Activation of NADPH oxidase involves the dissociation of p21^{rac} from its inhibitory GDP/GTP exchange protein (rhoGDI) followed by its translocation to the plasma membrane

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Activation of the NADPH oxidase of phagocytes involves the small GTP-binding protein p21^{rac}. In this paper we report that neutrophil cytosol contains predominantly p21^{rac2} rather than p21^{rac1}, and that the p21^{rac2} is almost entirely complexed with rhoGDI (GDP dissociation inhibitor) to form a heterodimer with a molecular mass of 45–50 kDa. Activation of superoxide production by phorbol 12-myristate 13-acetate or formyl-methionyl-leucyl-phenylalanine in whole cells, and by SDS in the cell-free assay, led to the dissociation of some of the p21^{rac2} from rhoGDI and its movement to the plasma membrane together with p47^{phox} and p67^{phox}. The appearance of these proteins at the

plasma membrane was related to the dose of the agonist and to the rate of superoxide generation. The nucleotide bound to p21^{rac2} in this complex following isolation was almost exclusively GDP, with less than 2% GTP, and the complex was active in the cell-free assay. Although the rac/GDI complex could activate the NADPH oxidase in the absence of exogenous GTP, the rate of superoxide production was increased 3-fold by the addition of GTP and was almost completely inhibited by GDP. Our findings confirm that rhoGDI serves as GDP dissociation inhibitor and that the release of p21^{rac2} from this inhibitor is an important step in activation of the NADPH oxidase.

INTRODUCTION

Phagocytic cells such as neutrophils and macrophages produce superoxide radicals by an electron-transport chain known as NADPH oxidase that is essential for the efficient killing and digestion of ingested micro-organisms. The specialized components of this NADPH oxidase system include a low-potential flavocytochrome *b* in the membrane, and two cytosolic factors, p47^{phox} and p67^{phox}. In activated cells, these cytosolic proteins translocate to the membrane to associate with the flavocytochrome *b* (reviewed [1,2]). An additional factor was subsequently isolated from guinea pig macrophages which was essential for the activation of the NADPH oxidase [3]. This factor was identified as a heterodimeric complex of a small GTP-binding protein, p21^{rac1}, and rhoGDI (GDP dissociation inhibitor) [4]. Subsequently p21^{rac2} was isolated from human neutrophils [5,6] and HL-60 cells [7] in studies that used a similar approach. The complex of p21^{rac1} and GDI could be replaced in the cell-free assay by recombinant p21^{rac1}, but only when it was exchanged into the GTP-bound form [4].

Pure flavocytochrome *b*, together with recombinant p47^{phox}, p67^{phox} and p21^{rac1}, were able to fully reconstitute the activity of the NADPH oxidase in the cell-free assay system [8]. Recently it was demonstrated that p21^{rac1} is involved in membrane ruffling in Swiss 3T3 cells [9], suggesting an involvement of this protein in membrane-associated events.

In order to understand the sequence of events involved in forming an active NADPH oxidase complex, several approaches were taken: the distribution of p21^{rac1} and p21^{rac2} was determined in a number of different cell types, the state of association of p21^{rac} with rhoGDI in the neutrophil cytosol was determined, and the nucleotide bound to p21^{rac2} in this complex was investigated in resting and activated cells. In addition, the effect of

activating the NADPH oxidase on the association of the oxidase cytosolic proteins with the plasma membrane was determined.

MATERIALS AND METHODS

Chemicals and reagents

SDS, cytochrome *c* (type III), NADPH, GTP, FAD, superoxide dismutase, di-isopropyl fluorophosphate, Tos-Lys-CH₂Cl ('TLCK'), phenylmethanesulphonyl fluoride (PMSF), dextran, formyl-methionyl-leucyl-phenylalanine (fMLP) and phorbol 12-myristate 13-acetate (PMA) were all obtained from Sigma. Ficoll/Hypaque (Lymphoprep) was from Nycomed Pharma (Oslo, Norway). Anti-rabbit IgG conjugated to alkaline phosphatase was from Promega, and Mono HR 5/5, Superose 12 and Sephadex G-25M PD 10 columns were from Pharmacia LKB Biotechnology Inc.

