Activation of the Phagocyte NADPH Oxidase by Rac Guanine Nucleotide Exchange Factors in Conjunction with ATP and Nucleoside Diphosphate Kinase*

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Phagocytes are bearers of an enzyme complex capable of responding with the production of microbicidal oxygen-derived radicals to a variety of stimuli acting on membrane receptors. This multicompartment complex, referred to as the NADPH oxidase (briefly, “oxidase”) catalyzes the formation of superoxide anions (\(\text{O}_2^-\)) by NADPH-driven one-electron reduction of molecular oxygen (reviewed in Refs. 1 and 2). In resting phagocytes, the components of the future complex exist as distinct entities, and these are a membrane-associated flavocytochrome \(b_{559}\) (a heterodimer of two subunits, gp91\(^{phox}\) and \(p22\)^{phox}) and four cytosolic components, \(p47\)^{phox}, \(p67\)^{phox}, \(p40\)^{phox}, and the small GTPase Rac (Rac1 or 2) (reviewed in Ref. 3). Oxidase activation is the consequence of the interaction of cytochrome \(b_{559}\) with the cytosolic components, involving translocation of the latter to the membrane. The process comprises a complex set of protein-protein interactions and is defined as oxidase assembly (reviewed in Refs. 3 and 4).

Oxidase assembly in the intact cell can be reproduced \textit{in vitro} in a cell-free system, consisting of phagocyte membranes or purified cytochrome \(b_{559}\) with the cytosolic components \(p47\)^{phox}, \(p67\)^{phox}, and Rac, supplemented with an anionic amphiphile serving as activator (5–9). Subsequently, it was found that oxidase activation \textit{in vitro} can be achieved in the absence of \(p47\)^{phox} but not in the absence of \(p67\)^{phox} and Rac (10, 11). This supports a model of oxidase assembly in which \(p67\)^{phox} functions as the paramount cytosolic “activator,” interacting with \(gp91\)^{phox} to initiate electron flow from NADPH to oxygen, with \(p47\)^{phox} and Rac serving as “organizers” of the \(p67\)^{phox}, \(gp91\)^{phox} interaction (12, 13).

Rac is absolutely required for the activation of the phagocyte oxidase. This was demonstrated by the ability of Rac1, purified from macrophages (14), and Rac2, purified from neutrophils (15), to support oxidase activation \textit{in vitro} and by the finding that recombinant Rac1 (14, 16) and Rac2 (17, 18) could substitute for the purified material. There is also a large body of evidence for a key role of Rac in the regulation of phagocyte oxidase \textit{in vivo} (19–21). The physiologic form of both Rac1 and Rac2 in phagocytes is that of a C-terminally prenylated (geranylgeranylated) protein (22). In the cytosol, both isomers are found exclusively as heterodimers with the regulatory protein GDP dissociation inhibitor for Rho (RhoGDI) (14, 17, 23). In accordance with the general rule applying to the role of small GTPases as on/off switches, the oxidase-activating function of both Rac1 (14, 16) and Rac2 (17, 24) was found to be dependent

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1 The abbreviations used are: \(\text{O}_2^-\), superoxide radical; AMPPNP, adenylylimidodiphosphate; ATP-g\(\delta\), adenosine-5’-O-(3-thiotriphosphate); GMPPNP, guanylylimidodiphosphate; GTP-g\(\gamma\), guanosine-5’-O-(3-thiotriphosphate); NDPK, nucleoside 5’-diphosphate kinase; mS, milliSieverts; RhoGDI, GDP dissociation inhibitor for Rho; GEF, guanine nucleotide exchange factor; GST, glutathione S-transferase; DH, Dbl homology domain; PH, pleckstrin homology domain.
tathione S-transferase (GST) fusion proteins and purified as described in Ref. 35, with the modification that induction was by 0.4 mM isopropyl-β-D-thiogalactopyranoside, at 18 °C for 12–16 h. p67^{phox} was isolated from E. coli transformed with full-length p67^{phox} DNA cloned into the expression vector pGEX-2T (Amersham Biosciences). The cells were induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside at 25 °C for 6 h. The GST-p67^{phox} fusion protein was purified and cleared by thrombin, as described in Ref. 35. To obtain higher purity preparations, the material was further purified by anion exchange chromatography on a Mono Q HR 5/5 fast performance liquid chromatography column (Amersham Biosciences). The column was equilibrated with 50 mM Tris-HCl, pH 7.5, supplemented with 0.1 mM 1,4-dithioerythritol and p67^{phox} was eluted by a 30-mL linear gradient of NaCl in equilibration buffer, from 0 to 0.5 M, at a flow of 0.5 mL/min. The highest purity fractions (>95%), eluting at a conductivity of 15–17 mS/cm, were used in all the experiments to be described. p67^{phox} was truncated at residue 212 was purified as described before (36). An enzymatically active human Trio, containing the N-terminal DbI homology domain and pleckstrin homology (PH) domains (residues 1225–1537), to be referred as TrioN, was expressed and purified as described earlier (32). CDNA corresponding to a fragment of mouse Tiam1, encompassing the DH domain and the adjacent C-terminal PH domain (residues 1033–1406) and carrying an N-terminal His_{6} tag, was cloned in the expression vector pProEX HT (37) and used to transform BL21-CodonPlus™ competent cells (Stratagene). The cells were induced with 1 mM isopropyl-β-D-thiogalactopyranoside at 37 °C for 3 h. The His_{6}-tagged protein, to be referred as Tiam1 DH/PH, was purified using the procedure used for His_{6}-tagged TrioN.

