Participation of Rac GTPase Activating Proteins in the Deactivation of the Phagocytic NADPH Oxidase†

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ABSTRACT: The aim of the present study was to investigate possible mechanisms that could be involved in the deactivation of the assembled, catalytically active NADPH oxidase of phagocytic cells and thereby lead to termination of $O_2^{\bullet-}$ production. Our major findings are the following: (1) Addition of GDP to the active oxidase is able to reduce $O_2^{\bullet-}$ production both in the fully purified and in a semirecombinant cell-free activation system. (2) $p67^{phox}$ inhibits GTP hydrolysis on Rac whereas $p47^{phox}$ has no effect on Rac GTPase activity. (3) Soluble regulatory proteins (GTPase activating protein, guanine nucleotide dissociation inhibitor, and the Rac-binding domain of the target protein p21-activated kinase) inhibit activation of the NADPH oxidase but have no effect on electron transfer via the assembled enzyme complex. (4) Membrane-associated GTPase activating proteins (GAPs) have access also to the assembled, catalytically active oxidase. Taken together, we propose that the GTP-bound active form of Rac is required for sustained enzyme activity and that membrane-localized GAPs have a role in the deactivation of NADPH oxidase.

The superoxide- ($O_2^{\bullet-}$-) producing NADPH oxidase of phagocytic cells is a central factor in the antimicrobial defense of the organism. In resting phagocytes the enzyme is inactive, but it can be rapidly activated by various stimuli. Failure of $O_2^{\bullet-}$ generation results in impaired killing of microorganisms as in the case of chronic granulomatous disease (*1*−*3*). On the other hand, production of toxic oxygen metabolites by neutrophil granulocytes was shown to contribute to tissue damage occurring in postischaemic states or low-rate perfusion in critical organs such as brain and heart (*4*, *5*). Thus, tight regulation of both the site and intensity of activation of phagocytic NADPH oxidase is a basic requirement for physiological functioning of mammalian organisms.

Activation of the NADPH oxidase involves translocation of several subunits ($p47^{phox}$, $p67^{phox}$, $p40^{phox}$, and the small GTPase Rac) from the cytosol and assembly of the catalytically active enzyme complex in the plasma (or phagosomal) membrane of the phagocytic cell. The proteins minimally required for $O_2^{\bullet-}$ production have been cloned, and their molecular interactions as well as the kinetic properties of the activation have been analyzed in various cell-free systems (for review see refs *6*−*10*). However, information is still lacking on the mechanisms of fine-tuning of $O_2^{\bullet-}$ production which could provide an explanation for the wide differences in the intensity and duration of $O_2^{\bullet-}$ production induced by various stimuli.

In our previous papers we provided experimental support for the involvement of Rac GTPase activating proteins (Rac-GAPs)[1] in the regulation of the activation phase of the NADPH oxidase and characterized Rac-GAP proteins localized in the membrane and cytosol of neutrophil granulocytes (*11*−*14*). The aim of the present study was to investigate the fate of the assembled, catalytically active enzyme complex. We show that (1) GDP is able to inhibit electron transfer via the active oxidase and (2) membrane-localized Rac-GAP(s) is (are) able to interact with the assembled oxidase complex. We propose that membrane-associated Rac-GAP(s) has (have) a significant role also in the deactivation of the enzyme and in termination of $O_2^{\bullet-}$ production, determining thus both the intensity and the duration of the oxidative burst.

EXPERIMENTAL PROCEDURES

Materials. Recombinant $p47^{phox}$ and $p67^{phox}$ were produced as GST fusion proteins in *Escherichia coli* and purified as described in ref *15*. Cytochrome b_{558} was purified and relipidated as described in ref *16*. Prenylated Rac was isolated from the membrane fraction of *Sf*9 cells by extraction with 1% Chaps and purified as described in ref *17*. Nonprenylated Rac1, Rho guanine nucleotide inhibitory factor (Rho-GDI),

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[1] Abbreviations: DTT, dithiothreitol; GAP, GTPase activating protein; GDI, guanine nucleotide dissociation inhibitor; GST, glutathione *S*-transferase; GDPβS, guanosine 5′-*O*-(2-thiodiphosphate); GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); OAG, 1-oleoyl-2-acetyl-*sn*-glycerol; PA, phosphatidic acid; PAK, p21-activated kinase; PBS, phosphate-buffered saline; phox, *ph*agocyte *ox*idase; PMN, polymorphonuclear granulocyte; RBD, Rac-binding domain.

