Mechanisms of NADPH oxidase activation in human neutrophils: p67^{phox} is required for the translocation of rac 1 but not of rac 2 from cytosol to the membranes

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NADPH oxidase is the enzyme complex responsible for the production of oxygen radicals in phagocytes. On neutrophil stimulation, the cytosolic components of NADPH oxidase, p67^{phox} and p47^{phox}, as well as the Ras-related G-protein rac 2, are translocated from the cytosol to cell membranes where they associate with a flavocytochrome *b* to form a functional complex. Besides rac 2, rac 1 G-protein is also involved in the activation of the NADPH oxidase, but, to date, it has not been documented whether it is also translocated in activated neutrophils. In this paper we show that: (a) in neutrophils stimulated with formylmethionyl-leucylphenylalanine, concanavalin A or phorbol 12-myristate 13-acetate, both rac 1 and rac 2 are translocated from cytosol to the membranes; (b) in neutrophils from

a patient with a form of chronic granulomatous disease in which p67^{phox} is absent, rac 2 and p47^{phox} were translocated as in normal neutrophils on stimulation with the above agonists, but rac 1 failed to be translocated from the cytosol to the membranes. This is the first demonstration that, in activated neutrophils, rac 1 is translocated from the cytosol to the membranes and this translocation requires p67^{phox}. These results, coupled with those showing that rac 2 is not translocated in activated neutrophils lacking p47^{phox} [El Benna, Ruedi and Babior (1994) *J. Biol. Chem.* **269**, 6729–6734], may suggest that the assembly of the cytosolic components of NADPH oxidase on the plasma membrane takes place through selective coupling of activated rac 1 and rac 2 with p67^{phox} and p47^{phox} respectively.

INTRODUCTION

The Ras-related small G-proteins are ubiquitous proteins that possess an intrinsic GTPase activity. Their activation results from the exchange of bound GDP for GTP, promoting a conformational change and an interaction with putative effector molecules, thus generating signals in the cell [1]. However, these signals are largely unknown. Recently, it has been reported that the Ras-related G-proteins, rac 1 and rac 2, are essential components of NADPH oxidase, the enzyme system responsible for the generation of the bactericidal oxygen free radicals in phagocytes [2–10]. NADPH oxidase is composed of several proteins, some located on the plasma membrane, e.g. cytochrome *b*-558, and others in the cytosol, e.g. p47^{phox}, p67^{phox} and the small G-proteins, rac 1 and rac 2 [11–17]. The importance of these components for NADPH oxidase function is illustrated by the fact that, in the inherited condition chronic granulomatous disease, the absence of cytochrome *b* (X-linked form), p47^{phox} or p67^{phox} (autosomal forms) results in a clinical condition typified by severe infections [18,19].

Activation of NADPH oxidase involves the translocation of cytosolic factors p47^{phox} and p67^{phox} to the plasma membrane, where they associate with cytochrome *b*-558, forming an electron-transport chain responsible for the reduction of molecular O₂ to O₂^{•-} [11–17]. Recently, it has been reported that rac 2 is also translocated from the cytosol to the plasma membrane on neutrophil stimulation with formylmethionyl-leucylphenylalanine (fMLP) or phorbol 12-myristate 13-acetate (PMA) [17,20,21], but until now, translocation of rac 1 has not been demonstrated. Little is known, however, about the interactions

that occur between the cytosolic factors in the translocation process. It has been shown that, in the absence of p47^{phox}, p67^{phox} is not translocated, whereas in the absence of p67^{phox}, p47^{phox} is normally translocated to the membranes [22]. Conversely, a recent study in which the translocation of recombinant p67^{phox} and p47^{phox} was investigated in a cell-free system demonstrated that maximal translocation of either protein is achieved only in the presence of the other [23]. Finally, it has been reported that rac 2 fails to be translocated to the membranes of PMA-stimulated neutrophils in the absence of p47^{phox} [17], but it is not known whether the translocation of rac 2 directly depends on interaction with p47^{phox} or p67^{phox}.

In the present paper we have investigated the translocation of p47^{phox}, p67^{phox}, rac 1 and rac 2 in neutrophils from normal subjects, and that of p47^{phox}, rac 1 and rac 2 in neutrophils from a patient with a rare form of chronic granulomatous disease in which p67^{phox} is absent (for the characteristics of these deficient neutrophils see ref. [24]) stimulated with fMLP, PMA or concanavalin A (ConA). The results show that (i) in normal neutrophils, in addition to p47^{phox} and p67^{phox}, both rac 1 and rac 2 are translocated from the cytosol to the membrane and (ii) in p67^{phox}-lacking neutrophils p47^{phox} and rac 2, but not rac 1, are translocated.