**Fractionation of Bacularovirus-derived p67^{phox}**—Bacularovirus-derived p67^{phox} was prepared and purified on a Q-Sepharose column (Amersham Biosciences) by a modification (35) of the procedure of Leto et al. (36). Fractions of highest purity eluted from the column at a buffer conductivity of 15–17 mS/cm. However, material with lower p67^{phox} content and lesser purity was also present in fractions eluting at a lower buffer conductivity range (13–15 mS/cm) and was present is some batches of p67^{phox}.

**Fractionation of Mock-infected S9 Cell Cytosol**—S9 cells were infected with wild-type baculovirus (BD Pharmingen) and grown for 72 h under conditions identical to those employed for growing cells infected with p67^{phox} recombinant baculovirus. A cytosolic fraction was prepared and subjected to fractionation on a Q-Sepharose column by the procedure used for the fractionation of p67^{phox}. Fractions eluting in a wide buffer conductivity range (9–19 mS/cm) were collected. These fractions were expected to contain substrates derived from the S9 cells, with an isoelectric point close to that of p67^{phox}. Material derived from fractionation of S9 cells cytosol on Q-Sepharose was further characterized by gel filtration on a Superdex 75 column (Amersham Biosciences).

**Protein Concentration**—This was estimated by the method of Bradford (37) and modified for use with 96-well microplates, using the Bio-Rad protein assay dye reagent concentrate and bovine γ-globulin as standard.

**Enzymatic Prenylation of Rac1**—Recombinant non-prenylated Rac1 was purified in vitro by recombinant geranylgeranyltransferase type L, as described before (38). The level of prenylation was assessed as described in Ref. 39.

**Cell-free NADPH Oxidase Activation Assay**—Oxidase activation 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 (29, 32).

**Identification and Quantification of Nucleotides**—Free nucleotides (ATP, ADP, AMP, GTP, and GDP) as well as bound nucleotides released from recombinant Rac1 were identified and quantified by high pressure liquid chromatography on a Partisil 10 SAX anion exchange column (250 × 4.6 mm) (Whatman), as described before (32).

**Enzymatic Activity of Protein-bound Nucleotides and Separation of Nucleotides from Mixtures Containing Proteins**—In the course of the experiments to be described, it was required to isolate and quantify GDP or GTP bound to Rac1 or nucleotides generated in the course of enzymatic reactions containing proteins. For these purposes, we applied the method described in Ref. 40. The protein was precipitated by the addition of perchloric acid, to result in a final concentration of 1 N. The acid was neutralized to pH 7 by the addition of a solution containing 1.5 M KOH and 0.5 M KHCO_{3}, and the precipitated protein sedimented by centrifugation at 12,000 × g, for 30 min at room temperature. The supernatant was filtered using a Microcon centrifugal filter device with a membrane cut-off of 10 kDa (YM-10, Millipore), centrifuged at 12,000 × g, also at room temperature, and the filtrate was injected in the Partisil 10 SAX column.

**Measurement of NDPK Activity**—NDPK activity was measured by
following the formation of ADP from ATP in a coupled pyruvate kinase-lactate dehydrogenase system (41). The reaction mixtures contained 3 mM phosphoenol pyruvate, 2 mM ATP, 1.25 units/ml pyruvate kinase, 2.25 mM lactate, 10 mM MgCl₂, 25 mM KCl, 0.3 mM NADH, 0.4 mM deoxythymidine 5’-diphosphate, and the material to be assayed for NDPK activity, in 80 mM Tris acetate buffer, pH 7.5, in a final volume of 1 ml. All components of the mixture, with the exception of thymidine 5’-diphosphate, were incubated for 5 min at 25 °C in a quartz glass cuvette and placed in a double-beam spectrophotometer (Uvikon 943, Kontron), and the reaction was started by the addition of deoxythymidine 5’-diphosphate. The rate of oxidation of NADH, reflecting the formation of ADP from ATP, was followed by measuring the decrease in absorbance at 340 nm, for 2–4 min, and calculations were based on an extinction coefficient for reduced NADH of 6.22 mM⁻¹ cm⁻¹ (42). Activities were expressed as micromoles of ADP/min/mg of protein. The measured values were corrected for background rates by running assays in the absence of deoxythymidine 5’-diphosphate and of the NDPK-containing material (41). As a control for the specificity of the reaction, measurements were also performed in mixtures in which ATP was replaced by AMPPNP, which cannot serve as a substrate for NDPK.

**SDS-PAGE and Immunoblotting—**SDS-PAGE and immunoblotting were performed as described in Ref. 43. NDPK was detected with two types of antibody. The first was a rabbit polyclonal antibody, raised against a synthetic peptide corresponding to an inner region shared by human NDPK isoforms A and B (nm23/NDPK kinase Ab-1, Labvision). This antibody was used at a dilution of 1:1000 in blocking buffer. The other was a mouse monoclonal antibody produced by immunization with purified human NDPK isoform A (nm23-H1 (NM301, Santa Cruz Biotechnology) and was used at dilution of 1:250, also in blocking buffer. The antibodies were an affinity-purified goat anti-rabbit IgG conjugated with alkaline phosphatase (Sigma-Aldrich), diluted 1:2000 in blocking buffer, to follow the rabbit polyclonal antibody, and an affinity-purified goat anti-mouse IgG conjugated with alkaline phosphatase (Sigma-Aldrich), diluted 1:1000 in blocking buffer to follow the mouse monoclonal antibody. Exposure of blots to the first antibodies was for 2 h and to the second antibodies, for 1 h. Alkaline phosphatase activity on the blots was detected as described in Ref. 44.