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the GAP domain of p50Rho-GAP (amino acids 198−439), and the Rac-binding domain (RBD) of p21-activated kinase (PAK) were produced in the form of GST fusion proteins and purified as described in ref *18*. The *E. coli* clones producing Rac1, Rho-GDI, and p50Rho-GAP were a generous gift of A. Hall, the clone producing RBD was obtained from G. Bokoch, and the clones producing $p47^{phox}$ and $p67^{phox}$ were from F. Wientjes. Rac mutant A27K G30S was made using the Quickchange site-directed mutagenesis kit (Stratagene) according to the manufacturer's protocol, and the sequence was checked. Ficoll-Paque and nitrocellulose were purchased from Pharmacia, glutathione, glutathione−agarose, ampicillin, components of the LB medium, BSA, aprotinin, pepstatin, PMSF, phosphatidic acid (PA), 1-oleoyl-2-acetylglycerol (OAG), and ferricytochrome *c* (horse heart, type VI) were from Sigma, IPTG was from Promega, NADPH, superoxide dismutase, GTPγS, and GTP were from Boehringer Mannheim, and [γ-^{32}P]GTP and [^{35}S]GTPγS were from Izotóp Intézet, Hungary. All other reagents were of the highest available quality. Incubations were carried out in 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, and 1.5 mM KH_2PO_4, pH 7.4 (PBS).

Preparation of Neutrophils and Subcellular Fractions. Human neutrophil granulocytes were prepared from buffy coats of healthy volunteers as described in ref *19*, were suspended in PBS, and were treated with 1 mM diisopropyl 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, and 0.1 mM phenylmethanesulfonyl fluoride. Cells were broken by ultrasonic treatment, and membrane and cytosolic fractions were prepared on a discontinuous sucrose gradient as described in ref *20*.

Measurement of the GTPase Activity of Rac. This was performed by the nitrocellulose filter binding assay as described in ref *21*. Loading of Rac1 (1−4 μg of *E. coli* or *Sf*9 cell protein) was performed with a high specific activity of [γ-^{32}P]GTP (>5000 Ci/mmol) in the following solution: 16 mM Tris-HCl, pH 7.5, 20 mM NaCl, 0.1 mM DTT, 5 mM EDTA, and 100 nM [γ-^{32}P]GTP (5 μCi) for 10 min at room temperature. Thereafter, $MgCl_2$ was added to a concentration of 20 mM in order to block further nucleotide exchange, and the solution was kept on ice. The GTPase reaction was initiated by addition of 3 μL of Rac loaded with [γ-^{32}P]GTP to 27 μL of a warmed (30 °C) buffer containing 16 mM Tris-HCl, pH 7.5, 0.1 mM DTT, and the indicated proteins. Bovine serum albumin (1 mg/mL) was included as a carrier protein. Aliquots of 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 presented as the decrease of protein-bound radioactivity in time.

Measurement of Superoxide Generation in the Cell-Free System. The rate of superoxide generation was determined as the superoxide dismutase-sensitive portion of ferricytochrome *c* reduction measured at 550 nm in a Labsystems iEMS microplate reader. A two-step activation system was applied as described in ref *22*.

In the semirecombinant system membranes (5 μg) and recombinant $p47^{phox}$ (0.5 μg) and recombinant $p67^{phox}$ (0.5 μg) were preincubated for 90 min in the presence of 375 μM PA, 250 μM OAG, and 125 μM cytochrome *c* in a final volume of 120 μL of PBS containing 1 mM $MgCl_2$. The membrane fraction isolated from human PMN contained sufficient Rac, as addition of either prenylated or nonprenylated Rac to the system did not influence the maximal rate of $O_2^{\bullet-}$ production. Nucleotides (GTP, GTPγS, GDP, UDP) or regulatory proteins were added in the indicated concentrations. Superoxide ($O_2^{\bullet-}$) production was initiated by the addition of 100 μM NADPH and followed for 10 min. The initial linear portion of the absorption curves (lasting for 2−4 min) was used for calculation of the rate of $O_2^{\bullet-}$ production. Parallel samples were run in the presence of 100 μg of superoxide dismutase (SOD). The absorption coefficient of ferricytochrome *c* of 21000 M^{-1} cm^{-1} was used for calculation of the $O_2^{\bullet-}$ production.