Preparation of neutrophil membranes and cytosol

Neutrophils were prepared from 400–500 ml of human blood by dextran sedimentation of erythrocytes followed by centrifugation on Ficoll/Hypaque as described previously [10]. The neutrophil pellet was freed of erythrocytes and resuspended at a concentration of 2×10^8 cells/ml in break buffer, consisting of 6 mM Pipes, pH 7.3, 6% (w/w) sucrose, 60 mM KCl, 1.8 mM NaCl, 2.3 mM MgCl₂, 1 μM di-isopropyl fluorophosphate, 1 mM PMSF, 1 μg/ml Tos-Lys-CH₂Cl and 1 μg/ml pepstatin. The cells were sonicated at 4 °C for 2 × 5 s using an MSE 150 W sonicator. The broken cells were loaded on a discontinuous sucrose gradient of 0.5 ml of 15% sucrose on 0.5 ml of 34% (w/w) sucrose made up in the break buffer, and centrifuged at 200 000 *g* for 15 min in a TLX Beckman ultracentrifuge using a TLS55 swing-out rotor.

Abbreviations used: GDI, GDP dissociation inhibitor; fMLP, formyl-methionyl-leucyl-phenylalanine; PMSF, phenylmethanesulphonyl fluoride; DTT, dithiothreitol; PMA, phorbol 12-myristate 13-acetate; GDS, GDP dissociation stimulator.

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Figure 1 Distribution of p21^{rac1} and p21^{rac2} in different cell types

Post-nuclear supernatants (60 µg of protein) from the various cells were subjected to SDS/PAGE, blotted and probed with affinity-purified anti-p21^{rac1} and -p21^{rac2} as described in the Materials and methods section. Lane 1, COS cells transfected with p21^{rac1}; 2, HFG-1 cells; 3, Raji cells; 4, Jurkat cells; 5, non-differentiated U937 cells; 6, macrophage; 7, non-differentiated HL-60 cells; 8, differentiated HL-60 cells; 9, neutrophils; 10, recombinant p21^{rac1}.

The cytosol was collected above the 15% sucrose and the membranes were collected at the 15%/34% interface. The membranes were diluted 1:4 (v/v) in cold break buffer and pelleted by centrifugation as above.

In some experiments the membranes and cytosol were analysed for translocation of p47^{phox}, p67^{phox}, rhoGDI and p21^{rac} by Western blotting with affinity-purified antibodies, as described below.

Neutrophil activation

Intact neutrophils were resuspended in PBS (2 × 10⁷ cells/ml) and were preincubated at 37 °C for 5 min. fMLP and PMA were solubilized in dimethyl sulphoxide (DMSO) and were added to the neutrophil mixture at final concentrations of 1 µg/ml and 100 ng/ml respectively.

Samples of 20 µl were tested for superoxide production by measuring the superoxide dismutase-inhibitable reduction of cytochrome *c*. After cell stimulation the cells were diluted 1:10 in ice-cold PBS and pelleted by centrifugation at 200 *g* for 5 min at 4 °C. Cytosol and membranes fractions were prepared as mentioned above.

Determination of superoxide production in the cell-free assay

Superoxide production was determined by measuring the rate of superoxide dismutase-inhibitable cytochrome *c* reduction. Solubilized membranes prepared as described before [8] (5–15 µg of protein) were preincubated for 2 min at room temperature with cytosol (200–300 µg of protein) or 10 µl (1–3 µg of protein) of pure recombinant p67^{phox}, p47^{phox}, p21^{rac1} [8] or partially purified fractions containing the rac/GDI complex in the presence of an optimal concentration of SDS (100–200 µM). The activated mixture was then transferred into 0.9 ml of assay buffer consisting of 65 mM sodium phosphate buffer, pH 7.0, 1 mM EGTA, 1 mM MgCl₂, 10 µM FAD and 100 µM cytochrome *c*. The reaction was initiated by the addition of 200 µM NADPH. Superoxide production was measured for 90 s in a double-beam spectrophotometer (Uvikon 860; Kontron); then superoxide dismutase (50 µg/ml) was added to the reference sample and the reaction was monitored for an additional 90 s.