**RESULTS**

**Difficulty in Repeating the Finding that TrioN-dependent Oxidase Activation Does Not Involve GDP to GTP Exchange on Rac—**TrioN is a member of the Dbl family of GEFs and contains two distinct guanine nucleotide exchange domains, specific for Rac and Rho, respectively (45). A recombinant N-terminal segment of Trio (residues 1225–1537), which includes the Rac-specific DH and PH domains (TrioN), was found to stimulate GDP to GTP exchange on Rac1 (46). We recently reported that TrioN activates the oxidase in a cell-free system consisting of macrophage membranes, p67<sub>phox</sub>, and prenylated Rac1-GDP, in the absence of amphiphile and p47<sub>phox</sub> (32). We, initially, found that activation occurred in the absence of exogenous GTP and proposed that the effect of TrioN did not involve GDP to GTP exchange on Rac1 but was explained by a Trio-induced conformational change in Rac1-GDP. This conclusion was based on experiments in which the source of p67<sub>phox</sub> was recombinant protein produced in baculovirus-infected SF9 cells, purified by anion exchange chromatography on Q-Sepharose. Attempts to confirm this finding by using different batches of baculovirus-derived p67<sub>phox</sub> revealed that some were unable to support GDP-independent oxidase activation. We also found that recombinant p67<sub>phox</sub> produced in <i>E. coli</i> uniformly failed to support amphiphile- and p47<sub>phox</sub>-independent oxidase activation by TrioN and prenylated Rac1-GDP, in the absence of added GTP. Therefore, we suspected that a material present in some but not all baculovirus p67<sub>phox</sub> preparations and absent in p67<sub>phox</sub> preparations from <i>E. coli</i> was required for TrioN-dependent oxidase activation by prenylated Rac1-GDP. Thus, we subjected baculovirus p67<sub>phox</sub> preparations, capable of supporting GDP-independent oxidase activation by TrioN, to heat inactivation at 80 °C or filtration through a membrane rejecting molecules larger than 3 kDa. We found that both forms of "inactivated" baculovirus p67<sub>phox</sub> preparations reconstituted the deficient oxidase activation by p67<sub>phox</sub> from <i>E. coli</i> to levels reported previously (32) with active baculovirus p67<sub>phox</sub>.

We suspected that the material originated in the cytosol of SF9 cells. To identify the responsible component, we prepared cytosol from SF9 cells infected with wild-type baculovirus and subjected this to fractionation on Q-Sepharose by mimicking the conditions used to purify p67<sub>phox</sub>. We did, indeed, identify a material eluting at a buffer conductivity of 13–15 mScm, capable of restoring the defective ability of p67<sub>phox</sub> from <i>E. coli</i> to support GTP-independent oxidase activation by prenylated Rac1-GDP and TrioN. Size exclusion chromatography on Superdex 75 demonstrated that the one or more active components were of a size of < 1 kDa. Applying this material to a Partisil 10 SAX column, under conditions allowing the separation and identification of nucleotides, demonstrated that it contained GDP and ATP and smaller amounts of GDP and ADP (results not shown). Measurements made on several samples of baculovirus-derived p67<sub>phox</sub> demonstrated that, contrary to the statement made in Ref. 32, that no nucleotides could be detected, increasing the amount of material subjected to analysis led to the detection of up to 0.2 μM GTP in some batches, containing 10–15 μM p67<sub>phox</sub>.

**The Inability of p67<sub>phox</sub> from <i>E. coli</i> to Support TrioN-dependent Oxidase Activation by Prenylated Rac1-GDP Is Corrected by Both GTP and ATP—**The experiments described in the previous section suggested that TrioN-dependent oxidase activation by prenylated Rac1-GDP does require the presence of GTP and/or ATP. Therefore, we ran a series of experiments in which we compared oxidase activation in a system consisting of macrophage membranes, p67<sub>phox</sub> from <i>E. coli</i>, prenylated Rac1-GDP and TrioN, in the absence and presence of GTP, ATP, and analogs of these. As seen in Fig. 1, oxidase activation was markedly stimulated by GTP, and to a lesser degree, by ATP.

**FIG. 1.** The TrioN-dependent activation of NADPH oxidase by prenylated Rac1-GDP and recombinant p67<sub>phox</sub> from <i>E. coli</i>, in the absence of amphiphile and p47<sub>phox</sub>, requires the presence of exogenous GTP or ATP. NADPH oxidase activation was assayed in a cell-free system consisting of macrophage membrane vesicles (equivalent of 5 μM cytochrome b<sub>559</sub> heme), p67<sub>phox</sub> from <i>E. coli</i> (300 nM), prenylated Rac1-GDP (300 nM), and TrioN (300 nM), in the absence of amphiphile and p47<sub>phox</sub>. The reactions were run in the absence or presence of the listed nucleotides and nucleotide analogs, at concentrations ranging from 10 nM to 10 μM. The results are means ± S.E. of three experiments.

