Mechanisms of NADPH oxidase activation: translocation of p40_{phox}, Rac1 and Rac2 from the cytosol to the membranes in human neutrophils lacking p47_{phox} or p67_{phox}

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On neutrophil stimulation, the cytosolic components of NADPH oxidase, p67_{phox}, p47_{phox}, p40_{phox}, as well as the Ras-related G-proteins Rac1 and Rac2, are translocated from the cytosol to cell membranes where they associate with a flavocytochrome *b*, forming a functional complex responsible for the production of oxygen radicals in phagocytes. In this paper we show that (a) in neutrophils from a patient with a form of chronic granulomatous disease (CGD) in which p67_{phox} is absent, p47_{phox} and Rac2, but not p40_{phox} and Rac1 were translocated from the cytosol to the membrane on stimulation with formylmethionyl-leucylphenylalanine (fMLP) or phorbol 12-myristate 13-acetate

(PMA); (b) in neutrophils from a patient with a form of CGD in which p47_{phox} is absent, p67_{phox}, p40_{phox} and Rac1 failed to associate with the membrane on stimulation with fMLP or PMA, whereas Rac2 was translocated as in normal neutrophils. We also show that in neutrophils from a patient lacking p67_{phox}, the amount of cytosolic p40_{phox} was decreased by about 40%. These findings indicate that, on neutrophil stimulation, p67_{phox} mediates the translocation of p40_{phox} and Rac1 from the cytosol to cell membranes and that Rac2 associates with the membranes independently of p47_{phox} and p67_{phox}.

INTRODUCTION

NADPH oxidase, the enzyme system that generates bactericidal oxygen free radicals in phagocytes [1–7], is composed of several proteins, some located on the plasma membrane, i.e. cytochrome *b*-558, and others in the cytosol, i.e. p47_{phox}, p67_{phox} and the small G-proteins, Rac1 and Rac2 [2–7]. Recently, a novel 40 kDa protein, named p40_{phox}, has been identified as a new cytosolic component of NADPH oxidase [8,9]. Although p40_{phox} is not required for activity of NADPH oxidase in the cell-free system [10,11], it has been suggested to be important in the regulation of the oxidase complex [8,9,12].

On neutrophil activation, p47_{phox}, p67_{phox} [13–16], p40_{phox} [8], Rac1 [17] and Rac2 [18–20] move to the plasma membrane and associate with cytochrome *b*-558, forming an electron-transport chain responsible for the reduction of molecular oxygen to O₂^{•−} [1–7].

To investigate the relationships between the various NADPH oxidase components during the assembly of the enzyme, two different approaches were used. One involved the study of the interactions between normal and mutated recombinant NADPH oxidase components *in vitro*. Such investigations have shown that, whereas the src homology (SH3) domain of p47_{phox} binds to a proline-rich cytoplasmic region of the small subunit of cytochrome *b*-558, the SH3 sequence of p67_{phox} and p40_{phox} binds to the proline-rich region present on the C-terminus of p47_{phox} [21 and references contained therein, 22,23]. It has also been demonstrated that the C-terminus of p40_{phox}, but not its SH3 domain, is responsible for the tight association of p40_{phox} with p67_{phox} in the cytosol of resting neutrophils [22,23].

The second involved the study of NADPH oxidase assembly in

phagocytes derived from patients affected by different forms of chronic granulomatous disease (CGD), i.e. having a selective lack of NADPH oxidase components. It has been shown that, in the absence of p47_{phox}, p67_{phox} is not translocated to the membrane, whereas in the absence of p67_{phox}, p47_{phox} is normally translocated [14,15,24,25]. Moreover, p40_{phox} [8], like p47_{phox} and p67_{phox} [14,15,24,25], is not translocated to the membrane of cytochrome *b*-558-lacking neutrophils. These findings demonstrate that p67_{phox} is translocated only after the association of p47_{phox} with cytochrome *b*-558, but they do not clarify whether the translocation of p40_{phox} depends on interaction with p47_{phox}, p67_{phox} or cytochrome *b*-558. In patients with a CGD form characterized by a lack of p67_{phox}, but not in those lacking p47_{phox} or cytochrome *b*-558, the p40_{phox} was absent [12] or present but in reduced levels [8,23]. This suggests that the presence of, and perhaps the binding to, p67_{phox} is required to maintain the stability of p40_{phox} [8,12].