Translocation of cytosolic NADPH oxidase proteins in the cell-free assay

Translocation in the cell-free system was performed by preincubating 200 µl of neutrophil membranes (40–60 µg of protein) with 300 µl of cytosol (4 mg) at room temperature in the presence of an optimal concentration of SDS (100–200 µM). Superoxide

production was determined in an aliquot (50 µl) of the activated mixture and the test was immediately transferred on to a discontinuous sucrose gradient and centrifuged as described above. The collected plasma membranes at the 15%/34% sucrose interface was diluted 1:4 in assay buffer lacking cytochrome *c* and FAD and pelleted by centrifugation. The membranes and cytosol fractions were analysed for cytosolic components of the oxidase by Western blotting.

Antibody production

Synthetic peptides specific for p21^{rac1} (CPPPVKKRKRK) and p21^{rac2} (CPQPTRQKRA) were purchased from Alta Bioscience (University of Birmingham, U.K.). Full-length recombinant rhoGDI (a gift from Dr. John Hancock, Onyx Pharmaceuticals). p47^{phox} and p67^{phox} proteins were prepared in *Escherichia coli*, and purified to homogeneity [8]. The peptides and proteins were conjugated to keyhole-limpet haemocyanin with glutaraldehyde. Samples of 1 mg of the conjugated peptides or 0.5 mg of the proteins were homogenized in Freund's complete adjuvant by sonication and injected subcutaneously into rabbits. The rabbits were boosted at 2-week intervals with 1 mg of conjugated peptide, or 0.5 mg of protein, in Freund's incomplete adjuvant.

Peptides and proteins were coupled to an NHS-activated column (Hitrap; Pharmacia) and affinity antibodies were purified according to the manufacturer's instructions.

SDS/PAGE and Western blotting

SDS/PAGE was performed essentially as described by Laemmli on 12.5% acrylamide [11]. Proteins were transferred on to nitrocellulose membranes in a semi-dry apparatus for 1 h and probed with affinity-purified antibodies against p67^{phox}, p47^{phox}, p21^{rac1}, p21^{rac2} and rhoGDI. Bound antibodies were detected with alkaline phosphatase-conjugated anti-rabbit antibodies (Promega) or with ¹²⁵I-labelled Protein A (Amersham) followed by autoradiography, and in some cases quantification with a PhosphorImager (Molecular Dynamics). Cells used for the analysis of p21^{rac1} and p21^{rac2} distribution were gifts from Dr. Dianna Wallace (Jurkat, HFG-1 and Raji cell lines), Dr. Pam Roberts (U937, macrophages and HL-60 cells) and Dr. Anthony Best (COS cells transfected with p21^{rac1}).

Partial purification of p21^{rac}/rhoGDI

Neutrophil cytosol (5–7 ml; 15–25 mg of protein) was desalted into a buffer containing 20 mM Tris/HCl, pH 7.4, 1 mM MgCl₂, 1 mM dithiothreitol (DTT), 1 mM PMSF and 1 µg/ml Tos-Lys-CH₂Cl on a PD-10 column equilibrated with the same buffer.

The desalted cytosol was purified by f.p.l.c. on a Mono Q column equilibrated with the same buffer, and eluted with a 30 ml gradient of 0–0.5 NaCl in the same buffer at a flow rate of 1 ml/min. Fractions of 1 ml were collected and the superoxide production was determined in the cell-free assay.

