Activation of NADPH oxidase of human neutrophils involves the phosphorylation and the translocation of cytosolic p67phox

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Activation of human neutrophil NADPH oxidase requires the interaction of cytosolic and membrane-associated components. Evidence has been accumulated that in phorbol 12-myristate 13-acetate (PMA)-stimulated neutrophils, the translocation to the plasma membrane of the cytosolic components p47phox and p67phox and the phosphorylation of p47phox are essential steps in activation of NADPH oxidase. No direct evidence has been presented to date as to whether p67phox is also phosphorylated. To address this problem we have immunoprecipitated p67phox from neutrophil cytosol and membrane fractions. The results indicate that, very soon after activation with PMA (20 s), p67phox was present in a phosphorylated form in the cytosol and in the membranes. At later times (1–3 min) the extent of p67phox phosphorylation continuously increased both in the

cytosol and in the membrane fraction, while oxygen consumption reached the maximal rate within 40 s, and then remained linear. p67phox was also phosphorylated in formyl-methionyl-leucyl-phenylalanine-activated neutrophils. That the phosphorylated p67 protein we identified in immunoprecipitation experiments was p67phox was confirmed by the observation that no phosphorylated band of 67 kDa was immunoprecipitated from the cytosol and membranes of PMA-stimulated neutrophils from a p67phox-deficient chronic granulomatous disease patient. In this case, p47phox was normally phosphorylated. These data demonstrate that: (1) the phosphorylation of p67phox is correlated with activation of NADPH oxidase, and (2) continuous phosphorylation of p67phox is required in order to maintain the linearity of the respiratory burst.

INTRODUCTION

Phagocyte NADPH oxidase is a multicomponent enzyme composed of a membrane-bound cytochrome b_{558} and at least three cytosolic factors: p47phox, p67phox and a small GTP-binding protein [1–4]. The importance of the oxidase components in the activation of the respiratory burst is underlined by the inherited condition chronic granulomatous disease (CGD), wherein the absence of cytochrome b (X-linked form) and of p47phox or p67phox (autosomal forms) results in a clinical condition typified by frequent and severe infections [5,6].

The specific functions of the cytosolic factors have not been established, but it is widely accepted that translocation of p47phox from the cytosol to the plasma membrane is essential for the activation of NADPH oxidase [7–9]. Moreover, in phorbol 12-myristate 13-acetate (PMA)-stimulated neutrophils, the phosphorylation of p47phox is a prerequisite for its translocation to the plasma membrane and for activation of NADPH oxidase [8,10–12], and continuous phosphorylation of this protein is necessary to maintain NADPH oxidase in an activated state [13,14]. The translocation of p67phox is also essential for the activation of NADPH oxidase [7,8], but, to our knowledge, no direct evidence has been presented to date as to whether this protein is also phosphorylated in the process of activation of this enzymic complex. In a recent paper [15] we reported that the phosphorylation of a 67 kDa protein on the neutrophil plasma membrane was correlated with the activation of NADPH oxidase and with the translocation of p67phox from the cytosol to the plasma membrane, but this 67 kDa phosphoprotein was not specifically identified. The present paper deals with this identification. By immunoprecipitation with an anti-p67phox specific antibody, a clear demonstration has been obtained that

the activation of NADPH oxidase is associated with phosphorylation of p67phox in the cytosol and its translocation to the plasma membrane. The time course of p67phox phosphorylation compared with that of the respiratory burst suggests that continuous p67phox phosphorylation is required in order to maintain the linearity of the respiratory burst. Moreover, data obtained with neutrophils from a CGD form lacking p67phox demonstrate that the presence of p67phox is not necessary for the translocation and phosphorylation of p47phox.

MATERIALS AND METHODS

Materials

Phorbol 12-myristate 13-acetate (PMA) and formyl-methionyl-leucyl-phenylalanine (FMLP) were purchased from Sigma Chemical Co., St Louis, MO, U.S.A. SDS, acrylamide, NN' -methylenebisacrylamide, tetramethylethylenediamine and blotting nitrocellulose membranes were purchased from Bio-Rad, Richmond, CA, U.S.A. Ficoll 400 and molecular mass standards were from Amersham.