In the fully purified system 0.005 μg of purified and relipidated cytochrome b_{558} was incubated with 0.5 μg of $p47^{phox}$, 0.5 μg of $p67^{phox}$, 0.12 μg of GTP-loaded prenylated Rac, 75 μM OAG, 112.5 μM PA, and 125 μM cytochrome *c* in a final volume of 120 μL for 10 min. Superoxide ($O_2^{\bullet-}$) production was initiated by the addition of 100 μM NADPH and followed for 10 min. Nucleotides (GTP, GTPγS, GDP, UDP) or regulatory proteins were added in the indicated concentrations.

In contrast to cell-free activation systems applying other amphiphiles (e.g., arachidonic acid or SDS), in this system $O_2^{\bullet-}$ production was independent of the applied concentration of PA and OAG in a broad range.

For loading of prenylated or nonprenylated Rac with guanine nucleotides, 1.8 μg of the small GTPase was incubated in a final volume of 22 μL of PBS containing 4 mM EDTA and 20 μM GTP or GTPγS for 10 min at 25 °C. Subsequently, 20 mM $MgCl_2$ was added, and the protein was kept on ice (*21*).

Measurement of Guanine Nucleotide Exchange. Incubation was carried out as described for measurement of superoxide generation in the cell-free system in the presence of 1.25 μM [^{35}S]GTPγS. After complete activation of the enzyme, GDP was added, and at the indicated time points the reaction mixture was filtered through nitrocellulose filters. Washing of the filters was carried out as for measurement of GTPase activity. Radioactivity was measured in toluene/Triton scintillation cocktail.

Protein Determination. The protein content was determined as described by Bradford (*23*) with bovine serum albumin as standard.

Statistical Analysis. Data are presented as mean ± SEM of the indicated number of determinations (*n*).

RESULTS

Effect of GDP on the Catalytically Active, Assembled Oxidase Complex. In previous studies (*11*−*13*) we showed that Rac GTPase activating proteins (Rac-GAPs) effectively counteract the activation phase of the NADPH oxidase, but the information available on their possible interaction with the assembled enzyme complex is scarce. In our earlier experiments, using crude membrane and cytosol fractions of porcine neutrophils, we could decrease oxidase activity

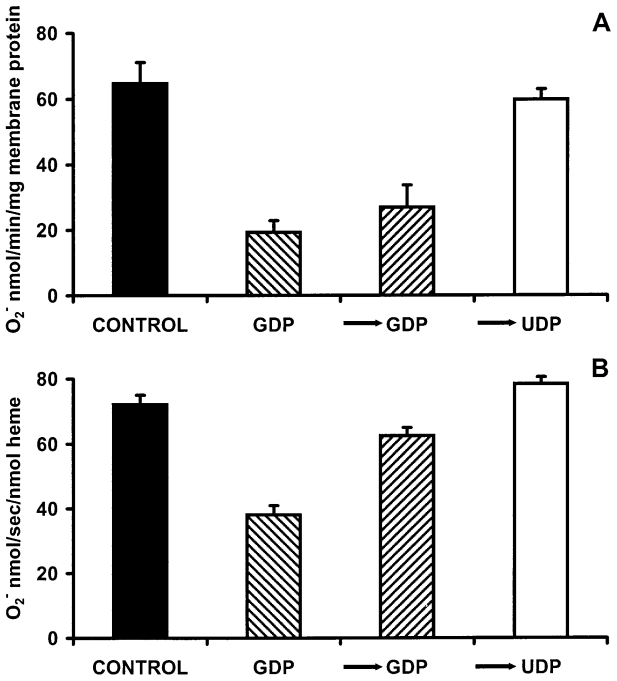

FIGURE 1: Effect of GDP on the activation and catalytic activity of NADPH oxidase. Control represents the rate of $O_2^{\bullet-}$ production obtained in the semirecombinant system in the presence of 10 μM GTP (A) or in the fully purified system with RacGTP (B). GDP was added at a concentration of 2 mM either at the beginning of the activation phase or after the completion of enzyme activation at 90 min (A) or 10 min (B) (→). Where present, UDP was applied in a concentration of 2 mM. Mean and SEM of 10 measurements are shown.