2 The level of "contamination" of individual batches of p67<sub>phox</sub> with GTP/ATP was found to be variable and, apparently, depended on the width of the p67<sub>phox</sub> elution peak chosen for pooling the p67<sub>phox</sub>-containing fractions. This situation was to be expected, considering the fact that the bulk of p67<sub>phox</sub> protein eluted from Q-Sepharose at a buffer conductivity of 15–17 mScm, whereas GTP and ATP eluted at a buffer conductivity of 13–15 mScm.
Amphiphile- and p47\textsuperscript{phox}-independent cell-free NADPH oxidase activation was performed as described under “Experimental Procedures.” The concentrations of components were as follows: membrane, equivalent of 5 nm cytochrome \textsubscript{b552} heme; p67\textsuperscript{phox}, 300 nm; prenylated Rac1-GDP, 300 nm, and TrioN, 300 nm. Results represent means \pm S.E. of three experiments for each combination of NADPH oxidase components. The concentration of GTP was 1 \mu M; the concentration of ATP was 10 \mu M.

| Components of the reaction | NADPH oxidase activity | No nucleotide added | + GTP | + ATP |
|----------------------------|------------------------|--------------------|------|------|
| Membrane                   | 0.97 \pm 0.17          | 0.75 \pm 0.30      | 0.82 \pm 0.35 |
| Membrane + prenylated Rac1-GDP | 1.75 \pm 0.25          | 1.49 \pm 0.31      | 1.34 \pm 0.52 |
| Membrane + p67\textsuperscript{phox} | 0.97 \pm 0.26          | 0.86 \pm 0.31      | 1.01 \pm 0.48 |
| Membrane + TrioN           | 0.92 \pm 0.30          | 0.79 \pm 0.35      | 0.86 \pm 0.41 |
| Membrane + prenylated Rac1-GDP + p67\textsuperscript{phox} + TrioN | 2.18 \pm 0.43          | 19.06 \pm 10.60    | 8.58 \pm 4.61 |
| Membrane + prenylated Rac1-GDP + TrioN | 1.61 \pm 0.22          | 1.73 \pm 0.19      | 1.73 \pm 0.30 |
| Membrane + p67\textsuperscript{phox} + TrioN | 1.07 \pm 0.25          | 1.03 \pm 0.48      | 0.96 \pm 0.40 |
| Membrane + prenylated Rac1-GDP + p67\textsuperscript{phox} + TrioN | 4.91 \pm 1.20          | 70.50 \pm 16.00    | 66.18 \pm 15.39 |
| Membrane + prenylated Rac1-GDP + p67\textsuperscript{phox},(1–212) + TrioN | 5.35 \pm 1.20          | 38.63 \pm 9.37    | 40.63 \pm 10.61 |
| Membrane + prenylated Rac1-GDP + p67\textsuperscript{phox}, superoxide dismutase | 2.93 \pm 1.58          | 2.01 \pm 0.25      | 2.22 \pm 0.15 |
| Membrane + nonprenylated Rac1-GDP + p67\textsuperscript{phox} + TrioN | 1.42 \pm 0.12          | 1.41 \pm 0.50      | 1.24 \pm 0.38 |
| Membrane + nonprenylated Rac1-GDP + p67\textsuperscript{phox},(1–212) + TrioN | 1.37 \pm 0.09          | 1.46 \pm 0.50      | 1.25 \pm 0.35 |

* 500 units/ml.

GMPPNP and GTP\textsubscript{s}. GDP was not stimulatory. It is also of interest that the dose-response curves for the guanine nucleotides were quite different, with GTP and GTP\textsubscript{s}S exhibiting lesser stimulatory activity at higher concentrations. ATP was a potent activator, with a dose-response curve similar to that of GMPPNP. AMPPNP and ATP\textsubscript{s}S lacked activating ability. The stimulatory ability of both GTP and the non-hydrolyzable ATP analogs suggests that these compounds act directly on a component of the oxidase complex. The experiments summarized in Table I show that, for the effect of GTP to be evident, all four participants in the reaction (membrane, p67\textsuperscript{phox}, prenylated Rac1-GDP, and TrioN) must be present. A low level of activation by GTP could also be achieved in the absence of TrioN; the most likely explanation for this is the presence in the membrane of a GEF acting on Rac1.\textsuperscript{3} In contrast to GTP, ATP, but none of its analogs, activated the oxidase, suggesting that the effect of ATP is indirect and that ATP hydrolysis or \gamma-phosphoryl transfer are necessary for the stimulatory effect to occur. The ATP preparation used in the assays was 99% pure, as specified by the manufacturer. In addition, it was checked for possible contamination by GTP by chromatography of 50-nmol samples of ATP on Partisil 10 SAX; no GTP was detected, and the only contaminant found was ADP (<1%).

No activation was elicited by ADP (results not shown). The experiments summarized in Table I also show that: 1) GTP- and ATP-dependent oxidase activation also takes place when full-length p67\textsuperscript{phox} is replaced by the truncated form p67\textsuperscript{phox}, (1–212); 2) no oxidase activation occurs when Rac1-GDP is non-prenylated, and 3) the assay by which oxidase activation was quantified accurately reflected \textsubscript{O2} production, as shown by the inhibitory effect of superoxide dismutase.

GTP- and ATP-dependent oxidase activation by p67\textsuperscript{phox} and prenylated Rac1-GDP was elicited not only by TrioN. Thus, a recombinant protein, consisting of the DH domain and the C-terminal PH domain of the Rac-specific GEF Tiam1 (47), also stimulated oxidase activation, and this exhibited the same absolute dependence on GTP or ATP as that of TrioN (Fig. 2). Therefore, the capacity of ATP to substitute for GTP appears to be a general characteristic of Rac GEF-elicited oxidase activation. This result is of special relevance, because Tiam1 was found to be expressed in macrophages (48), the cell type from which the membrane preparations used in these experiments were derived.