Fractions were probed on Western blots with affinity-purified antibodies to rhoGDI and p21^{rac2}. Concentrations of guanine nucleotides were measured in each fraction as described below. Fractions containing the p21^{rac2}/rhoGDI complex were combined, concentrated to 200 μ l (1–2 mg of protein) and further purified by gel filtration on a Superose 12 column equilibrated with 100 mM sodium phosphate buffer, pH 7.4, 1 mM MgCl₂, 1 mM DTT and 1 mM PMSF.

Determination of the nucleotide bound to p21^{rac}/GDI

Guanine nucleotides in fractions containing p21^{rac2} and GDI purified by Mono Q anion-exchange and/or Superose 12 chromatography were determined by h.p.l.c. Samples (typically 200 μ l) were acidified by the addition of 68 μ l of 5% (v/v) HClO₄. This was partially neutralized by the addition of 28 μ l of 1.4 M NaOH/0.6 M sodium acetate and 200 μ l of the h.p.l.c. buffer. The denatured protein was removed by centrifugation at 12000 *g* for 4 min and the solution was applied to an anion-exchange column (Whatman SAX; 25 mm $\times$ 4 mm) equilibrated in the running buffer, 0.45 M (NH₄)₂HPO₄, adjusted to pH 4.0 with concentrated HCl. Nucleotides were eluted isocratically and monitored at 254 nm. The amounts of nucleotide were determined by comparison with a standard solution of GMP/GDP/GTP treated in the same way. After analysis, the same fractions were tested for activity in the cell-free assay.

RESULTS

p21^{rac2} rather than p21^{rac1} is expressed in haemopoietic cells

Figure 1 shows a Western blot demonstrating the distribution of p21^{rac1} and p21^{rac2} in different cell types. p21^{rac2} is highly expressed in neutrophils, differentiated HL-60 cells and macrophages. It was present at lower levels in HFG-1 and Raji B cell lines, Jurkat cells and undifferentiated HL-60 and U937 cells. In contrast, p21^{rac1} was not detected in these cell lines with an antibody against p21^{rac1} that recognized the non-modified recombinant p21^{rac1} and cross-reacted with COS cells transfected with p21^{rac1}.

p21^{rac2} is almost entirely complexed to rhoGDI

p21^{rac1} was initially purified from guinea pig macrophage cytosol in a complex with GDI [3]. It was not clear whether the p21^{rac} was mainly present in this complex, or whether the purification procedures selectively isolated it in the complexed form. p21^{rac2} co-eluted in approximately equimolar amounts with GDI when neutrophil cytosol was chromatographed, initially by anion exchange on Mono Q Sepharose (Figure 2a) and then by gel filtration, from which it eluted with an apparent molecular mass of 45–50 kDa (Figure 2b).

The complexed p21^{rac2} is almost exclusively in the GDP-bound form

The nucleotide bound in this complex prepared from either resting or stimulated neutrophils was almost exclusively GDP, with less than 2% GTP, and the GDP was present in roughly equimolar concentrations with the two proteins (Figures 3b and 3c). The elution of GDP was strongly correlated with the p21^{rac}/GDI complex and with the ability of this complex to stimulate superoxide production (Figure 3c). The cell-free assay

confirmed our initial observations that this complex of p21^{rac2} with GDI produced NADPH oxidase activity in the absence of added GTP. However, simply adding GTP to the incubation mixture, without attempting to exchange the nucleotide into the protein, rapidly increased the rate of superoxide production by approx. 3-fold. In contrast, recombinant unmodified p21^{rac} alone was inactive in the GDP-bound form, and GTP had to be

Figure 2 Distribution of p21^{rac}/GDI in neutrophil cytosol fractions

(a) Neutrophil cytosol (15–20 mg) was chromatographed on a Mono Q anion-exchange column as described in the Materials and methods section. Fractions were analysed by Western blotting and probed with affinity-purified anti-p21^{rac2} and anti-rhoGDI. Peak fractions (9–12) containing p21^{rac2} complexed to rhoGDI were pooled, concentrated and (b) chromatographed on Superose 12; fractions (0.5 ml) were analysed for p21^{rac2} and GDI by Western blotting.

