The Guanine Nucleotide Exchange Factor Trio Activates the Phagocyte NADPH Oxidase in the Absence of GDP to GTP Exchange on Rac

"THE EMPEROR'S NEW CLOTHES"**

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Natalia Sigal‡§, Yara Gorzalczany‡§, Rive Sarfstein‡§, Carolyn Weinbaum‡, Yi Zheng‡, and Edgar Pick‡**

From the Julius Friedrich Cohnheim-Minerva Center for Phagocyte Research and the Ela Kodesz Institute of Host Defense against Infectious Diseases, Sackler School of Medicine, Tel Aviv University, Tel Aviv 69978, Israel, the Julius Friedrich Cohnheim-Minerva Center for Phagocyte Research, Children's Hospital Research Foundation, Cincinnati, Ohio 45229, and the Department of Pharmacology and Cancer Biology, Duke University Medical Center, Durham, North Carolina 27710

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The superoxide-generating NADPH oxidase complex of phagocytes consists of a membrane-associated flavocytochrome b$_{559}$ and four cytosolic components as follows: p47$^{	ext{phox}}$, p67$^{	ext{phox}}$, p40$^{	ext{phox}}$, and the small GTPase Rac (1 or 2). Activation of the oxidase is the result of assembly of the cytosolic components with flavocytochrome b$_{559}$ and can be mimicked in vitro by mixtures of membrane and cytosolic components exposed to an anionic amphiphile, serving as activator. We reported that prenylation of Rac1 endows it with the ability to support oxidase activation in conjunction with p67$^{	ext{phox}}$ but in the absence of amphiphile and p47$^{	ext{phox}}$. We now show the following 6 points. 1) The Rac guanine nucleotide exchange factor Trio markedly potentiates oxidase activation by prenylated Rac1-GDP. 2) This occurs in the absence of exogenous GTP or any other source of GTP generation, demonstrating that the effect of Trio does not involve GDP to GTP exchange on Rac. 3) Trio does not potentiate oxidase activation by prenylated Rac1-GTP, by nonprenylated Rac1-GDP in the presence or absence of amphiphile, and by a prenylated [p67$^{	ext{phox}}$-Rac1] chimera in GDP-bound form. 4) Rac1 mutants defective in the ability to bind Trio or to respond to Trio by nucleotide exchange fail to respond to Trio by enhanced oxidase activation. 5) A Trio mutant with conserved Rac1-binding ability but lacking nucleotide exchange activity fails to enhance oxidase activation. 6) The effect of Trio is mimicked by displacement of Mg$^{2+}$ from Rac1-GDP. These results reveal the existence of a novel mechanism of Rac activation by a guanine nucleotide exchange factor and suggest that the induction by Trio of a conformational change in Rac1, in the absence of nucleotide exchange, is sufficient for enhancing its effector function.

Phagocytes utilize oxygen radicals for the killing of engulfed microorganisms (reviewed in Ref. 1). Oxygen radicals also serve as signal transduction messengers in a variety of nonphagocytic cells (reviewed in Ref. 2). In phagocytes, the primordial oxygen radical, superoxide (O$_{2}^{-}$) is produced by the NADPH-driven reduction of molecular oxygen catalyzed by a membrane-localized flavocytochrome (cytochrome b$_{559}$), comprising two subunits, gp91$^{	ext{phox}}$ and p22$^{	ext{phox}}$ (reviewed in Ref. 3). All redox stations involved in electron flow from NADPH to O$_{2}$ are found on gp91$^{	ext{phox}}$, and it is assumed that the redox cascade is initiated by a conformational change induced in gp91$^{	ext{phox}}$ by interaction with one or more cytosolic proteins. These are p47$^{	ext{phox}}$, p67$^{	ext{phox}}$, p40$^{	ext{phox}}$, and the small GTPase Rac (Rac1 or -2). Upon stimulation of the phagocyte, they translocate to the membrane, resulting in the assembly of what is known as the NADPH oxidase complex (referred to as "oxidase") (reviewed in Ref. 4). The identity of the cytosolic component(s) responsible for causing the conformational change in gp91$^{	ext{phox}}$ is controversial. The principal candidate is p67$^{	ext{phox}}$, based on the identification of an "activation domain" in p67$^{	ext{phox}}$, consisting of residues 199–210 (5), and on direct evidence of binding of p67$^{	ext{phox}}$ to gp91$^{	ext{phox}}$, an interaction enhanced by Rac1 (6). This hypothesis also implies that p47$^{	ext{phox}}$ and Rac serve either as "carriers" for p67$^{	ext{phox}}$ from the cytosol to the membrane or, following their own translocation, as membrane "anchors" for the correct positioning of p67$^{	ext{phox}}$ in the assembled complex.