Neutrophil preparation

Human neutrophils were prepared from the venous blood of healthy donors and of a patient with CGD as previously described [16]. Cells, suspended in PBS, were treated at 4 °C for 10 min with 2 mM di-isopropyl fluorophosphate (Sigma Chemical Co.), washed and suspended to a final concentration of 60×10^6 /ml in Hank's balanced salt solution containing 20 mM Hepes, 0.5 mM CaCl_2 and 5.6 mM glucose (pH 7.4).

Abbreviations used: PMA, phorbol 12-myristate 13-acetate; FMLP, formyl-methionyl-leucyl-phenylalanine; CGD, chronic granulomatous disease; DOC, sodium deoxycholate.

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Metabolic studies

Oxygen consumption was measured at 37 °C with a Clark oxygen electrode using 25×10^6 neutrophils/ml in Hanks' balanced salt solution containing 2 mM $\text{Na}_2\text{S}_2\text{O}_3$ and 0.5 mM CaCl_2 .

Neutrophil activation and fractionation

Human neutrophils (60×10^6 /ml) were suspended in phosphate-free Hank's balanced salt solution containing 1 mg/ml glucose and 0.025 % (w/v) BSA, and were incubated for 60 min, in the presence or in the absence of 500 $\mu\text{Ci}/\text{ml}$ [^{32}P]P_i (Amersham). At the end of incubation the cells were washed twice and resuspended in Hank's balanced salt solution containing 0.5 mM CaCl_2 and 1 mg/ml glucose.

Neutrophils were then stimulated with PMA (100 ng/ml) or FMLP (100 nM) in Hank's balanced salt solution containing 2 mM $\text{Na}_2\text{S}_2\text{O}_3$ and 0.5 mM CaCl_2 in a water bath with continuous shaking. After different time periods, cells were diluted with a 10-fold excess of ice-cold Hank's balanced salt solution containing 0.5 mM CaCl_2 , centrifuged (4 °C) at 500 *g* for 7 min, and finally suspended in 0.4 ml of relaxation buffer (100 mM KCl, 3 mM NaCl, 3.5 mM MgCl_2 , 1.25 mM EGTA, 3 mM phenylmethanesulphonic acid, 10 mM Pipes, pH 7.3, containing 20 $\mu\text{g}/\text{ml}$ leupeptin, 20 $\mu\text{g}/\text{ml}$ pepstatin, 5 mM EDTA, 10 mM NaF and 0.5 mM okadaic acid). The cells were then disrupted by two 15 s cycles of sonication at 200 W (4 °C) using a Labsonic 1510 Sonicator. Unbroken cells and nuclei were pelleted by centrifugation at 500 *g* for 5 min at 4 °C. The supernatant was then centrifuged at 100 000 *g* for 12 min at 4 °C in a Beckman L5-50B ultracentrifuge. The high-speed supernatant, representing the soluble cytosolic fraction, was mixed 1:1 (v/v) with a 2-fold-concentrated solubilization buffer [50 mM Tris, pH 7.5, 300 mM NaCl, 2 % (v/v) Triton X-100, 2 % (w/v) sodium deoxycholate (DOC), 0.2 % (w/v) SDS]. 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 the membrane fraction, was resuspended in 400 μl of solubilization buffer [25 mM Tris, pH 7.5, 150 mM NaCl, 1 % (v/v) Triton X-100, 1 % (w/v) DOC, 0.1 % (w/v) SDS].