Figure 3 Analysis of the guanine nucleotides bound to the p21^{rac2}/GDI complex

The nucleotides were extracted from fractions containing the p21^{rac2}/GDI complex and were separated on h.p.l.c. as described in the Materials and methods section. (a) Separation of a standard mixture containing 0.06 nmol of GMP, 0.12 nmol of GDP and 0.25 nmol of GTP on h.p.l.c. (b) A typical separation of nucleotides extracted from the p21^{rac2}/GDI complex; (c) relationship between the nucleotide content and the oxidase activity in the cell-free assay of fractions collected after Mono Q separation of cytosol prepared from stimulated and resting cells. Oxidase activity: ○ activated cytosol, ● resting cytosol. GDP content: □ activated cytosol, ■ resting cytosol.

exchanged into the protein for its activity to be expressed (Figure 4). In addition, GTP was not detected in the solubilized membranes used in the cell-free assay (results not shown).

p21^{rac2} dissociates from GDI and is translocated to the membrane in the cell-free assay and in intact activated cells

p47^{phox} and p67^{phox} are translocated to the plasma membrane after activation of the NADPH oxidase by SDS in the cell-free system and by activating neutrophils by fMLP or PMA. To assess the role of rac/GDI in the activation complex, we examined the movement of these proteins to the plasma membrane upon activation of the oxidase. Figure 5 shows that p47^{phox}, p67^{phox} and p21^{rac2}, but not GDI, are translocated to the membrane after activation of intact neutrophils with fMLP and PMA, and by SDS activation in the cell-free assay. This indicates that p21^{rac2} and GDI dissociate upon activation of the oxidase, and that the p21^{rac2} moves to the membranes where it might associate with p67^{phox}, p47^{phox} and flavocytochrome b.

Translocation of p21^{rac2}, p67^{phox} and p47^{phox} to the plasma membrane coincides with the activation of the NADPH oxidase and is agonist-dependent

Superoxide production in the cell-free system is absolutely

dependent on a critical concentration of the activator SDS or arachidonic acid. The pattern of translocation of p67^{phox}, p47^{phox} and p21^{rac2} was determined at various concentrations of SDS and related to the rates of superoxide generation induced by the same mixture of proteins. Maximum superoxide production and translocation were obtained at 135 μ M SDS (Figure 6). Concentrations of SDS below 86 μ M induced the movement of p21^{rac2} and p47^{phox}, but not of p67^{phox}, to the membrane, and did not stimulate superoxide production. The translocation of p67^{phox} coincided with the activation of oxidase activity. At concentrations of SDS above the optimal dose required for superoxide production (169–200 μ M), although all the components of the oxidase complex had moved to the membrane, a limited amount of superoxide was produced, possibly because these higher concentrations of SDS denature the component proteins.

The pattern of translocation of these cytosolic proteins to the membranes on stimulation of intact cells with fMLP and PMA was then determined. The same three proteins were observed to be translocated (Figure 7). The response to fMLP was dose-dependent and was maximal (83 nmol of superoxide/min per 10⁷ cells) at approx. 100 nM, at which concentration 2.7%, 2.0% and 0.9% of the total cytosolic p47^{phox}, p67^{phox} and p21^{rac2} respectively was associated with the membrane. Interestingly, at lower concentrations of fMLP (10 nM), which resulted in little

Figure 4 Effect of GTP on the NADPH oxidase activity in the cell-free system assayed with rac/GDI complex or recombinant p21^{rac}

Partially purified p21^{rac}/GDI complex or recombinant p21^{rac} was added to saturating amounts of p47^{phox}, p67^{phox} and solubilized neutrophil membranes with an optimal concentration of SDS. rac/GDI with 100 μM GTP (○) or without GTP (□); recombinant p21^{rac}1 preloaded with 100 μM GTP (●) or without GTP (■).