Oxidase assembly can be elicited in vitro in a cell-free system consisting of phagocyte membranes and the cytosolic components p47$^{	ext{phox}}$, p67$^{	ext{phox}}$, and Rac, exposed to an anionic amphiphile (7, 8). The role of the amphiphile is to induce a conformational change in p47$^{	ext{phox}}$, resulting in its binding to p22$^{	ext{phox}}$ and the passive translocation of p67$^{	ext{phox}}$, by virtue of its affinity for p47$^{	ext{phox}}$, to the vicinity of gp91$^{	ext{phox}}$. The in vivo equivalent of amphiphile action is the phosphorylation of critical serines in p67$^{	ext{phox}}$ in the assembled complex.

Under certain conditions, oxidase activation in vitro is also possible in the absence of p47$^{	ext{phox}}$. Originally, this was achieved in an amphiphile-dependent cell-free system by having recom-

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§ These authors contributed equally to this work.

** To whom correspondence and should be addressed: Dept. of Human Microbiology, Sackler School of Medicine, Tel Aviv University, Tel Aviv 69978, Israel. Tel.: 972-3-640-7872, Fax: 972-3-642-9119; E-mail: epick@post.tau.ac.il.
binant p67^{phox} and Rac present at micromolar concentrations (9, 10). A characteristic of earlier versions of the amphiphile-activated cell-free system is the use of bacterially expressed recombinant Rac, which did not undergo C-terminal geranylgeranlylation (prenylation). We recently described oxidase activation in vitro in a system consisting of phagocyte membranes, p67^{phox} and prenylated Rac1, in the absence of amphiphile and p47^{phox} (11). Amphiphile- and p47^{phox}-independent oxidase activation could also be achieved in mixtures of membrane and a chimeric [p67^{phox}-Rac1] construct predicted to have the C-terminal fragment of the Rac1 segment (12). These findings support the existence of a second “Rac-initiated” pathway of oxidase activation (13–16), and evidence is accumulating in support of a guanine nucleotide exchange factor (GEF), responsible for GDP to GTP exchange on Rac, being the key link in this pathway. In the present report we show that a minimal functional module of Trio, a Rac-specific GEF (17), markedly potentiates oxidase activation by prenylated Rac1 in vitro in the absence of an activator and of p47^{phox}. Surprisingly, the effect of Trio on Rac does not involve GDP to GTP exchange.

EXPERIMENTAL PROCEDURES

**Chemicals**—The following nucleotides were purchased from Sigma (product numbers appear in parentheses following the description of the degree of purity): GTP (lithium salt, 97%, G5884); GDP (sodium salt, type 1, 98%, G7127); GMP (disodium salt, 99%, G6377); ATP (disodium salt, 99%, A7699); ADP (sodium salt, 95–99%, A2754); and AMP (sodium salt, 99%, A1752). The hydrolysis-resistant GTP analog, GTP\(\cdot\)S (lithium salt, 97%, 100 mM solution), was purchased from Roche Molecular Biochemicals. The fluorescent GTP analog mant-GTP was obtained from Molecular Probes, Eugene, OR. Lithium dodecyl sulfate (LDS, >99% pure) was purchased from Merck. Potassium dihydrogen orthophosphate and potassium chloride, both 99.5% pure, were used for preparing the buffers used in the separation of nucleotides by high pressure liquid chromatography (HPLC) were obtained from BDH, Poole, United Kingdom (Aristar grade).

