of large protein aggregates containing Rho-GAP has been given as the reason for the poor recovery of the isolated 28 kDa fragment [52]. (2) Alternatively, other proteins with stronger Rac-GAP activity might be present in the neutrophil membrane fraction that are not renatured in the overlay experiments but under native conditions surpass the effect of p50Rho-GAP so that its depletion does not seriously decrease the total Rac-GAP activity.

With the use of the overlay technique, two more GAP activities were detected at 35–40 kDa that reacted with Rac, Rho and Cdc42. There are several arguments to suggest that they are independent proteins and not proteolytic fragments of p50RhoGAP: (1) they appeared consistently, in spite of the application of various combinations of protease inhibitors; (2) although they were detected on the basis of their GAP activity, they were not recognized by the antibody raised against the GAP domain of p50RhoGAP; (3) their estimated molecular mass was higher than that of the proteolytic fragment of p50RhoGAP purified in two different laboratories from two different human tissues [50,51]; (4) they appeared mainly in the membrane fraction, although p50RhoGAP was also present in the cytosol. Their molecular mass does not correspond to the 30 kDa β-chimaerin [54] but it is near to that of the 38 kDa α_1-chimaerin. However, chimaerins were shown to react mainly with Rac and to be expressed only in brain and testis [54].

Applying the overlay technique to human granulocyte fractions, we could not detect any GAP activity at 45 kDa, corresponding to α_2-chimaerin. However, this protein was also shown to be specific for brain and testis [43]. No GAP activity could be revealed at approx. 160 and 190 kDa corresponding to Bcr and p190RhoGAP respectively, although both proteins could be detected by immunoblotting. As their quantity was significantly lower than in the brain extract, they probably remained below the detection limit of the overlay assay.

Using the semirecombinant and the fully purified activation systems, we revealed an inverse relationship between the Rac-GAP activity of the membrane fraction and the oxidase activating potency of Rac-GTP. Furthermore, the enhancing effect of fluoride on oxidase activation paralleled the GAP activity (Figure 2). The highest rate of $O_2^{-\bullet}$ production that could be elicited with Rac-GTP in the absence of GAPs (with purified cytochrome b_{558}) amounted to 75–80 % of the maximal rate attained with Rac-GTP[S] (Figure 2C). This value corresponds to the high endogenous GTPase activity of Rac that resulted in the hydrolysis of approx. 25 % of bound GTP within 5 min (Figure 1). In the presence of Rac-GTP comparable rates of $O_2^{-\bullet}$ production were obtained in the fully purified system, from which Rac-GAP activity was absent, and in the semirecombinant system, in which GAP action was inhibited by fluoride. Thus our results show clearly that the Rac-GAP activity associated with the membrane fraction of resting neutrophil granulocytes is able to interact with Rac-GTP and that it is involved in the regulation of the assembly of NADPH oxidase. On the basis of the overlay and immunoprecipitation experiments, the membrane-localized intense Rac-GAP activity could be ascribed either to the GAP activities detected at 35–40 kDa or to one or more other GAP proteins that were not renatured after the electrophoretic separation and were therefore undetectable in the overlay assay.

Exogenous GAP domains decreased $O_2^{-\bullet}$ production in both the semirecombinant and the fully purified cell-free activation systems (Figure 3). Thus, independently of the presence of membrane-associated GAP, soluble (cytosolic) GAPs have access to Rac involved in the activation of NADPH oxidase. As revealed in our immunodetection experiments, Bcr, p50Rho-GAP and p190Rho-GAP are obvious candidates for the role of cytosolic GAPs. Our observation that both Bcr and p50Rho-GAP were more effective in the semirecombinant system than in the fully purified system might indicate that the effect of membrane-associated and soluble GAPs is additive. In this view, GAP functions could be overlapping and redundant, as for example the actions of Src kinase are.

Finally, it should be noted that in the system of NADPH oxidase activation *in vitro* used in the present study, both the inhibition (or elimination) of endogenous Rac-GAP activity and the addition of exogenous Rac-GAP resulted in a significant alteration in the rate of $O_2^{-\bullet}$ production. Thus in intact cells modulation of Rac-GAP activity (e.g. by covalent modification or complex formation) can be an effective method of both increasing and decreasing Rac activity and thereby down-regulating or up-regulating processes affected by Rac.

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