\textsuperscript{3} A. Mizrahi and E. Pick, unpublished observations.
The presence of a Trp residue at position 56 in Rac was shown to be the critical determinant for discrimination by a subset of Rac-specific GEFs, including Trio and Tiam1 (49). We have recently shown that the Rac1 mutant W56F, although fully capable of supporting oxidation activate in the canonical amphiphile- and p47phox-dependent system, was inactive in the TrioN-activated amphiphile- and p47phox-independent system (32). We then expanded these studies to a cell-free system in which oxidation activate by GEFs was GTP- or ATP-dependent, consisting of membrane (5 nm cytochrome b559 heme), p67phox from E. coli (300 nM), wild-type or mutant (W56F) prenylated Rac1-GDP (both, 300 nM), TrioN or Tiam1 DH/PH (both, 300 nM), and GTP (1 μM) or ATP (10 μM). We found that replacing wild-type prenylated Rac1-GDP by the Rac1 W56F mutant resulted in the practical elimination of the stimulation of oxidation activate by both TrioN and Tiam1 DH/PH, in the presence of GTP or ATP (results not shown). These results emphasize again that we are dealing with an effect probably shared by all Rac GEFs and that an interaction between GEF and Rac is a precondition for both GTP- and ATP-dependent oxidation activate to take place.

Is the Effect of ATP on Oxidase Activation Mediated by the Conversion of GDP to GTP?—The similarity in the effects of GTP and ATP on TrioN and Tiam1-dependent oxidation activate suggested a common mechanism of action. However, the finding, that both GTP and non-hydrolyzable GTP analogs were active whereas the ATP analogs AMP/PNP and ATP-S were inactive (Fig. 1), indicated that the effect of ATP was indirect. A likely mechanism was that ATP served as the substrate for a NDPK present in the membrane (the only non-recombinant component of the cell-free system), capable of converting GDP to GTP, in accordance to the reaction, ATP + GDP → ADP + GTP (50). Because the only source of GDP in the system was prenylated Rac1, we hypothesized that a membrane NDPK acted on Rac1-derived GDP, to produce GTP, which was then bound to Rac1, to form Rac1-GTP. To confirm this hypothesis, we designed experiments in which membrane preparations were incubated with a mixture of ATP and GDP or AMP/PNP and GDP. As illustrated in Fig. 3A, in the presence of membrane, ATP, and GDP, there was significant conversion of GDP to GTP; no GTP generation was found in the absence of membrane and in the presence of only one of the nucleotides. As seen in Fig. 3B, no significant GTP generation was found when ATP was replaced by AMP/PNP, an ATP analog that cannot serve as a substrate for NDPK and that was also incapable of supporting oxidation activate by TrioN (Fig. 1).

Biochemical and Immunologic Proof for the Presence of NDPK in Macrophage Membranes—The existence of a NDPK in macrophage membranes was confirmed by measuring its enzymatic activity by the conventional coupled pyruvate kinase-lactate dehydrogenase assay (41). As seen in Table II, membranes expressed levels of activity of ~0.13 μmol/min/mg of protein. If ATP was replaced by AMP/PNP in the coupled pyruvate-lactate dehydrogenase assay, the NDPK activity in the membrane was 1/10 of that detected with ATP (results not shown). NDPK activity measured in total macrophage cytosol was about 9 times higher than in the membrane. We calculated that the amount of NDPK present in the assay (5 nmol of cytochrome b559 heme/liter) will carry over a quantity of NDPK capable of converting about 5 μmol of GDP to GTP/min, which is largely in excess of the amounts of Rac1-bound GDP present in the assay (0.3–0.5 μM).

Purified and reactivated cytochrome b559 can fully substitute for whole membrane in the amphiphile- and p47phox-independent cell-free oxidation assay (29). We made use of this fact and tested the effects of GTP and ATP on the ability of purified, reactivated, and reactivated cytochrome b559 (2.5 nM cytochrome b559 heme) to exhibit oxidation activate in a cell-free system containing p67phox from E. coli (300 nM), prenylated Rac1-GDP (300 nM), and TrioN (300 nM). We found that when total membrane was replaced by purified cytochrome b559/TrioN-dependent oxidation activate maintained its GDP dependence but lost its ability to respond to ATP. Thus, oxidation activate, as expressed by turnover (moles of O2/mole of cytochrome b559 heme), increased from 12.93 ± 1.67, in the absence of nucleotides, to 42.07 ± 3.08, in the presence of GTP (1 μM), but only to 15.18 ± 1.21, in the presence of ATP (10 μM) (means ± S.E. of three experiments). Partially purified cytochrome b559, with a specific content of 1176 pmol heme/mg of protein, exhibited more than 10-fold lower NDPK activity than that of total membrane, on a per milligram of protein basis, and 100-fold lower, on a per mole of heme basis (Table II). When performing the calculation applied earlier to total membrane, we found that the amount of cytochrome b559 present in the oxidase assay (2.5 nmol of cytochrome b559 heme/liter) will carry over a quantity of NDPK capable of converting about 0.025 μmol of GDP to GTP/min, which is markedly inferior to the amounts of Rac1-bound GDP present in the assay (0.3–0.5 μM). These results suggest a link between the amount of NDPK present in a certain membrane preparation (total or enriched in cytochrome b559) and its responsiveness to ATP, and support a model in which membrane-bound NDPK utilizes added ATP to convert GDP to GTP.

Further support for this model was provided by experiments based on the knowledge that NDPK requires Mg2+ for activity (50). In these, oxidation activate was assessed in mixtures containing membrane, p67phox from E. coli, prenylated Rac1-GDP, and TrioN in the presence of GTP or ATP, in the virtual absence of Mg2+ (the concentration of free Mg2+ in the assay buffer was reduced to 0.13 μM by the addition of EDTA). Under these conditions, GTP-supported activation by TrioN was unchanged, whereas activation by ATP was reduced to one-half of the level seen in the presence of 1 mM Mg2+ (results not shown).