**Preparation of Phagocyte Membrane Vesicles**—Phagocyte membranes were prepared from guinea pig peritoneal macrophages, as described (7). The membranes were solubilized by 40 mM n-octyl-\(\beta\)-D-glucopyranoside, and membrane vesicles were prepared by extensive dialysis against detergent-free buffer, as described (18).

**Preparation of Recombinant Proteins**—p47^{phox} and p67^{phox} were prepared in baculovirus-infected SF9 cells, as described before (19). p67^{phox}, truncated at residue 212 (p67^{phox}1–212), was produced in Escherichia coli, as described before (11). Nonprenylated Rac1 and Cdc42Hs were expressed in E. coli, as described earlier (19). Rac1 point mutants were generated and expressed in E. coli, as recently described (20). The N-terminal fragment of Trio (residues 1225–1537) and the N1406A/D1407A mutant of this protein were expressed in E. coli as N-terminal His_{6}-tagged fusion proteins and purified by metal affinity chromatography, as described before (21), except for the fact that a Co2+-based resin (TalonTM; BD Biosciences) was used instead of Ni^{2+}-agarose. Chimera [p67^{phox}-1–212]-Rac1 (1–192), referred to as chimera 3 in Ref. 22, was expressed in E. coli and purified as described (22). Recombinant geranylgeranyltransferase type I was produced in baculovirus-infected SF9 cells (23).

**Identification of Nucleotides Present on Recombinant Rac1**—Bound nucleotides were liberated from recombinant Rac1 preparations as described before (24). They were identified and quantified by HPLC on a Partisil 10 SAX anion exchange column (250×4.6 mm) (Whatman), as described in Ref. 25. Briefly, nucleotide standards or nucleotides liberated from recombinant or other proteins were injected into the column in a volume of 0.5 ml. This was followed by delivery of 0.007 M KH_{2}PO_{4}, pH 4.0, at 1.5 ml/min for 15 min, followed by a linear gradient from 0.007 M KH_{2}PO_{4}, pH 4.0, to 0.25 M KH_{2}PO_{4} and 0.5 M KCl, pH 4.5, at 1.5 ml/min over a period of 45 min, and continuing the high molarity buffer for an additional 40 min at the same flow rate. The column eluate was monitored by passage through a diode array detector, set at 220–450 nm and monitoring the absorbance at 280 nm, as described elsewhere (24). The concentrations of nucleotides were determined by peak integration (based on absorbance at 254 nm) and related to nmol amounts of the following nucleotide standards: AMP, GMP, ADP, GDP, ATP, and GTP.

**Enzymatic Prenylation of GTPases**—Nonprenylated Rac1, Cdc42Hs, and [p67^{phox}-Rac1] chimera 3 were prenylated in vitro by recombinant geranylgeranyltransferase type I, as recently described (12).

**Nucleotide Exchange**—Prenylated Rac1 was subjected to nucleotide exchange to GTP\(\cdot\)S, as described before (11).

**Cell-free NADPH Oxidase Assay**—Activation of oxidase in vitro supported by prenylated Rac1 was assessed by measuring NADPH-dependent \(O_{2}\) production in a semi-recombinant cell-free system, in the absence of an amphiphilic activator and of p47^{phox}, essentially as described earlier (11, 26). The components of the assay were added to 96-well microplates, in the following order: TrioN or buffer, prenylated Rac1, a mixture of solubilized membrane and p67^{phox}, and assay buffer in a total volume of 200\(\mu\)l. The mixture was incubated for 90 s at 25° C, and 240\(\mu\)M NADPH was added to initiate \(O_{2}\) production. The concentration of membrane was constant throughout all the experiments and corresponded to 5 nm cytochrome b_{558}, heme; the concentrations of TrioN, prenylated Rac1, and p67^{phox} were varied and are indicated under “Results.” Most experiments were performed in the assay buffer used by this laboratory before (18), which contains 1 mM MgCl_{2}. In some experiments, assay buffers containing 0.4 mM and 4 mM free Mg^{2+} were used; these were prepared by adding EDTA and MgCl_{2} to the basic assay buffer in amounts calculated as described in Ref. 27.