Conflicting results have been reported on the interactions occurring between rac G-proteins and the other NADPH oxidase components. Some authors have shown that Rac2 could interact with p47_{phox} or p67_{phox} [19], whereas others have suggested an interaction between Rac2 and cytochrome *b*-558 [26]. It has been demonstrated that, in an *in vitro* system using recombinant peptides, Rac1 interacts specifically with p67_{phox} in a GTP-dependent manner [27,28]. We have recently extended these findings reporting that Rac1 fails to move to the membranes of cells from a patient lacking p67_{phox} [17].

In the present work we have re-investigated the translocations of NADPH oxidase cytosolic components in neutrophils from normal subjects and from two patients affected by CGD forms in which p67_{phox} or p47_{phox} was absent. Our results demonstrate

Abbreviations used: CGD, chronic granulomatous disease; p47_{phox}, p67_{phox} and p40_{phox}, cytosolic phagocyte oxidase factors of 47, 67 and 40 kDa respectively; fMLP, formylmethionyl-leucylphenylalanine; PMA, phorbol 12-myristate 13-acetate; HBSS, Hanks balanced-salt solution.

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that, on stimulation with formylmethionyl-leucylphenylalanine (fMLP) or phorbol 2-myristate 13-acetate (PMA): (i) in $p67_{phox}$ -deficient neutrophils, $p47_{phox}$ and Rac2, but not $p40_{phox}$ and Rac1 are translocated from the cytosol to the membrane; (ii) in $p47_{phox}$ -lacking neutrophils Rac2, but not $p67_{phox}$, $p40_{phox}$ and Rac1, is translocated.

MATERIALS AND METHODS

Materials

PMA and fMLP were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A.; anti-Rac1, anti-Rac2, anti- $p67_{phox}$, anti- $p40_{phox}$ and anti- $p47_{phox}$ rabbit polyclonal antisera were a gift from Dr. F. B. Wientjes, Department of Medicine, University College, London, U.K. [8,20,29,30].

Neutrophil preparation

Human neutrophils were prepared from venous blood of healthy donors as previously described [31]. Cells were resuspended in PBS, treated at 4 °C for 5 min with 1 mM di-isopropyl fluorophosphate (Aldrich), washed and resuspended in Hanks balanced-salt solution (HBSS) buffered with 20 mM Hepes, pH 7.4, and containing 0.5 mM $CaCl_2$ and 5.6 mM glucose.

Metabolic studies

Oxygen consumption was measured at 37 °C with a Clark oxygen electrode using 3×10^7 neutrophils/ml in HBSS containing 2 mM NaN_3 and 5 μ g/ml cytochalasin B when fMLP was used as stimulus.

Neutrophil activation and fractionation

Human neutrophils (3×10^7 /ml) were treated with different stimuli at 37 °C, in a water bath with continuous shaking. Reactions were stopped at the required time points by diluting the cells with a 10-fold excess of ice-cold HBSS containing 1 mM PMSF, 2 mM Na_3VO_4 and 10 μ M phenylarsine oxide. Neutrophils were then centrifuged (500 g; 7 min; 4 °C), resuspended in relaxation buffer (100 mM KCl, 3 mM NaCl, 3.5 mM $MgCl_2$, 1 mM EGTA, 1 mM PMSF, 10 mM Pipes, pH 7.3, containing 2 mM Na_3VO_4 , 10 μ M phenylarsine oxide, 5 μ g/ml pepstatin, 5 μ g/ml leupeptin, 1 mM EDTA and 10 mM NaF), sonicated and subjected to ultracentrifugation at 100 000 g as previously described [13,16,17]. The high-speed supernatant, referred to as the soluble cytosolic fraction, was mixed 4:1 (v/v) with 4 $\times$ electrophoresis sample buffer [60 mM Tris/HCl, pH 6.8, 20% (v/v) glycerol, 4% (w/v) SDS, 2% (v/v) 2-mercaptoethanol]. The pellet was resuspended in 0.4 ml of ice-cold relaxation buffer with vigorous mixing and centrifuged again at 100 000 g for 12 min at 4 °C. The supernatant was discarded and the final pellet, representing cell membranes, was resuspended in 100 μ l of 1 $\times$ sample buffer.