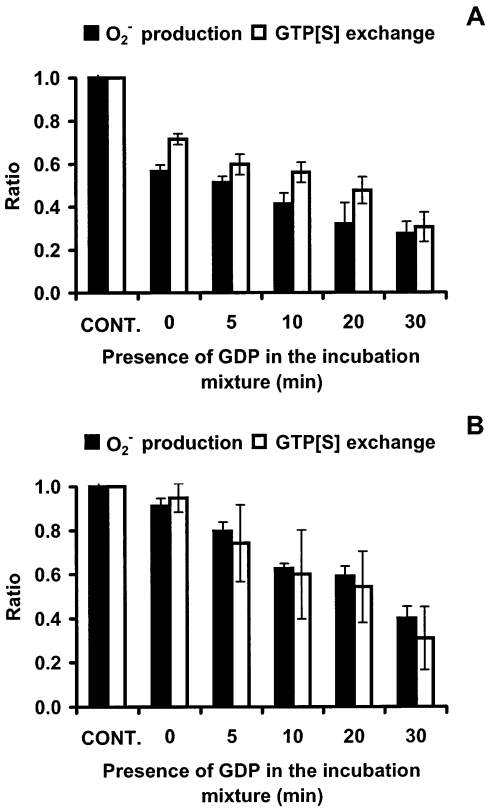

FIGURE 2: Kinetics of the effect of GDP on $O_2^{\bullet-}$ production and [^{35}S]GTPγS binding. Activation of the enzyme was achieved in the presence of 10 μM GTP or 1.25 μM [^{35}S]GTPγS in 90 min (in the semirecombinant system, shown in part A) or with RacGTP/Rac[^{35}S]GTPγS in 10 min (in the fully purified system, shown in part B). GDP (2 mM) was added at the end of the activation phase, and after the indicated delay $O_2^{\bullet-}$ production has been initiated by addition of NADPH (black columns) or nucleotide binding was determined by filtration (empty columns). Mean and SEM of five ($O_2^{\bullet-}$ production) or four ([^{35}S]GTPγS exchange) measurements are shown.

by adding GDP to the assembled enzyme (*12*). However, the crude preparations contained several GTP-binding proteins so that the site of action of GDP could not be unequivocally determined. Therefore, in the present study we first investigated whether replacement of GTP by GDP has any effect on the catalytically active oxidase complex in more purified versions of the in vitro activation system. In the experiments summarized in Figure 1, complete activation of the enzyme was achieved in the semirecombinant and in the fully purified systems in 90 and 10 min, respectively (data not shown). Thereafter, GDP was added simultaneously with NADPH. In the semirecombinant system (Figure 1A) GDP significantly reduced $O_2^{\bullet-}$ production also when it was added after the completion of the activation phase. In contrast to our findings in the semirecombinant and crude activation systems, GDP added together with NADPH had only weak effect on $O_2^{\bullet-}$ production in the fully purified system (Figure 1B).

As our membrane preparation contains Rac exchange factor activity (P. Moskwa and E. Ligeti, unpublished observation), the difference between the crude and semirecombinant versus the fully recombinant system may be due to different kinetics of GDP exchange. Therefore, in the next experiment we followed the effect of GDP in time by varying the interval between addition of GDP and initiation of $O_2^{\bullet-}$ production by NADPH. As shown in Figure 2 (black columns), in the semirecombinant system a reduction of $O_2^{\bullet-}$ production to 60% of the control value was achieved at the first measurable time point (Figure 2A) whereas in the fully recombinant system only after 10 min (Figure 2B). The time required for 50% inhibition was <5 min and >20 min in the two versions of the cell-free activation system. After 30 min in both systems approximately 35% of the control value was detectable. Similar results were obtained when GTP or GTPγS was used for activation of the enzyme. As control, we applied UDP or GTP, but none of these nucleotides had an inhibitory effect.

In parallel experiments activation of the enzyme was carried out in the presence of [^{35}S]GTPγS, and decrease of protein-bound radioactivity was followed after addition of GDP (Figure 2, empty columns). Good correlation was found between decline of enzyme activity and nucleotide exchange in both types of the cell-free system.

Thus, independently of the type of the cell-free system applied, replacement of the activating guanine nucleotide by GDP was able to reduce electron transfer via the catalytically active enzyme complex. This finding indicates that, in addition to its role in the activation phase, the participation of the active, GTP-bound form of Rac in the complex is required also for sustained enzyme activity.