**Guanine Nucleotide Exchange Assay**—The ability of TrioN to perform guanine nucleotide exchange on Rac1 and Rac1 mutants was assayed by the increase in fluorescence (excitation = 361 nm; emission = 440 nm), consequent to the uptake of the fluorescent GTP analog mant-GTP by Rac1, as described in Ref. 28. Briefly, 1 μM Rac1 or Rac1 mutants were incubated with 0.375 μM mant-GTP at 20° C in a magnetically stirred thermostated cuvette in a model FP-750 spectrofluorometer (Jasco, Easton, MD) in 20 mM Tris-HCl buffer, pH 7.4, also containing 150 mM NaCl and 4 mM MgCl_{2}. This was followed by the addition of 0.5 μM TrioN, and the kinetics of change in fluorescence was recorded over time.

**RESULTS**

**Nucleotide Content of Native Rac1**—It is generally assumed that recombinant small GTPases produced in E. coli are in the GDP-bound form. We put this hypothesis to a direct test by determining the nature and amount of nucleotide bound to native recombinant Rac1. As illustrated in Fig. 1B, the only nucleotide identified on Rac1 was GDP, and it was present at a ratio of 1.046 ± 0.064 mol GDP/mol Rac1 (mean ± S.E. of five experiments). We have shown before that the procedure employed to liberate the nucleotides bound to Rac did not lead to degradation of GTP to GDP (24). Further proof for the reliability of the procedure was offered by the finding that Rac1 mutant Q61L, known to hydrolyze GTP poorly (29), was found indeed to contain 0.8 mol of nucleotide/mol of Rac1, of which 83.5% was GTP and 16.5%, GDP (mean of two determinations) (Fig. 1C). The fact that a major part of mutant Q61L shall remain in the GTP-bound state throughout protein purification was predicted by Xu et al. (30) and is now proven unequivocally. All other recombinant Rac1 mutants, used in the experiments to be described, were found to contain exclusively bound GDP (results not shown).

**Effect of Trio on Oxidase Activation by Prenylated Rac1-GDP**—We found earlier that prenylated Rac1 exchanged to either GTP\(\cdot\)S or GDP\(\cdot\)S supported oxidase activation in the amphiphile- and p47^{phox}-independent cell-free system with equal potency (11). In those experiments, Rac1 was subject to nucleotide exchange at low Mg^{2+} concentration achieved by metal chelation by EDTA. The original purpose of the experiments, having led to the findings described in this report, was to study the effect of “enzymatic” as opposed to “chemical” nucleotide exchange on prenylated Rac1 to GTP on its ability to support oxidase activation. We first examined the ability of prenylated native Rac1 (found to contain only GDP and, therefore, to be referred to as Rac1-GDP) to activate the oxidase in the amphiphile- and p47^{phox}-independent cell-free system. As seen in Fig. 2A, only a modest, dose-dependent, activation was found.

We next assessed the effect of inducing nucleotide exchange on Rac1 to GTP, in a manner closer to the physiological mechanism, by using a GEF. For this purpose, we chose Trio, a GEF...
containing two distinct guanine nucleotide exchange domains, specific for Rac1 and Rho (17, 31). A recombinant N-terminal segment of Trio (residues 1225–1537), including the Rac1-specific N-terminal DBL homology (DH) and pleckstrin homology (PH) domains, to be referred to as TrioN, was found to stimulate GDP to GTP exchange on Rac1 (21). The ability of TrioN to modulate oxidase activation by prenylated Rac1-GDP was assayed in the presence of exogenous GTPyS and, as a control, in its absence. Surprisingly, TrioN exhibited a pronounced potentiating effect on oxidase activation by prenylated Rac1-GDP in the absence of added GTPyS (Fig. 2A). Oxidase activation by prenylated Rac1-GDP, in the presence of TrioN and in the absence of exogenous GTPyS, reached a $V_{\text{max}}$ of $69.5 \pm 2.9$ mol O$_2$/mol cytochrome b$_{559}$ heme, compared with $31.3 \pm 1.4$, in the absence of TrioN; EC$_{50}$ values were $5.1 \pm 2.1$ nM Rac1, in the presence of TrioN, and $69.8 \pm 12.1$, in its absence (means $\pm$ S.E. of three experiments for each group).