Immunoprecipitation, electrophoresis and immunoblotting

Cytosol and membranes (50×10^6 cell equivalents) were incubated for 60 min on ice with solubilization buffer and then centrifuged at 15 000 *g* for 5 min to remove insoluble material. The samples were then incubated for 60 min at 4 °C under rotation with 50 μl of 10 % (w/v) *Staphylococcus aureus* (Pansorbin; Calbiochem Co., La Jolla, CA, U.S.A.) to minimize non-specific binding. *Staphylococcus* cells were then pelleted and discarded, and each supernatant was incubated with 3 μl of specific anti-p67phox rabbit antibodies for 60 min on ice. The antibodies were kindly provided by Dr. A. W. Segal (Department of Medicine, University College, London, U.K.) [17,18]. The samples were then incubated for another 60 min at 4 °C under rotation after addition of 10 μl of 10 % (w/v) Pansorbin. After centrifugation (5 min in Microfuge at full speed), the supernatant was discarded and the pellet was washed three times with 700 μl of solubilization buffer. The immunoprecipitated proteins were eluted by boiling the *Staphylococcus aureus* pellet for 3 min at 95 °C in 50 μl of electrophoresis sample buffer [60 mM Tris/HCl, 20 % (v/v) glycerol, 4 % (w/v) SDS, 2 % (v/v) 2-mercaptoethanol, pH 6.8]. The *Staphylococcus* cells were pelleted by centrifugation and the supernatant was subjected to SDS/10 %-PAGE according to

Laemmli [19]. The slab gel containing the ^{32}P -labelled immunoprecipitated material was dried under vacuum in a Bio-Rad 583 gel dryer and autoradiographed using Kodak X-Omat AR film.

Proteins immunoprecipitated from unlabelled neutrophils were transferred from the gels to nitrocellulose membranes (Bio-Rad, Richmond, CA, U.S.A.) using a Bio-Rad Trans Blot Cell apparatus; blotting was performed at 500 mA for 180 min in 25 mM Tris, 192 mM glycine and 20 % (v/v) methanol, pH 8.3. To confirm that comparable amounts of proteins had been transferred to nitrocellulose membranes, proteins were revealed on the nitrocellulose membranes by staining with 0.02 % (v/v) Ponceau S (Sigma) for 1 min. The blots were rinsed with water and incubated for 120 min in blocking buffer [3 % (w/v) BSA and 0.025 % (v/v) Tween 20 in PBS, pH 7.4], before incubation overnight at 4 °C with purified rabbit anti-p67phox antibodies, which were diluted 1:300 in blocking buffer. The blots were rinsed with three changes of PBS containing 0.1 % (v/v) Tween 20, then twice with PBS alone, and then incubated for 60 min in horseradish peroxidase-labelled donkey anti-rabbit IgG (Amersham) at a dilution of 1:15 000 in blocking buffer (room temperature). After further washing, bound antibodies were detected using enhanced chemiluminescence (ECL) Western blotting detection reagents (Amersham).

Studies of p67phox phosphorylation in a patient with CGD

The patient was a 31-year-old woman, with a clinical history characteristic of CGD (recurrent infectious diseases, including a latero-cervical lymphadenitis and fungal abscesses). The production of H_2O_2 and O_2^- did not occur on stimulation of CGD neutrophils with PMA (100 ng/ml) or FMLP (100 nM). The NitroBlue Tetrazolium reduction test was negative. The amount of cytochrome b_{558} was calculated by measuring the difference spectrum (oxidized minus reduced) of the membrane fraction, as previously reported [20]. For immunoblot analysis of gp91phox, p22phox, p67phox and p47phox, normal and CGD neutrophils were pelleted and resuspended directly in boiling electrophoresis sample buffer (see above). After warming at 100 °C for 5 min, samples (150 $\mu\text{g}/\text{well}$) were subjected to SDS/PAGE and immunoblotted as described above, with the difference that the anti-gp91phox and anti-p22phox antibodies were used at a dilution of 1:500 and were detected by horseradish peroxidase-labelled anti-mouse antibodies (Amersham) diluted 1:3000 in blocking buffer. The anti-gp91phox and anti-p22phox antibodies were kindly provided by Dr. A. J. Verhoeven (Laboratory for Experimental and Clinical Immunology, University of Amsterdam, The Netherlands); the anti-p47phox antibodies were generously provided by Dr. A. W. Segal. This latter antibody was raised to the whole p47phox recombinant protein, while the anti-p67phox antibody was against the C-terminal peptide.

Northern blot analysis demonstrated that p67phox mRNA expression was normal in CGD neutrophils (results not shown). The immunoprecipitation of p47phox and p67phox from CGD neutrophils was performed as described above.