We next sought additional proof for the presence of a NDPK in macrophage membranes by immunologic detection. For this purpose, we made use of antibodies directed against the A and B isoforms of human NDPK. The rodent equivalents of human NDPK isoforms A and B are 95–98% identical to their human counterparts (reviewed in Ref. 51). As apparent from the immunoblot illustrated in Fig. 4, an antibody recognizing both NDPK isoforms A and B reacted with a protein of about 17–18 kDa in macrophage membranes, whereas an antibody recognizing only isoform A did not react with any protein. Although no antibody recognizing specifically the B isoform was available to us, we interpret these results as indicating that guinea pig macrophage membranes contain the B isoform of NDPK. The B isoform of NDPK was recently reported to be present in phagosomes of a murine macrophage cell line having phagocytosed latex particles (52).

The Conversion of GDP, Originating from Rac1-GDP, to GTP by NDPK Is GEF-dependent—We next investigated the origin of GDP serving as target for the γ-phosphoryl transfer by membrane NDPK. The most likely explanation is that it originated from the GDP bound to Rac1. To test this hypothesis, we made use of purified NDPK from bovine liver, by incubating it with non-prenylated Rac1-GDP and ATP, in the absence and presence of TrioN, and analyzing the products of the reaction by chromatography on Partisil 10 SAX. As seen in Fig. 5, incubation of purified NDPK with Rac1-GDP, ATP, and TrioN...
led to the generation of GTP. In the absence of TrioN, a much smaller amount of GTP was produced; in the absence of NDPK, no significant amounts of GTP were produced, both in the absence and presence of TrioN. There was no generation of GTP in the absence of ATP (results not shown). These results indicate that NDPK converts Rac1-bound GDP to GTP by an

TABLE II

Nucleoside diphosphate kinase content of macrophage subcellular fractions and of partially purified cytochrome b559

Guinea pig peritoneal macrophages were sonically disrupted and separated into membrane and cytosol fractions. The membrane fraction was solubilized by octyl glucoside and reconstituted into detergent-free membrane vesicles. Cytochrome b559 was purified from the solubilized membrane fraction before reconstitution. Nucleoside diphosphate kinase activity was assayed by the coupled pyruvate kinase-lactate dehydrogenase system. Methodologic details for these procedures are provided under “Experimental Results.” Results represent means ± S.E. of three measurements.

| Subcellular fraction       | Nucleoside diphosphate kinase activity | Nucleoside diphosphate kinase activity |
|----------------------------|---------------------------------------|---------------------------------------|
|                            | µmol/min/mg protein                   | µmol/min/mmol cytochrome b559 heme   |
| Membrane                   | 0.1343 ± 0.0220                       | 0.9526 ± 0.1560                      |
| Cytochrome b559 (partially purified) | 0.0113 ± 0.0017   | 0.0096 ± 0.0015                        |
| Cytosol                    | 1.2033 ± 0.0390                       | NA                                    |

a Specific cytochrome b559 heme content = 0.141 nmol/mg protein.

b Specific cytochrome b559 heme content = 1.176 nmol/mg protein.

c NA, not applicable.
ATP-dependent reaction, which is dependent on the prior dissociation of GDP from Rac1 by a GEF, such as TrioN.

A Membrane-bound NDPK Is Responsible for the Generation of GTP in Mixtures of Membrane, Prenylated Rac1-GDP, TrioN, and ATP—In the experiments to be described in this section, we tested the generation of GTP under conditions that are closest to those of the cell-free oxidase assay. We, thus, incubated macrophage membranes with prenylated Rac1-GDP, and ATP, in the absence and presence of TrioN. Similarly to the conditions in the cell-free assay, prenylated Rac1-GDP and TrioN were present in equimolar amounts, with ATP, in a 5-fold molar excess. As shown in Fig. 6, GTP was generated when membranes, ATP, and Rac were present; the presence of TrioN augmented the amount of GTP produced 2-fold. Replacing membrane by purified NDPK, in an amount 7.5-fold higher in enzyme units than that present in the membrane, and in the presence of TrioN, also led to GTP generation (Fig. 6, bottom tracing). Another feature of these results was that GTP was also produced, albeit in lesser quantities, in the absence of TrioN. This is in good agreement with the finding that small amounts of O2 are produced in a cell-free system consisting of membrane, prenylated Rac1-GDP, p67phox, and ATP, in the absence of TrioN (Table I), a finding that is compatible with the presence in the membrane of a Rac-specific GEF.3

Exogenous Purified NDPK Enhances TrioN-dependent Oxidase Activation by ATP but Not by GTP and Is Independent of Exogenous Free GDP—The ability of exogenous NDPK to convert GDP, originally bound to prenylated Rac1, to GTP, suggested that the addition of NDPK to the TrioN-dependent cell-free oxidase activation system could enhance activation. Consequently, we tested the effect of adding purified bovine liver NDPK (1 unit, corresponding to the formation 1 μmol of ADP/min) was mixed with 100 nmol of ATP, 20 nmol of non-prenylated Rac1-GDP, and 20 nmol of TrioN, in 50 mM Tris-HCl buffer, pH 7.5, supplemented with 150 mM NaCl, 4 mM MgCl2, and 2 mM 1,4-dithioerythritol, in a total volume of 0.9 ml, and incubated for 5 min at 25 °C, with mixing at 500 rpm in a Thermomixer apparatus (Eppendorf). Nucleotides were extracted as described under “Experimental Procedures,” and aliquots corresponding to 75% of the amount of nucleotides present in the original mixture were applied to a Partisil 10 SAX column, which was eluted as described under “Experimental Procedures,” to identify and quantify the original and newly generated nucleotides. Recoveries of nucleotides eluted from the columns varied from 87 to 93% of the amount expected to be present in the nucleotide extracts injected into the column. The figure illustrates the results of one representative series of experiments, including control mixtures, to demonstrate the need for all four components (purified NDPK, ATP, non-prenylated Rac1-GDP, and TrioN) for maximal generation of GTP. Quantitative data appear in the table at the bottom of the panel.
DISCUSSION