Paradoxically, in the presence of exogenous GTPyS (5 $\mu$M), TrioN did not potentiate oxidase activation by prenylated Rac1-GDP (Fig. 2A). Oxidase activation by prenylated Rac1-GDP and TrioN, in the presence of added GTPyS, was not significantly different from that in the absence of TrioN and GTPyS ($V_{\text{max}} = 37.7 \pm 2.4$ mol O$_2$/mol cytochrome b$_{559}$ heme; EC$_{50} = 168.7 \pm 28.3$ nM Rac1, representing means $\pm$ S.E. of three experiments).
experiments). Dose-response studies indicated that inhibition of TrioN-enhanced oxidation by GTPγS was evident at 0.5 mM GTPγS and was complete at 5 mM (results not shown).

The enhancing effect of TrioN was seen only in the presence of both prenylated Rac1 and p67phox (Table I) and was related to the relative concentrations of Rac1 and p67phox present in the reaction. Thus, significantly higher activities were obtained when p67phox was in excess over Rac1 than when the two components were present in equimolar amounts (results not shown). TrioN was inactive when heat-denatured and had no effect on prenylated Cdc42Hs-GDP, a Rho subfamily GTPase lacking oxidase-activating ability (Table I). p67phox effect on prenylated Cdc42Hs-GDP, a Rho subfamily GTPase shown). TrioN was inactive when heat-denatured and had no presence of non-truncated p67phox in the absence of GTP (Table I). p67phox could be replaced by p67phox truncated at residue 212, although both the basal and the TrioN-enhanced activity were lower than in the presence of non-truncated p67phox (Fig. 2B). This suggests that the tetra-tripeptide repeat region, involved in binding of p67phox to Rac (32), and the activation domain (5) play important but not exclusive roles in TrioN-dependent activation.

TrioN exhibited no enhancing effect on oxidation activation by prenylated Rac1, exchanged to GTPγS and freed of unbound nucleotide on a desalting column (Fig. 2C). It is of interest that although the level of oxidation activation achieved with prenylated Rac1-GTPγS was higher than that measured with native Rac1 (Rac1-GDP), it was considerably lower than that found with Rac1-GDP combined with TrioN, in the absence of free GTPγS. This suggests that the conformational change(s) occurring in Rac1 as a consequence of nucleotide exchange to GTPγS might, at least in part, be different from those taking place under the influence of TrioN, although they both lead to an enhancement in the oxidation activating capacity of Rac1.

TrioN had only a minor enhancing effect on oxidation activation by nonprenylated Rac1-GDP in the canonical amphiphile- and p47phox-dependent system and did not convey an activating potential to nonprenylated Rac1-GDP, known to be inactive in the absence of amphiphile and p47phox (both situations are shown in Fig. 2D). This was not due to a requirement for prenylation for interaction of Rac1 with TrioN to take place because nonprenylated Rac1 was found to bind TrioN (20).

We recently showed that a chimera consisting of the N-terminal 212 residues of p67phox and full-length Rac1, when prenylated and in the GDP-bound form, was capable of moderate oxidation activation in the absence of an amphiphile and p47phox (12). In marked contrast to its enhancing effect on a combination of prenylated Rac1-GDP and p67phox (1–212) (Fig. 2B), TrioN did not potentiate the activity of a [p67phox (1–212)-

TrioN (or TrioN mutant), 300 nM. Results represent means ± S.E. of three to seven experiments for each combination of NADPH oxidase components.

**Table I**

| Combination of NADPH oxidase components | NADPH oxidase activity |
|----------------------------------------|------------------------|
| Membrane                               | 0.31 ± 0.13            |
| Membrane + p67phox                      | 0.67 ± 0.17            |
| Membrane + prenylated Rac1              | 3.26 ± 0.38            |
| Membrane + TrioN                        | 0.90 ± 0.07            |
| Membrane + p67phox + prenylated Rac1    | 8.72 ± 1.24            |
| Membrane + p67phox + TrioN              | 2.45 ± 0.73            |
| Membrane + prenylated Rac1 + TrioN      | 1.99 ± 0.41            |
| Membrane + p67phox + prenylated Rac1 + TrioN | 58.08 ± 5.30 |
| Membrane + p67phox + prenylated Rac1 + TrioN + superoxide dismutase<sup>a</sup> | 6.49 ± 0.51 |
| Membrane + p67phox + prenylated Rac1 + denatured TrioN<sup>b</sup> | 8.10 ± 0.46 |
| Membrane + p67phox + prenylated Rac1 + TrioN mutant N1406A/D1407A | 7.35 ± 0.26 |
| Membrane + p67phox + prenylated Cdc42Hs + TrioN | 3.77 ± 0.12 |
| Membrane + p67phox + prenylated Rac1 + EDTA<sup>c</sup> | 62.59 ± 3.86 |