Figure 5 Translocation of p21^{rac2}, p47^{phox} and p67^{phox} to the plasma membrane

NADPH oxidase was activated by SDS in the cell-free system, and in intact cells by fMLP, PMA and SDS (activation indicated by +). The cytosol (C) and plasma membrane (M) fractions were separated and analysed by probing Western blots with affinity-purified antibodies to p67^{phox}, p47^{phox}, p21^{rac2} and rhoGDI.

superoxide production, p21^{rac} and p47^{phox} were still translocated to the membranes at similar concentrations to those observed with maximal activation (100% and 60% of maximal respectively), whereas the concentration of p67^{phox} was only 20% of that observed with maximal activation.

Stimulation with PMA led to the association of the oxidase components with the membrane in a time-dependent manner. Maximum translocation of all three proteins (p47^{phox}, p67^{phox} and p21^{rac2}) occurred at about 150 s at which time approx. 10% of the total cellular p47^{phox}, p67^{phox} and p21^{rac} was associated with the membrane (Figure 8). Interestingly, a linear rate of superoxide production (50 nmol of superoxide/min per 10⁷ cells) was observed at about 60 s, at which time approx. 75% of the maximal translocated p67^{phox}, p47^{phox}, and p21^{rac2} was associated with the membrane. In contrast to the other three cytosolic proteins, no evidence was observed of the translocation of GDI

Figure 6 Effect of SDS concentration on NADPH oxidase activity in the cell-free system (a) and on the translocation of the cytosolic factors to the plasma membrane (b)

Cytosol and membranes were incubated in the presence of various concentrations of SDS followed by the separation of the cytosol and the membranes and analysis by Western blots with affinity-purified antibodies.

to the membranes in intact cells after stimulation with PMA or fMLP, or in the cell-free system after activation with SDS.

DISCUSSION

The first direct evidence for the possible involvement of a small GTP-binding protein in the regulation of NADPH oxidase came from the observation that p21^{rac1} and rhoGDI would be co-purified, and together constituted the σ1 cytosolic factor that combined together with p47^{phox}, p67^{phox} and membranes to produce an active NADPH oxidase in the cell-free assay [4]. Initially this complex was purified from the cytosol of guinea pig macrophages [3]. Subsequently p21^{rac2} from human neutrophils was found to play a similar role [5–7]. It had been demonstrated by Northern blot analysis that p21^{rac2} mRNA is expressed in haemopoietic cells and appeared to be more abundant in cells of myeloid origin, whereas the expression of p21^{rac1} was found to be ubiquitous [12].

We thus undertook a survey of the expression of these two proteins in a limited number of human bone marrow-derived cell lines and discovered that p21^{rac2} was expressed in all the cells examined, whereas p21^{rac1} was undetectable. It is possible that the anti-p21^{rac1} antibody failed to recognize the isoprenylated p21^{rac1} form in these cells. It did detect p21^{rac1} transfected into COS cells, in which the protein would have been modified, although it is possible that a small proportion of the protein might have been incompletely modified. It remains to be determined whether the properties of the p21^{rac1} initially purified from guinea pig peritoneal macrophages reflect species specificity. It is not surprising that both p21^{rac}s can induce the same

Figure 7 Effect of activating neutrophils with fMLP on the translocation of the cytosolic components to the plasma membrane

Neutrophils were activated at various concentrations of fMLP for 1 min, and cytosol and membrane fractions were prepared and analysed by Western blotting (b). 125 I-Protein A labelling of bound antibody was quantified in a Phosphor Imager (a). □, p67^{phox}; ■, p47^{phox}; ○, p21^{rac2}; ●, rhoGDI.