In the introductory part of this report we show that our initial assertion (32), that the Rac-specific GEF Trio activates the phagocyte oxidase by a mechanism not involving nucleotide exchange on Rac, is incorrect. We now find that the apparent GTP-independent activation is explained by contamination of baculovirus-derived p67phox preparations with GTP and/or ATP. When p67phox, free of these trinucleotides, is used, the presence of exogenous GTP or ATP becomes a sine qua non condition for oxidase activation. This demonstrates that caution must be exercised when a GTP-independent activity of Rac is proposed, by making certain that an unsuspected source of GTP (or ATP) is not present. The particular circumstances in which Rac-GDP is capable of oxidase activation in vitro were discussed in the introductory section of this report. In vivo, such circumstances are probably exceptional, and the GTP-bound form of Rac represents the physiologic mediator of oxidase activation in the intact phagocyte.

In the first part of this investigation, we re-examined the effect of guanine and adenine trinucleotides on TrioN-activated amphiphile- and p47phox-independent oxidase activation, under conditions that assured absolute freedom from "carried-over" nucleotides. This system offers a number of advantages...
compared with earlier studies of similar nature (53–57). These are: 1) the components of the system, with the exception of the membrane, are highly purified recombinant proteins; 2) no free nucleotides are present, with the exception of those added, and the only protein-associated nucleotide is Rac1-bound GDP, and 3) the system is free of activator and p47\textsuperscript{phox} and is, consequently, fully dependent on the interplay of GEF, Rac1, and p67\textsuperscript{phox}.

Oxidase activation by ATP was first reported by Clark et al. (54) in a neutrophil-derived cell-free system and was subsequently linked to the activity of a cytosolic or membrane NDPK, converting GDP to GTP by \gamma\textsuperscript{-}phosphoryl transfer from ATP (55–57). All earlier reports stressed the fact that an endogenous source of GDP was an obligatory requirement for ATP-dependent activation and proposed that this was represented by GDP present in the cytosol. It was generally assumed that this was free GDP (55, 56), although the finding that only 60% of GDP was removable by dialysis suggested that part of it was tightly bound to a cytosolic protein (57).

We found that oxidase activation by TrioN was not sustained by the ATP analogs AMPPNP and ATP\textsuperscript{S}, lending further support to the idea that ATP acts in conjunction with a NDPK. AMPPNP cannot support phosphotransferase activity by NDPK (58), and ATP\textsuperscript{S} supports the NDPK-dependent transfer of \gamma\textsuperscript{-}thiophosphate to nucleoside diphosphates at 1/1000 of the equivalent \gamma\textsuperscript{-}phosphoryl transfer rate (59). The lower effectiveness and different dose-response curves of non-hydrolyzable GTP analogs GMPPNP and GTP\textsuperscript{S}, compared with GTP, are yet unexplained. It is possible that this is the result of a two-pronged mechanism of action of GTP; in addition to its direct Rac-activating effect, via TrioN-stimulated GDP to GTP exchange on Rac, GTP could also serve as a source for \gamma\textsuperscript{-}phosphoryl transfer to GDP by NDPK, a function that is not shared by GMPPNP and GTP\textsuperscript{S}.

We present firm evidence for the involvement of a B-type NDPK, localized in the macrophage membrane, in ATP-dependent activation of the oxidase. To the best of our knowledge, this is also the first description of a B-type NDPK in guinea pigs; an A-type NDPK was described in guinea pig cardiac endothelial cells (60). In earlier reports linking NDPK to oxidase function, the subcellular location of NDPK could not be established with certainty, due to the presence of both membrane and cytosol in the assays (55–57), and a cytosolic location was proposed in one report (57). In resting cells, the bulk of both A and B isoforms of NDPK are cytosolic (61), as we also found in macrophages (Table 1D). A membranal NDPK was described in hepatocytes and found to be identical to the cytosolic enzyme by biochemical, enzymatic, and immunologic criteria (62). However, the subcellular localization of NDPK is a dynamic process. Thus, stimulation of fibroblasts with growth factors led to translocation of cytosolic NDPK to the plasma membrane by a process which, interestingly, was linked to Rac1 activation (63). Translocation of cytosolic NDPK to the membrane was also reported to occur in intact human neutrophils, in response to stimulation with chemotactic peptides (64).

Our findings clearly establish that Rac1-GDP is the only source of GDP serving as substrate for NDPK. This is shown by the ability of highly purified NDPK or macrophage membrane to generate GTP from either non-prenylated or prenylated Rac1-GDP, in the absence of free GDP. Further proof for Rac-derived GDP being the exclusive target of both exogenous and intrinsic NDPK is offered by functional assays demonstrating that ATP-dependent oxidase activation, whether in the presence or absence of exogenous purified NDPK, is not enhanced by the addition of free GDP.