Electrophoresis and immunoblotting

Aliquots of samples from resting and activated neutrophil cytosol (30 μ g/lane) and membranes (50 μ g/lane) were subjected to SDS/PAGE on 12% gels. Proteins were electroblotted as previously described [16,17]. The blots were incubated for 120 min in TBS (50 mM Tris, pH 7.5, 170 mM NaCl) containing 5% (w/v) BSA and 0.2% (v/v) Nonidet P40 (blocking buffer) before incubation overnight with 2–5 μ g/ml anti- $p47_{phox}$, anti- $p67_{phox}$, anti- $p40_{phox}$, anti-Rac1 or anti-Rac2 antibodies in

blocking buffer. After various washes with TBS containing 0.2% (v/v) Nonidet P40, the blots were incubated for 60 min with horseradish peroxidase-labelled anti-rabbit IgG (Amersham), diluted 1:15 000 in blocking buffer. After further washes, bound antibodies were revealed by enhanced chemiluminescence detection reagents (Amersham). When required, blots were stripped as previously described [16].

Studies in patients with CGD

The $p47_{phox}$ - and $p67_{phox}$ -deficient patients were a 29-year-old man and a 33-year-old woman respectively, sharing a clinical history characteristic of CGD (recurrent infectious diseases). No production of H_2O_2 and O_2^- occurred on stimulation of neutrophils from both patients with PMA (100 ng/ml) or fMLP (1 μ M). The Nitro Blue Tetrazolium reduction test was negative in both cases. An immunoblot analysis with specific antibodies demonstrated that both gp91 $_{phox}$ and p22 $_{phox}$ subunits of cytochrome b-558 were normally present in neutrophils from both patients.

RESULTS

To compare the translocation of cytosolic factors from cytosol to the membrane in normal and $p67_{phox}$ - or $p47_{phox}$ -lacking neutrophils, cytosolic and membrane fractions from neutrophils stimulated or not with fMLP (1 μ M; 1 min) or PMA (100 ng/ml; 5 min) were subjected to electrophoresis and immunoblotted with specific anti- $p67_{phox}$, anti- $p47_{phox}$, anti- $p40_{phox}$, anti-Rac1 and anti-Rac2 antibodies. The time points at which the translocation was studied were those at which a maximal rate of NADPH oxidase activity was observed in response to these agonists (results not shown). The results reported in Figure 1 show that, on stimulation: (i) in normal neutrophils, $p47_{phox}$, $p67_{phox}$, $p40_{phox}$, Rac2 and Rac1 were all translocated from the cytosol to the membrane; (ii) in $p47_{phox}$ -deficient cells, Rac2, but not $p67_{phox}$, $p40_{phox}$ and Rac1, associated with the membrane; (iii) in $p67_{phox}$ -deficient neutrophils, $p47_{phox}$ and Rac2, but not $p40_{phox}$ and Rac1, were translocated from the cytosol to the membrane.

Figure 1 also shows that the amount of $p40_{phox}$ was reduced in $p67_{phox}$ -deficient cytosol, but not in that lacking $p47_{phox}$. A densitometric analysis of the bands recognized by anti- $p40_{phox}$ antibody in whole cell lysates showed that in $p67_{phox}$ -deficient neutrophils, $p40_{phox}$ was reduced by 40% compared with normal or $p47_{phox}$ -lacking cells (results not shown). These findings are in agreement with previous reports [8,12,23].

DISCUSSION

In this paper we report that, in neutrophils from a patient lacking $p47_{phox}$, the cytosolic components of NADPH oxidase $p67_{phox}$ and $p40_{phox}$ are not translocated from the cytosol to the membrane on stimulation with fMLP or PMA. These findings suggest that $p40_{phox}$ is unable to associate directly with cytochrome b and that $p47_{phox}$ or $p67_{phox}$ is required for the binding of $p40_{phox}$ to the membrane. Moreover, we also show that, in activated neutrophils lacking $p67_{phox}$, whereas $p47_{phox}$ is translocated, $p40_{phox}$ is not. This might be because of the reduced levels of $p40_{phox}$ seen in these patients, caused perhaps by the instability of this molecule in the absence of $p67_{phox}$ [8,12,23], or because $p67_{phox}$ is required for the association of $p40_{phox}$ with the membrane. If this is the case, the absence of $p40_{phox}$ translocation in $p47_{phox}$ -deficient neutrophils may be due to the inability of $p67_{phox}$ to bind to the membrane when $p47_{phox}$ is missing [15,24,25]. The hypothesis of a requirement of $p67_{phox}$ for $p40_{phox}$

Figure 1 Translocation of p67_{phox}, p47_{phox}, p40_{phox}, Rac1 and Rac2 from cytosol to the membranes of normal, p47_{phox}⁻ and p67_{phox}⁻ deficient human neutrophils