Figure 8 Time course of the association of the cytosolic factors with the membrane

Neutrophils were activated with PMA (100 ng/ml) and at various times aliquots were taken; subsequently cytosol and membrane fractions were prepared and analysed by Western blotting. 125 I-Protein A labelling of bound antibodies was quantified in a Phosphor Imager. ●, p67^{phox}; □, p47^{phox}; ○, p21^{rac2}. The time course of superoxide generation by the cells under the same conditions is also shown (broken line).

functional response in the cell-free assay, as they both belong to the rho subfamily of the ras-related GTP-binding proteins, and their amino acid sequences show 92% identity [12]. In agreement with our results, Heyworth et al. [13] have recently shown that the processed and unprocessed forms of both p21^{rac1} and p21^{rac2} were able to activate NADPH oxidase in the cell-free system.

When p21^{rac1} was initially purified from guinea pig macrophage cytosol in a complex with GDI, it was not clear whether the p21^{rac} was mainly present in this complex or whether the purification procedure selectively isolated it in the complexed form. Similarly, in the initial studies in which GDI was shown to inhibit the dissociation of GDP from rho [14], complex formation was induced *in vitro* but no association was demonstrated with p21^{rac}, although it was implied by the inhibition by GDI of the dissociation of GDP from p21^{rac1} [15]. In addition, when p21^{rac2} was purified from human neutrophils [5], it was not associated with GDI. The studies described here show that the p21^{rac2} in neutrophil cytosol is not present as the free protein, but that it is associated with GDI in a soluble heterodimeric complex with an apparent mass of about 45–50 kDa. Our data indicate that activation of the NADPH oxidase both *in vivo* and *in vitro* is associated with the movement of p21^{rac}, unaccompanied by GDI, to the plasma membrane. The dissociation of p21^{rac} from GDI may be stimulated by either phosphorylation or dephosphorylation of p21^{rac} or GDI, or by lipid released by activation of phospholipases A2, C or D. Further work is required in order to understand the control of this process. It is most likely that, upon the release of rac protein from GDI, the exposed lipid moiety at the C-terminus anchors the protein to the membrane and may allow the interaction with exchange factors such as GDP dissociation stimulator (GDS) to catalyse the release of GDP and consequently the binding of GTP. Recently, Phillips et al. [16] have shown that carboxymethylated p21^{rac2} was translocated to the membrane when neutrophil cytosol was incubated with membranes in the presence of guanosine 5-[γ-thio]-triphosphate. In addition, the response of neutrophils to fMLP, but not to PMA, was blocked by treating the cells with a carboxymethylation inhibitor. In contrast, our study demonstrated the membrane association of p21^{rac} upon activation of neutrophils by PMA. This indicates either that the carboxymethylation of p21^{rac} is not essential for superoxide production and membrane association, or that the effect of the inhibition of superoxide production by the carboxymethylation inhibitor in the previous study was an effect on the heterotrimeric G-protein linked to the fMLP receptor or some other protein, rather than on p21^{rac2}.

It has been previously demonstrated that p47^{phox} and p67^{phox} are translocated to the membrane upon neutrophil activation by PMA and fMLP [17,18], and in the cell-free system by SDS [19], which is in agreement with our current findings. Translocation of p47^{phox}, which was found to be associated with extensive phosphorylation of this protein, did not take place in neutrophils from X-linked chronic granulomatous disease patients, which lack flavocytochrome *b* [20]. In addition, a synthetic peptide derived from the C-terminus of flavocytochrome *b* was capable of blocking the translocation of p47^{phox} and p67^{phox} [21]. Interestingly, our translocation study suggests that the first events in activation are associated with the movement of p47^{phox} and p21^{rac2} to the plasma membrane, followed by the appearance of p67^{phox} at the membrane to initiate superoxide production. It is possible that p47^{phox} and p21^{rac2} serve to facilitate the interaction of p67^{phox} with flavocytochrome *b*. In agreement with this, Uhlinger et al. [22] have shown that translocation of p67^{phox} to the membrane is dependent upon the presence of p47^{phox}. Moreover, we recently demonstrated, by kinetic analysis of