Effect of the Oxidase Subunits on the GTPase Activity of Rac. In living cells the concentration of GTP exceeds that of GDP; thus nucleotide exchange brings G-proteins in their active form. However, acceleration of GTP hydrolysis can rapidly transform the active form of the GTP-binding protein into its inactive form. In the case of several heterotrimeric

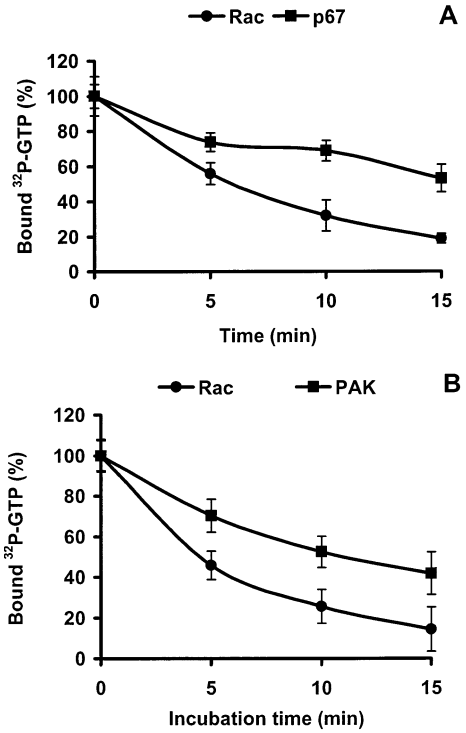

FIGURE 3: Effect of p67^{phox} (A) and the Rac-binding domain of PAK (B) on GTPase activity of Rac. Decrease of protein-bound [γ-^{32}P]GTP in time is shown in (A) in the presence of 0.1 μg of prenylated Rac only (●) or Rac plus 2.0 μg of p67^{phox} (■) and in (B) in the presence of Rac only (●) or Rac plus 0.6 μg of PAK-RBD (■). Mean and SEM of six (A) or three (B) measurements are shown.

G-proteins interaction with the target protein induces drastic increase in the rate of GTP hydrolysis, promoting thereby the termination of the biological process regulated by the G-protein (*24*, *25*). Acceleration of GTP hydrolysis by Rac assembled in the oxidase complex could present a mechanism leading to termination of $O_2^{\bullet-}$ production.

Following this rationale we investigated the effect of soluble oxidase components on GTPase activity of Rac. As shown in Figure 3A, addition of p67^{phox} clearly inhibited hydrolysis of radiolabeled GTP by Rac: the half-time of GTP hydrolysis was increased from 5 to 15 min. In contrast, addition of p47^{phox} to Rac had no inhibitory effect, and the presence of PA and OAG in similar concentrations as used for oxidase activation had no effect either. Simultaneous addition of p47^{phox} and p67^{phox} had the same effect as p67^{phox} alone (Table 1.). These data are in accordance with previous findings indicating that only p67^{phox} and not p47^{phox} was a Rac-interacting protein (*26*, *27*). Identical data were obtained when prenylated or nonprenylated Rac was tested (data not shown).

GTP hydrolysis by Rac was shown earlier to be inhibited by the Rac target protein p21-activated kinase (PAK) (*28*). Therefore, we compared the inhibitory effect of p67^{phox} to that of the Rac-binding domain (RBD) of PAK. According to the data of Figure 3, the inhibitory action of the two proteins was comparable.

The inhibitory effect of p67^{phox} was specific for Rac as no change in the hydrolysis rate could be observed either with Rho or with Cdc42 (Table 1). In the Rac mutant A27K + G30S two critical amino acids of the domain interacting with p67^{phox} have been replaced by the corresponding amino acids of Cdc42. p67^{phox} had no inhibitory effect on the hydrolysis rate of this mutant (Table 1).