Neutrophils (3×10^7 /ml) from normal donors (N) or from CGD patients lacking p47_{phox} (—p47) or p67_{phox} (—p67) were incubated at 37 °C with agitation with fMLP (1 μ M; 1 min) or PMA (100 ng/ml; 5 min); the reactions were stopped and the cells were sonicated and fractionated as described in the Materials and methods section. The membrane and cytosol fractions were subjected to SDS/PAGE on a 12% gel, and immunoblotted with anti-p67_{phox}, anti-p47_{phox}, anti-p40_{phox}, anti-Rac1 and anti-Rac2 polyclonal antibodies.

translocation is also supported by the previous findings that in resting neutrophils cytosolic p40_{phox} is complexed with p67_{phox} [8,9,12,23]. On this basis, one might speculate that p67_{phox} could function as a chaperone which, on neutrophil stimulation, carries p40_{phox} from the cytosol to the membrane. The complex p40_{phox}–p67_{phox} could then associate with the membrane via interaction between p67_{phox} and cytochrome-bound p47_{phox}. Our findings, however, do not exclude the possibility that p40_{phox} might also directly interact with p47_{phox}. In fact, it may be that, as mentioned above, in the absence of its partner protein p67_{phox}, the cytosolic free p40_{phox}, besides being decreased, is also conformationally changed so that it becomes unable to bind to p47_{phox}. The results obtained with recombinant NADPH oxidase components showing that p40_{phox} can bind p47_{phox} through its SH3 domain [22] are consistent with this hypothesis. Also in this case, however, p67_{phox} would be required to maintain p40_{phox} in the correct shape for its interaction with p47_{phox}. Such interaction may take place in both resting, where p47_{phox}, p67_{phox} and p40_{phox} could form a cytosolic complex [22,23], and activated neutrophils during NADPH oxidase assembly.

In this paper we also report that Rac1 and Rac2 are normally expressed in neutrophils from CGD patients lacking p67_{phox} or p47_{phox}, and we confirm our previous findings that, in p67_{phox}-deficient neutrophils stimulated with fMLP or PMA, Rac1 does not associate with the membrane in spite of normal translocation of p47_{phox} [17]. Moreover, we show that Rac1 is not translocated in neutrophils that lack p47_{phox}, a condition under which p67_{phox} cannot bind to the membrane. This further demonstrates that Rac1 indeed interacts *in vivo* directly with p67_{phox}, as suggested by previous *in vitro* experiments with recombinant peptides [27,28]. In contrast, Rac2 is normally translocated to the membrane in the absence of either p47_{phox} or p67_{phox}. These results are in agreement with those of others [26], showing that neither p47_{phox} nor p67_{phox} is required for the association of Rac2 with the membrane. The same authors [26] report that, in the absence of cytochrome *b*-558, Rac2 translocation is greatly depressed, suggesting an association between this G-protein and cytochrome *b*.

Collectively, our data and those of others allow us to propose two models of interaction between NADPH oxidase components

Figure 2 Two models of interaction between NADPH oxidase components on the membranes of activated neutrophils

Both models are discussed in the text. p22 and gp91 represents the α - and β -subunits of cytochrome *b*-558 of 22 kDa and 91 kDa respectively.

on the membranes of activated neutrophils (Figure 2). In the first model, p40_{phox} is complexed with p67_{phox}, and this latter protein binds p47_{phox}, which in turn associates with cytochrome *b*-558 [14,15,23–25,32]. This model is substantiated by recent evidence that recombinant p47_{phox} binds p67_{phox} but not p40_{phox} on blots of neutrophil cytosol and PMA-activated membranes [23]. In the second model (Figure 2), on neutrophil activation, p40_{phox} binds both p47_{phox} and p67_{phox}, and this complex is bound to cytochrome *b*-558 through p47_{phox}. This model is supported by the

findings of Fuchs et al. [22] that p40_{phox} can interact *in vitro* with both p47_{phox} and p67_{phox}. If this is true, our results showing that, in p67_{phox}-lacking neutrophils, p47_{phox} is translocated but p40_{phox} is not suggest that in this case also p67_{phox} is required to mediate the interaction between p40_{phox} and p47_{phox}.

Regarding the Rac G-proteins, in both models (Figure 2), once activated, Rac1 binds specifically to p67_{phox} [17,27,28] before [33] or after [27,34] translocation of p47_{phox} and p67_{phox}. In contrast, Rac2 interacts directly with cytochrome *b-558*, i.e. independently of p47_{phox} and p67_{phox} translocation [26,32].

Further investigations are required to elucidate fully the complicated interactions occurring among the different NADPH oxidase factors, and to clarify their functional relevance.

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