MATERIALS AND METHODS

Materials

PMA, fMLP and ConA were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. SDS, acrylamide, *NN'*-methylenebisacrylamide, tetramethylenediamine and nitrocellulose mem-

branes were obtained from Bio-Rad, Richmond, CA, U.S.A. Ficoll 400 and molecular-mass standards were from Amersham International, Amersham, Bucks., U.K. Anti-rac 1, anti-rac 2, anti-p67^{phox} and anti-p47^{phox} rabbit polyclonal antisera were kindly given by Dr. A. W. Segal, Department of Medicine, University College, London, U.K. [21,25,26]. Anti-rac 1 (C-11) and anti-rac 2 (C-11) affinity-purified rabbit polyclonal antibodies raised against a peptide mapping within the C-terminal domain of the predicted human *rac1* and *rac2* gene product respectively, and anti-rac 1/rac 2 (C-13), an affinity-purified rabbit polyclonal antibody raised against a C-terminal peptide common to human *rac1* and *rac2*, were from Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.

Neutrophil preparation

Human neutrophils were prepared from venous blood of healthy donors as previously described [27]. Cells were resuspended in PBS, treated at 4 °C for 5 min with 1 mM di-isopropyl fluorophosphate (Sigma), washed and resuspended in Hanks balanced-salt solution buffered with 20 mM Hepes (pH 7.4) and containing 0.5 mM CaCl₂ and 5.6 mM glucose.

Metabolic studies

O₂ consumption was measured at 37 °C with a Clark O₂ electrode using 3 × 10⁷ neutrophils/ml in Hanks balanced-salt solution containing 2 mM NaN₃ and 5 µg/ml cytochalasin B. When PMA was used as stimulus, cytochalasin B was omitted.

Neutrophil activation and fractionation

Human neutrophils (3 × 10⁷/ml) were treated with different stimuli for various lengths of time at 37 °C, in a water-bath with continuous shaking. Reactions were stopped by diluting the cells with a 10-fold excess of ice-cold Hanks balanced-salt solution containing 1 mM PMSF, 2 mM Na₃VO₄ and 10 µM 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 MgCl₂, 1 mM EGTA, 1 mM PMSF, 10 mM Pipes, pH 7.3, containing 2 mM Na₃VO₄, 10 µM phenylarsine oxide, 5 µg/ml pepstatin, 5 µg/ml leupeptin, 1 mM EDTA, 10 mM NaF), sonicated and subjected to ultracentrifugation at 100 000 g as previously described [24,28]. The high-speed supernatant (cytosolic fraction) was mixed 4:1 (v/v) with 4 × 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 again centrifuged at 100 000 g for 12 min at 4 °C. The supernatant was discarded and the final pellet, representing cellular membranes, was resuspended in 100 µl of 1 × sample buffer.

Electrophoresis and immunoblotting

Aliquots of samples from resting and activated neutrophil cytosol (30 µg/lane) and membranes (50 µg/lane) were subjected to SDS/PAGE on 14% gels as described by Laemmli [29]. Proteins were electroblotted as previously described [24,28]. 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 2–10 µg/ml anti-p47^{phox}, anti-p67^{phox}, anti-rac 1, anti-rac 2 or anti-rac 1/rac 2 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 International), diluted 1:15000 in blocking buffer. After further washes, bound antibodies were revealed by enhanced chemiluminescence detection reagents (Amersham International). When required, blots were stripped as previously described [28].

RESULTS

In order to compare the translocation from cytosol to the membranes of cytosolic factors in normal and p67^{phox}-deficient neutrophils, cytosolic and membrane fractions from neutrophils resting or stimulated with fMLP (1 µM; 20 s and 1 min), PMA (100 ng/ml; 4 min) or ConA (300 µg/ml; 4 min) were subjected to electrophoresis and immunoblotted with specific anti-p67^{phox}, anti-p47^{phox}, anti-rac 1 and anti-rac 2 polyclonal antibodies. The time points at which the translocation was studied were chosen because they corresponded to those at which a maximal rate of NADPH oxidase activity was observed in response to these agonists (not shown). The results are shown in Figure 1. On stimulation with fMLP, ConA or PMA (i) in normal neutrophils, p47^{phox}, p67^{phox}, rac 2 and rac 1 were all translocated from the cytosol to the membranes, and (ii) in p67^{phox}-deficient neutrophils, p47^{phox} and rac 2, but not rac 1, were translocated from the cytosol to the plasma membrane.