Table 1: Effect of Soluble Oxidase Subunits on the GTP Hydrolytic Activity of Various Small GTPases of the Rho Subfamily[a]

interacting proteins	half-life of protein-bound [γ-^{32}P]GTP (min)
Rac	6.5 ± 0.41
+p47^{phox}	6.5 ± 0.35
+p67^{phox}	17.0 ±1.19
+p47^{phox} + p67^{phox}	17.5 ± 1.42
+p47^{phox} + PA/OAG	7.25 ± 0.26
Rho	17.5 ± 0.34
+p67^{phox}	18.0 ± 0.51
Cdc42	4.08 ± 0.08
+p67^{phox}	4.23 ± 0.04
Rac A27K + G30S	6.0 ± 0.66
+p67^{phox}	4.5 ± 0.26

[a] Half-life of protein-bound [γ-^{32}P]GTP is expressed in minutes. Interaction of the small GTPase (0.1 μg) with 1.4 μg of p47^{phox} and/or 2.0 μg of p67^{phox} was followed for 15 min as described in Experimental Procedures. Where indicated, 375 μM PA and 250 μM OAG were applied. Mean and SEM of four to six measurements are shown.

Thus, the soluble subunit p67^{phox} of the oxidase complex has the tendency to prolong the prevalence of Rac in the active, GTP-bound form, sustaining thereby continuous electron transfer.

Effect of Rac-Interacting Proteins on the Activity of the Assembled Oxidase. In these experiments we tested the ability of various Rac-interacting proteins to influence $O_2^{\bullet-}$ production by the activated oxidase complex. p50Rho-GAP is the dominant protein with Rac-GAP activity present both in the membrane and in cytosolic fractions of human PMN (*13*). Rho-GDI was shown to be present in the cytosol and to bind tightly to Rac (*29*). In addition to these regulatory proteins we investigated the Rac-binding domain (RBD) of the target protein PAK which has been shown to interfere with the activation of the oxidase (*30*). In Figure 4 the effect of the three Rac-interacting proteins is compared under two different conditions: added at the beginning of the preincubation phase or to the activated oxidase, simultaneously with NADPH. The GAP domain of p50Rho-GAP, Rho-GDI, and PAK-RBD intensively inhibited enzyme activation when they were present in the preincubation phase, indicating that soluble proteins had free access both to soluble (in the fully recombinant system) and to membrane bound (in the semirecombinant system) Rac. In contrast, none of the three proteins had any significant effect on $O_2^{\bullet-}$ production when they were added at the end of the activation phase, i.e., when the oxidase complex has been assembled. Prolongation of the interval between addition of regulatory proteins and NADPH up to 30 min had no influence on $O_2^{\bullet-}$ production at all. Identical results were obtained in all tested forms of the cell-free system. Apparently, in the assembled complex Rac is in an embedded position so that Rac-interacting proteins have no longer access to the small GTPase. A similar conclusion has been reached earlier using p190Rho-GAP and a crude form of the in vitro activation system (*31*).

The membrane fraction of human PMNs contains several Rac-GAP proteins which counteract the activation of the oxidase (*13*). Next we investigated whether this membrane-associated Rac-GAP activity can be involved in the termination of $O_2^{\bullet-}$ production by the assembled oxidase. We made

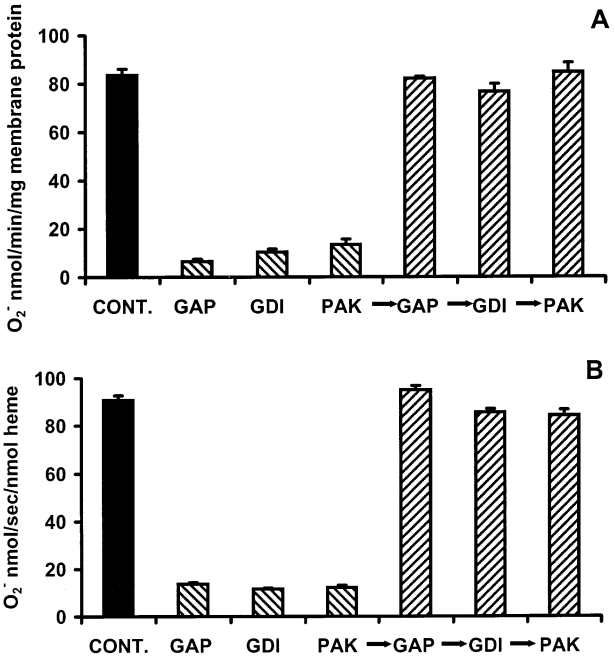

FIGURE 4: Effect of Rac-interacting proteins on the activation and catalytic activity of NADPH oxidase. Activation has been carried out in the semirecombinant system in the presence of 10 μM GTP (A) or in the fully purified system with RacGTP (B). The GAP domain of p50Rho-GAP (10 μg), Rho-GDI (10 μg), and PAK-RBD (10 μg) has been added at the beginning of the activation phase or to the activated enzyme (→). Mean and SEM of six measurements are shown.