It was originally proposed that NDPK can transfer the \gamma\textsuperscript{-}phosphate of nucleotide triphosphates directly to GDP bound to small GTPases and, consequently, convert these into the active GTP-bound form (65, 66). Structural constraints, evident in the crystal structure of NDPK, argue against such a possibility, and it appears well established that prior dissociation of GDP from the GTPase is a prerequisite for its conversion to GTP by NDPK plus ATP (67). The requirement for prior dissociation of GDP from Rac is also demonstrated by our results showing an enhancing effect of TrioN on ATP-dependent GTP generation by combinations of both purified NDPK and non-prenylated Rac1-GDP and endogenous membrane NDPK and prenylated Rac1.

To the best of our knowledge, this is the first report of a GEF seen as functionally linked to NDPK in mediating ATP-dependent GDP to GTP exchange on a small GTPase. A schematic rendering of this linkage in the particular context of Rac and the NADPH oxidase complex is illustrated in Fig. 8. At this point, we do not possess evidence for a physical association between GEF and NDPK. However, the actual binding of NDPK A to the N-terminal region of Tiam1 (66) and the formation of a tripartite complex of NDPK A, Tiam1, and the GTPase ARP6 (68), were recently described in two cell types.

Our findings are to be interpreted in the context of their relevance to oxidase function in the intact phagocyte. The concentration of GTP in the neutrophil cytosol was reported to be close to 200 \textmu{}M, and concentrations of GTP are about 8- to 14-fold lower (57, 69). This suggests that a lack of GTP, to serve as material for replacing GDP on small GTPases, is unlikely to be a common occurrence. On the other hand, the concentration
of ATP in the cytosol of neutrophils exceeds that of GTP, 4- to 25-fold (57, 69). Furthermore, it is significant that at least one of the two “model” GEFs used (Tiam1) is present in macrophages (48). It is, therefore, possible that the cooperation between GEF and NDPK could represent a physiologic pathway for the replenishment of GTP on Rac and play a role in the regulation of oxidase activation in phagocytes in vivo.

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Lucas, D. L., Webster, H. K., and Wright, D. G. (1983) J. Clin. Invest. 72, 1889–1900
Activation of the Phagocyte NADPH Oxidase by Rac Guanine Nucleotide Exchange Factors in Conjunction with ATP and Nucleoside Diphosphate Kinase
Ariel Mizrahi, Shahar Molshanski-Mor, Carolyn Weinbaum, Yi Zheng, Miriam Hirshberg and Edgar Pick

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Additions and Corrections

Vol. 279 (2004) 23882–23891

Acyl-CoA synthetase 2 overexpression enhances fatty acid internalization and neurite outgrowth.

Joseph R. Marzalek, Claire Kitidis, Ariya Dararutana, and Harvey F. Lodish

Page 23883, "Materials and Methods": Under the section headed "Isolation and Construction of ACS1 and ACS2," two errors occurred. First, the incorrect primer sequence was included for ACS1 antisense. It should read 5′-CCC GGAT-CCTAGGCTCCAAATCTTGATGGTGAG-3′, not 5′-CCC GG-ATCTTTAATCTTGATGGTTGGAGTAC-3′. Second, the GenBank™ accession number referenced for the rat ACS2 sequence used in the study should be AY625254, not D10041. These two sequences are alternatively spliced variants of rat Acs16, each containing a different variant of exon 13.

Although these minor corrections do not change the data or interpretation of the data in the paper, they are important for accuracy and reproducibility of the results by other investigators.

Vol. 279 (2004) 54881–54886

A crucial role for exopolysaccharide modification in bacterial biofilm formation, immune evasion, and virulence.

Cuong Vuong, Stanislava Kocianova, Jovanka M. Voyich, Yufeng Yao, Elizabeth R. Fischer, Frank R. DeLeo, and Michael Otto

The thesis of Oliver Schweitzer (1997) described S. epidermidis ΔicaB mutants and pTXicaB constructs similar to those described in this article, suggested a role for icaB in biofilm formation and cell aggregation, and noted its localization on the cell surface, with secretion when overexpressed. The thesis can be obtained from the international OPAC catalogue http://opac.ub.uni-tuebingen.de/.

Vol. 280 (2005) 2361–2369

Pro-angiogenic signaling by the endothelial presence of CEACAM1.

Nerbil Kilic, Leticia Oliviera-Ferrer, Jan-Henner Wurmback, Sonja Loges, Fariba Chalajour, Samira Neshat-Vahid, Joachim Weil, Malkanthi Fernando, and Suleyman Ergun

Dr. Neshat-Vahid's last name was misspelled. The correct spelling is shown above.

Vol. 280 (2005) 3605–3612

NMR structural comparison of the cytoplasmic juxtamembrane domains of G-protein-coupled CB1 and CB2 receptors in membrane mimetic dodecylphosphocholine micelles.

Xiang-Qun Xie and Jian-Zhong Chen

Page 3612: Add new Ref. 37, Wishart, D. S., Sykes, B. D., and Richards, F. M. (1992) Biochemistry 31, 1647–1651. The reference list will now include a total of 37 references. As a result, the following reference citation should be changed:

Pg. 3608, right column, line 11 from the top: “(26, 27)” should be “(37).”

Vol. 280 (2005) 3802–3811

Activation of the phagocyte NADPH oxidase by Rac guanine nucleotide exchange factors in conjunction with ATP and nucleoside diphosphate kinase.

Ariel Mizrahi, Shahar Molshanski-Mor, Carolyn Weinbaum, Yi Zheng, Miriam Hirshberg, and Edgar Pick

Pages 3803–3811: The word “on” was omitted from the running title. The correct running title should read as follows: NADPH Oxidase Activation Dependent on GEF, ATP, and NDPK.