<sup>a</sup> 500 units/ml.

<sup>b</sup> TrioN was heated at 60 °C for 30 min.

<sup>c</sup> EDTA was added to bring the concentration of free Mg<sup>2+</sup> to 0.4 μM.
We examined the importance of these residues in the oxidase activation enhancing effect of TrioN by using the Rac1 mutants Y32A (switch I) and W56F, Q61L, and Y64A (switch II). Rac1 Y32A was shown to retain the ability to bind TrioN but to lose its ability to respond to TrioN by GDP dissociation. The switch II mutants were found to be unable to bind TrioN; of special interest is mutant W56F because Trp56 appears to be the critical residue determining the Rac specificity of GEFs, including Trio (20). Among switch II mutants, Rac1 Q61L stands out because it was found to be predominantly in the GTP-bound form (Fig. 1C), unlike all the other mutants that contained only GDP. It was also reported to have a higher affinity for a yet unidentified oxidase component, most likely p67phox (29). We also examined the responsiveness of the negative dominant mutant T17N, known to possess a markedly lower affinity for GTP (34) but an unimpaired ability to bind GEFs (reviewed in Ref. 35).

As a preliminary to examining their responsiveness to TrioN in the oxidase assay, we assessed the ability of the Rac1 mutants to respond to TrioN by nucleotide exchange from GDP to mant-GTP. As seen in Fig. 3, wild type Rac1-GDP reacted to TrioN by a vigorous change in the slope of mant-GTP uptake. All Rac1 mutants, in GDP-bound form, were unresponsive to TrioN, as evident in the lack of change in the slope of mant-GTP uptake, following the addition of TrioN (Fig. 3). It is of interest that mutants T17N and Q61L also evidenced the lowest spontaneous uptake of mant-GTP, in accordance with the reported impairment in GTP binding of mutant T17N (34) and the slow nucleotide exchange rate of mutant Q61L (30).

We next examined the effect of the mutations on the capacity of nonprenylated Rac1 to support oxidase activation in the cell-free system. Mutants T17N, Y32A, W56F, Q61L, and Y64A (all at a concentration of 300 nM) were assayed in an amphiphile-dependent cell-free system consisting of membrane (5 nM cytochrome b559 heme), p47phox (300 nM), p67phox (300 nM), nonprenylated native (unexchanged) wild type or mutant Rac1 (300 nM), and LiDS (130 μM). All Rac1 mutants, with the exception of T17N and Q61L, exhibited unchanged oxidase activating ability, in the range of 103–114% that of wild type Rac1. Rac1 T17N was only 41% as active as wild type Rac1, whereas Rac1 Q61L was more active (128%) than wild type Rac1 (results represent means of two experiments). Finally, the influence of mutations on the ability of the prenylated form of Rac1 to support oxidase activation in the amphiphile and p47phox-independent cell-free system was examined. To achieve a maximal effect, prenylated wild type Rac1 and mutants were exchanged to GTP and assayed in a system consisting of membrane (5 nM cytochrome b559 heme), p67phox (300 nM), and prenylated wild type or mutant Rac1-GTP (300 nM) in the absence of LiDS. The activating abilities of the mutants were 39 (Y32A), 52 (W56F), and 72% (Y64A) that of wild type Rac1 (results represent means of two experiments). Mutant Q61L was 3.75 times more active than wild type Rac1, in support of the hypothesis that it has a higher affinity for p67phox.