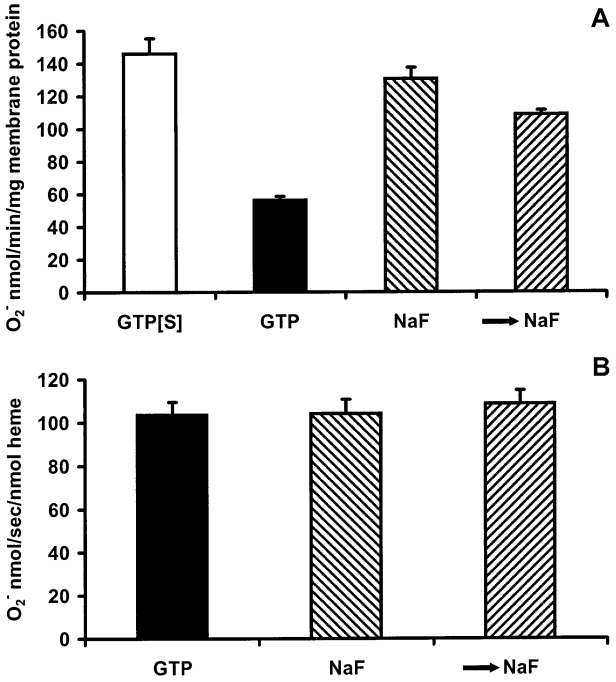

FIGURE 5: Effect of membrane-localized GAPs on the activation and catalytic function of NADPH oxidase. Activation of the enzyme was carried out in the presence of 1.25 μM GTPγS (empty column) or 10 μM GTP (all other columns) in the semirecombinant system (A) or with RacGTP in the fully purified system (B). NaF (30 mM) was added either at the beginning of the activation phase or after completion of the activation of the enzyme (→). Mean and SEM of three measurements are shown.

use of the previously characterized inhibitory action of fluoride ions on various Rac-GAP proteins (*11*, *14*). The effect of GAP is indicated by the difference in $O_2^{\bullet-}$ production achieved in the presence of GTPγS or GTP (Figure 5A). In the experiment presented in Figure 5, we compared the action of fluoride on the activation phase with its effect on the active oxidase complex by adding fluoride either at the beginning or at the end of the activation phase. In the semirecombinant system fluoride clearly augmented $O_2^{\bullet-}$ production also when it was added at the end of the activation phase (Figure 5A). Whereas GTP alone was able to evoke only approximately 35% of the maximal rate of $O_2^{\bullet-}$ production, addition of fluoride together with NADPH nearly doubled this value. When fluoride was present from the beginning of the activation phase, approximately 85% of the maximal rate could be achieved. A similar result was obtained in the crude system, too (data not shown). However, in the fully recombinant system, which contained no Rac-GAP activity, fluoride was without any effect (Figure 5B). This observation substantiates that under our experimental conditions the membrane-associated GAP activity was the target of the fluoride action.

Thus, in contrast to the externally added regulatory proteins, membrane-associated GAP(s) was (were) able to interact also with the assembled oxidase complex and thereby decrease the catalytic activity of NADPH oxidase and accelerate the termination of $O_2^{\bullet-}$ production.

DISCUSSION

The results presented in Figures 1 and 2 clearly indicate that GDP is able to inhibit electron transfer via the assembled, catalytically active oxidase complex. As in the fully purified system Rac is the only GTP-binding protein; the effect of GDP clearly represents the exchange of previously bound GTP or GTPγS for GDP added in excess. In the presence of membrane (in the crude and semirecombinant systems) GDP has an initial rapid effect. An obvious explanation is provided by accelerated nucleotide exchange due to exchange factor(s) associated to the membrane fraction. The possibility of any additional effect of GDP on other GTP-binding protein(s) present in the membrane fraction has to be investigated in future experiments.