activation in the cell-free system with recombinant proteins, that maximal activity was obtained with equimolar concentrations of p67^{phox} and flavocytochrome *b* [8]. Formation of the active oxidase complex involving p47^{phox}, p67^{phox} and p21^{rac} on the membrane-bound flavocytochrome *b* probably induces a conformational change which is favourable to electron transport and consequently the production of superoxide. It was shown previously that the detergents used to activate the cell-free assay cause p47^{phox} and p67^{phox} to associate and pellet upon centrifugation in the absence of membranes [18]. It is therefore important, when attempting to measure the association of these cytosolic proteins with the membranes, that they are separated not simply by pelleting but by using a sucrose cushion from a denser protein pellet. This was done in the present study, and clearly showed the association of the cytosolic proteins with the plasma membrane.

It has been shown that GDI inhibits the dissociation of GDP and the hydrolysis of GTP by p21^{rac} [23] and CDC42Hs (a ras-related small ϵ -protein with 70% amino acid sequence identity with p21^{rac}) [24]. However, it was uncertain as to which nucleotide was bound to p21^{rac} in the natural state. Small GTP-binding proteins are inactive in the GDP-bound form, and consequently it might be considered probable that they would be maintained in this form in the resting cell. In addition, Ando et al. have shown that recombinant p21^{rac1} loaded with GDP and complexed to rhoGDI suppresses NADPH oxidase activity [24]. However, because, as shown here, the p21^{rac2}/GDI complex is active in the absence of added GTP, it has been suggested that it might be present in the GTP-bound form in the resting cell, possibly maintained in this form by the bound GDI [23,25]. Interestingly, Hancock and Hall [26] have shown that the GTP-bound form of p21^{rac1} can bind rhoGDI at higher affinity than can the GDP-bound form.

We have confirmed, by direct measurement, that in the p21^{rac2}/GDI complex the protein is in the GDP-bound form. This confirms our initial observation that the purified p21^{rac1} complexed to rhoGDI could support the production of superoxide when reconstituted with partially purified NADPH oxidase components [3]. In our current study, no endogenous GTP was detected in the plasma membrane preparation used in the cell-free assay, indicating that p21^{rac} is not activated by nucleotide contaminating the assay from this source. What was unexpected was that the p21^{rac}/GDI complex was active in the cell-free assay in the absence of added GTP, but only after stimulation with SDS. This activity was enhanced approx. 3-fold by the addition of free GTP and almost completely inhibited by GDP. It is not clear why the rac/GDI complex was active in the absence of GTP. It is possible that the native p21^{rac} is held in an active conformation by rhoGDI, or that activation of the NADPH oxidase induces a GTP-like conformation for the lipid-modified p21^{rac}. Further work is required in order to understand the nature of this process. We hypothesize that, in the presence of SDS, the complex is destabilized, allowing p21^{rac2} to partially dissociate from GDI and move together with the other cytosolic proteins to the membrane. Exchange into the GTP form switches it to the fully active conformation, whereas GDP causes it to assume the inactive form. Whether the rate of exchange of GTP/GDP is accelerated by SDS or arachidonic acid, or whether one of the specialized component proteins of the oxidase acts as an exchange factor, remains to be determined. The non-modified recombinant protein cannot activate the oxidase in the absence

of GTP. With the protein in this form the nucleotide cannot be simply added, but must be exchanged in by the addition of EDTA to chelate Mg²⁺, which is then added in excess to lock the nucleotide into the bound form. p21^{rac} is sensitive to nucleotide exchange factors such as GDI and GDS only when it is modified at its C-terminus by an isoprenyl group. It is most likely that the isolated native p21^{rac2} exhibits a high affinity for guanine nucleotides in response to the activation of oxidase due to its lipid moiety at the C-terminus. In contrast, recombinant p21^{rac}, which is not modified at its C-terminus, can reach a high-affinity state for guanine nucleotide binding only at low Mg²⁺ concentrations.

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