As seen in Fig. 4A and B, TrioN was incapable of enhancing oxidase activation supported by prenylated Rac1 mutants T17N and Y32A. Because both mutants were expected to bind TrioN normally but did not respond to TrioN by mant-GTP uptake, it appears that an event, subsequent to binding and related to the catalytic action of TrioN, is required for the enhancement of oxidase activation. TrioN had little or no oxidase activation potentiating effect on Rac1 mutants W56, Q61L, and Y64A, known to be unable to bind TrioN (a minor effect was evident on mutant Y64A) (Fig. 4C–E). It appears
that the basal oxidase activating ability of the prenylated mutants, especially T17N and Y32A, was also lower than that of wild type Rac1 (with the notable exception of Q61L), as already noted in the course of preliminary testing of the mutants in GTP-bound form at a single concentration. The reason for this is not obvious.

A special case is mutant Q61L, which was found to contain predominantly GTP. Its basal oxidase activating ability (Fig. 4D) was found to exceed significantly that of wild type Rac1 in both native (GDP-bound) and GTP-bound (exchanged to GTPS-bound) forms (Fig. 2, A and C), and TrioN exerted no further enhancing effect. Thus, $V_{\text{max}}$ was 58.1 ± 0.7 and 57.3 ± 1.4 mol O2/s/mol cytochrome b559 heme, in the absence and presence of TrioN, respectively, and the corresponding EC50 values were 36.1 ± 2.3 and 34.8 ± 4.3 nM Rac1 mutant Q61L (means ± S.E. of three experiments for each group).

Recently, a double mutant of TrioN was described, the mutations being located at the C terminus of the DH domain (N1406A/D1407A).2 This mutant binds normally to Rac1 but fails to stimulate nucleotide exchange. We tested the ability of TrioN mutant N1406A/D1407A to enhance oxidase activation by prenylated Rac1-GDP in the presence of p67phox and in the absence of amphiphile and p47phox. As seen in Table I, the TrioN mutant failed to enhance oxidase activation. This finding offers additional support to the conclusion derived from the results obtained with Rac1 mutants T17N and Y32A that enhancement of oxidase activation by prenylated Rac1 requires, in addition to Rac1-TrioN interaction, yet another step associated with the catalytic action of TrioN.

Effect of TrioN Is Mediated by Mg2⁺ Displacement—It was shown that nucleotide exchange by Trio is effected principally through the displacement of bound Mg2⁺ (21). Therefore, we reasoned that the effect of TrioN on Rac1 could be mimicked by removal of Mg2⁺ from Rac1 by the divalent cation chelator EDTA. As shown in Fig. 5 and Table I, lowering the free Mg2⁺ concentration to 0.4 μM (open circles) or 4 μM (closed circles) free Mg2⁺ concentrations in the reaction buffer. Results are means ± S.E. of three experiments.

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2 B. Debreceni and Y. Zheng, submitted for publication.
Raising the concentration of free Mg\(^{2+}\) to 4 mM eliminated oxidase activation by prenylated Rac1-GDP, reaching a level \((V_{\text{max}} = 16.3 \pm 2.6\) mol O\(_2\)/mol cytochrome b\(_{559}\) heme; means ± S.E. of three experiments\) lower than that found under standard assay conditions, in which the concentration of free Mg\(^{2+}\) was 1 mM. In control experiments, in which EDTA was replaced by an equal concentration of the Ca\(^{2+}\)-specific chelator EGTA, no enhancement of oxidase activation was seen (results not shown). Enhancement of oxidase activation was also obtained when prenylated Rac1-GDP was treated with EDTA for 10 min at 30 °C, to bring the concentration of free Mg\(^{2+}\) to 0.27 μM, and then freed of EDTA and chelated Mg\(^{2+}\) by passage through a desalting column (results not shown). This indicates that enhancement of oxidase activation is mediated by displacement of Mg\(^{2+}\) from Rac1 and not from another component present in the cell-free assay. Cross et al. (36) first described \"spontaneous\" oxidase activation in the absence of Mg\(^{2+}\) in a cell-free system, consisting of purified cytochrome b\(_{559}\), recombinant p47\(^{phox}\) p67\(^{phox}\) and prenylated Rac2, in the absence of amphiphile.