rac 1 and rac 2 were identified in cytosol and membrane fractions by using two different anti-rac 1 and anti-rac 2 antibodies (see the Materials and methods section). Moreover, an anti-rac 1/rac 2 antibody, raised against a C-terminal peptide common to both proteins, recognized a 21–22 kDa protein doublet in immunoblots of cytosol and stimulated membrane fractions of normal neutrophils. A comparison of this doublet with the bands detected using single anti-rac 1 or anti-rac 2 antibodies indicated that the upper band represented rac 1 and the lower band rac 2. Figure 1 illustrates that this doublet was also recognized by anti-rac 1/rac 2 antibody in cytosol of p67^{phox}-deficient neutrophils. However, in PMA-, fMLP- and ConA-stimulated membranes of these neutrophils, this antibody recognized only the lower band (rac 2), confirming that in such defective cells, rac 2 was translocated in response to neutrophil stimulation, whereas rac 1 was not.

DISCUSSION

In this paper, we report that the stimulation of normal neutrophils with different agonists led to the translocation of rac 1 in addition to that of p67^{phox}, p47^{phox} and rac 2. This is the first demonstration that both small G-proteins are translocated during neutrophil activation. In the absence of p67^{phox}, however, rac 1 failed to move from the cytosol to the membrane on neutrophil stimulation, in spite of normal translocation of rac 2 and p47^{phox}. It has been recently shown *in vitro* using recombinant peptides that rac 1 interacted directly with p67^{phox} in a GTP-dependent manner [30]. Our finding that rac 1 was not translocated in the absence of p67^{phox} in a whole-cell system is consistent with these observations. It therefore seems reasonable to postulate, on the basis of the fact that the translocation of rac 1 requires the presence of p67^{phox}, that the two proteins either interact in the cytosol of resting cells or associate on neutrophil activation, thereby fulfilling the prerequisite for co-ordinated translocation. We favour the second possibility in view of the fact that association of rac 1 with p67^{phox} appears to depend on prior activation of rac 1 [30]. We have tried without success to co-immunoprecipitate rac 1 and p67^{phox} from the cytosol and membranes of resting and activated neutrophils.

Figure 1 Translocation of p67^{phox}, p47^{phox}, rac 1 and rac 2 from cytosol to the membranes of normal and p67^{phox}-deficient human neutrophils

Neutrophils (3×10^7 /ml) were incubated at 37 °C with agitation with fMLP (1 μ M), ConA (300 μ g/ml) or PMA (100 ng/ml); the reactions were stopped at the indicated times 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 14% gel, and immunoblotted with a mixture of anti-p67^{phox} and anti-p47^{phox} polyclonal antibodies (panel A), anti-rac 1 (panel B), anti-rac 2 (panel C) or anti-rac 1/rac 2 (panel D) polyclonal antibodies.

In the present paper we found that rac 2 was normally translocated to the membranes in the absence of p67^{phox}. It has previously been reported [17] that rac 2 was not translocated in neutrophils from a patient with chronic granulomatous disease, which lacked p47^{phox}. This might indicate that p47^{phox} is required for the translocation of rac 2. However, as in the absence of p47^{phox}, p67^{phox} also failed to be translocated [22], it could not be excluded that p67^{phox} might also be required for the translocation of rac 2. Nevertheless, our finding that the translocation of rac 2 took place independently of p67^{phox} rules out this latter possibility. Thus a direct role for p47^{phox} in the translocation of rac 2 is emerging. Our data, coupled with those of others [17,30], suggest that there may be a selective relationship between p47^{phox} and rac 2 and between p67^{phox} and rac 1. In this scenario, rac 1 or rac 2 could interact with specific NADPH oxidase components in the cytosol and then be translocated as a complex, or associate in the membrane after independent translocation. Whichever is the case, previous activation of the small G-proteins may be required for such interaction [30]. This suggests that an essential step of the signalling pathway for the assembly of the components and activation of NADPH oxidase might be the activation of rac 1 and rac 2. Further investigations are required to demonstrate directly a physical association between Rac G-proteins and the NADPH oxidase cytosolic factors, and to clarify the mechanisms of activation of rac 1 and rac 2.

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