The observation that GDP is able to terminate the catalytic activity of the NADPH oxidase indicates that Rac in its active, GTP-bound form is definitely required for sustained enzyme activity. In recent studies it has been suggested that $p67^{phox}$ by itself is able to bring about the critical conformational change on the cytochrome b_{558} and to initiate the electron flow through the membrane component (*32*−*34*). RacGTP and $p47^{phox}$ serve as important adaptor proteins, increasing the affinity of soluble subunits for the membrane component and allowing their correct positioning in the complex (*30*, *33*, *35*). The affinity of RacGTP for $p67^{phox}$ largely exceeds that of RacGDP (*26*, *27*). Thus, hydrolysis of GTP on Rac results in a significant decrease of the affinity of the small GTPase for $p67^{phox}$. The loosening of the interaction between these two proteins could slightly alter the position of $p67^{phox}$, moving it to a position less favorable for electron transfer. Recent experiments showed that certain chimera proteins in which various fragments of the soluble subunits (Rac and $p67^{phox}$ or $p67^{phox}$ and $p47^{phox}$) are covalently linked are able to increase both the rate of $O_2^{\bullet-}$ production and the lifetime of the active enzyme (*36*, *37*). These findings support the view that stabilizing or destabiliz-

ing the interaction between the individual subunits represents a decisive factor in positioning the activation domain of $p67^{phox}$ and thus in determination of the rate of electron flow through the complex.

Our data indicate that GTP hydrolysis by Rac is differentially regulated during the activation phase and in the assembled oxidase. In previous experiments we demonstrated that during the activation phase RacGTP was equally accessible for soluble and membrane-localized GAPs, and some experiments indicated that the two effects could be additive (*13*). In contrast, in the assembled oxidase RacGTP proved not to be accessible for soluble regulatory factors (Figure 4) whereas membrane-associated GAP(s) was (were) still able to downregulate the enzyme activity (Figure 5). Thus, GAP(s) has (have) to be localized in the vicinity of the active oxidase complex in the plasma (and intracellular) membrane. Details of their molecular interaction have to be determined in future investigations.

A comparison of the crystal structure of the Rac/$p67^{phox}$ and the Rho/RhoGAP complexes revealed that different amino acids are involved in the interaction of the small GTPase with the two partners; thus the formation of a ternary complex of Rac/$p67^{phox}$/GAP seems to be possible (*38*). Complex formation between Rho-GDI-bound Rac and two lipid kinases has been shown both in vitro and in vivo (*39*). Therefore, in the assembled complex the GTPase activity of Rac is probably influenced in opposing directions: interaction with $p67^{phox}$ prolongs whereas the action of membrane-associated GAP(s) decreases the lifetime of the active, GTP-bound form of Rac. Modification of $p67^{phox}$ and/or GAP could result in alteration of the Rac GTPase activity. $p67^{phox}$ was shown to become phosphorylated (*40*); however, the functional consequence of this modification has not been clarified. Selective modification of some GAPs has been reported earlier. Phosphorylation of p120Ras-GAP by p60-Src decreases its activity whereas phosphorylation by Lck is without any effect (*41*). ASAP1, a GAP acting on ARF proteins, is specifically activated by phosphatidylinositol 4,5-bisphosphate (*42*). However, presently no clear biochemical data are available on the functional alterations due to covalent or noncovalent modification of any Rac-GAP.

In the membrane fraction of PMN p50Rho-GAP has been identified as the major GAP affecting Rac whereas Bcr and p190Rho-GAP are localized mainly in the cytosol. All three proteins offer several potential sites of modification: p50Rho-GAP has two potential tyrosine phosphorylation sites, a polyproline region and an N-terminal Sec14p-like domain that is homologous to the phosphoinositide- (PI-) binding domain of PI-transfer proteins (*43*); Bcr possesses three potential tyrosine phosphorylation sites and a Ca^{2+}-dependent phospholipid binding site, whereas p190Rho-GAP has six potential tyrosine phosphorylation sites, an ATP- or GTP-binding site, and a myristoylation site. In vivo a functional role has been suggested for tyrosine phosphorylation (*44*) and ATP/GTP binding of p190Rho-GAP (*45*). Rac-GAPs could thus be a target of different modifications allowing selective alteration of the activation or deactivation of the NADPH oxidase, reflected by variations in the intensity and duration of $O_2^{\bullet-}$ production, respectively. In this way the abundance of GAPs affecting Rac and their apparently overlapping function could serve the fine-tuning of an essential physiological function.

Taken together, we propose that participation of RacGTP in the NADPH oxidase complex is required for sustained enzymatic activity and membrane-localized GAP(s) could be regarded as attached regulatory proteins of the oxidase which reduce both the intensity and the duration of $O_2^{\bullet-}$ production.

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