**DISCUSSION**

These results point toward a novel mechanism of Rac activation, the essence of which is the induction of a conformational change in Rac consequent to its interaction with a GEF. Based on findings made with Rac1 mutants W56F and Y64A, binding of TrioN to Rac1 is an absolute requirement for oxidase activation. The lack of effect of TrioN on Rac1 mutants T17N and Y32A demonstrates that binding to TrioN, although required, is not sufficient for activation and that an event normally related to the nucleotide exchange promoting effect of TrioN is involved. However, the fact that TrioN was fully effective when added to Rac1-GDP in the absence of exogenous GTP indicates that the effect of TrioN occurs independently of actual nucleotide exchange. Based on a model of GEF action (37), it is likely that, under these conditions, a [TrioN-Rac1-GDP] complex is formed. It was found that in the complex of the DH-PII module of the GEF Tiam1 with Rac1, the conformation of the switch I and II regions and their vicinities are altered (28). We suggest that TrioN induces a conformational change in Rac1-GDP, normally related to nucleotide exchange to GTP but now taking place in the absence of such an exchange. This conformational change appears to be related to the displacement by TrioN of Mg\(^{2+}\) bound to Rac1 (21), in agreement with the TrioN-mimicking effect of Mg\(^{2+}\) depletion by EDTA. It was, indeed, reported that a conformational change, represented by the opening of the switch I region, took place in RhoA-GDP as the direct result of Mg\(^{2+}\) dissociation induced by Li\(_2\)SO\(_4\) (38). Less pronounced conformational changes were also found to be induced by Mg\(^{2+}\) depletion in the switch II and insert regions. We propose that the conformational change in Rac1 consequent to interaction with TrioN, in the absence of exogenous GTP, results in an increased affinity of Rac1 for another oxidase component. The principal candidates are p67\(^{phox}\) (change in switch I) (39) or cytochrome b\(_{559}\) (change in the insert region) (40). We favor the hypothesis that a [TrioN-Rac1-GDP-p67\(^{phox}\)] complex is formed that translocates to the membrane. Once there, p67\(^{phox}\) interacts with cytochrome b\(_{559}\) and activates the oxidase. Support for this proposal is offered by the finding that conditions leading to high affinity binding of Rac to p67\(^{phox}\), such as a Q61L mutation in Rac (29) or chimerization of Rac with p67\(^{phox}\) (12, 22), are also the situations in which TrioN is incapable of further enhancement of oxidase activation. Support for the proposal that a [TrioN-Rac1-GDP-p67\(^{phox}\)] ternary complex is formed, is offered by the finding of a similar [GEF-GTPase-effector] ternary complex, consisting of the minimal functional domains of Dbl, Cdc42Hs, and p21-activated kinase 1 (PAK1), in which PAK1 was activated. An alternative to the [TrioN-Rac1-GDP-p67\(^{phox}\)] complex model is the causation by TrioN of a conformational change in Rac1, in the absence of complex formation with TrioN, leading to an increased affinity of Rac1 for another oxidase component. Further work is required for proving the veracity of one of the two models.

TrioN is a sequence module consisting of the N-terminal DH and PH domains of Trio. An issue to be clarified is the relative importance of the DH and PH domains in the effect of TrioN on Rac1. The finding that the TrioN mutant N1406A/D1407A is inactive indicates that the DH domain is involved in the oxidase activation enhancing activity of TrioN. It has been shown that the N-terminal PH domain of Trio binds to acidic phospholipids and might serve as a membrane localizing signal (41). We cannot yet establish the possible involvement of the DH domain in the potentiating effect of Trio, but it is likely that a [TrioN-Rac1-GDP] complex will express a high affinity for the membrane because of the presence of two groups binding to acidic phospholipids, the prenylated polybasic C terminus of Rac1 and the PH domain of Trio.

Finally, it remains to be established whether activation of Rac1 by Trio, in the absence of nucleotide exchange, represents but a particular example of a property shared by other Rac-specific GEFs and whether such a mechanism is at work in the intact cell. Recently, a concept is emerging looking upon Rac GEFs not merely as mediators of nucleotide exchange on Rac but also as factors directing Rac toward specific effector pathways (42).

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