RESULTS

In the present studies we have analysed the translocation and phosphorylation of cytosolic and membrane-associated p67phox by immunoprecipitation of this protein with specific antibodies. Figure 1 shows the results of a typical experiment that is representative of four. Figure 1(a) shows the translocation of p67phox immunoprecipitated from fractionated resting and PMA-stimulated neutrophils and detected by immunoblotting. In resting cells p67phox was present only in the cytosol fraction,

Figure 1 Translocation and phosphorylation of p67phox in human neutrophils

(a) Translocation of p67phox to the plasma membrane. The cells ($25 \times 10^6/\text{ml}$) were incubated at 37°C with PMA (100 ng/ml) for 3 min, then blocked, sonicated and fractionated. The membrane (M) and cytosol (C) fractions were immunoprecipitated with anti-p67phox as described in the Materials and methods section. The immunoprecipitated material was subjected to electrophoresis on SDS/10% PAGE. Proteins were electroblotted to nitrocellulose paper, probed with anti-p67phox antiserum, incubated with horseradish peroxidase-labelled donkey anti-rabbit IgG, exposed to enhanced chemiluminescence detection reagents and visualized on X-ray film. The location of a protein of 67 kDa molecular mass is indicated. UN, unstimulated cells. (b) Phosphorylation of p67phox in PMA-stimulated neutrophils. Some of the neutrophils from the same donor as in (a) were prelabelled with ^{32}P and then activated, blocked at 3 min, fractionated, immunoprecipitated with anti-p67phox or rabbit irrelevant antibodies (Control Ab), and subjected to SDS/PAGE as described in (a). The slab gel was then dried under vacuum and autoradiographed. The location of the 67 kDa band is indicated. (c) Phosphorylation of p67phox in FMLP-stimulated neutrophils. The protocol was the same as described for (b), except that neutrophils were stimulated with FMLP (100 nM) and blocked at 1 min, i.e. when the respiratory burst measured in a parallel assay was linear.

while in activated neutrophils this protein was present in both the cytosolic and membrane fractions. These data confirm previous reports that p67phox translocates from the cytosol to the membranes after stimulation of neutrophils [7,8].

To study the phosphorylation of p67phox, some of the neutrophils from the same donor as the unlabelled cells used for the above-described immunoblot were labelled with ^{32}P and then activated, fractionated, immunoprecipitated and subjected to SDS/PAGE (for details see the Materials and methods section). Figure 1(b) shows autoradiography of p67phox immuno-

Figure 2 Kinetics of p67phox phosphorylation in neutrophil membrane and cytosol fractions

(a) The protocol was as in Figure 1(b). The cells were activated with PMA (100 ng/ml), then blocked at the indicated times, fractionated, immunoprecipitated with anti-p67phox or rabbit irrelevant antibodies (Control Ab) from cytosol (C) and membrane (M) fractions, and subjected to electrophoresis. The gel was then dried and autoradiographed. The 67 kDa molecular mass protein is indicated. UN, unstimulated neutrophils. (b) Traces of oxygen consumption registered in a parallel assay are reported. Numbers indicate the time in min at which the sampling was carried out.

precipitated from the cytosol and membranes of ^{32}P -prelabelled neutrophils. It can be seen that p67phox was present in a phosphorylated form in both the cytosol and membrane fractions of PMA-activated neutrophils, while in the same fractions from unstimulated cells it was not phosphorylated. Similar results were obtained using FMLP as stimulant (Figure 1c).

Figure 2 shows the time course of p67phox phosphorylation in resting and PMA-stimulated neutrophils. The data demonstrate that, in resting cells, p67phox was not phosphorylated in either the cytosol or the membrane fraction over the entire observation period. On the other hand, in PMA-stimulated neutrophils the phosphorylation of p67phox in both cytosol and membranes was detectable 20 s after activation and this phosphorylation increased continuously with time up to 3 min. Contemporaneous measurement of the respiratory burst showed that this process started between 20 and 30 s, reached a maximal rate within 40 s and then continued linearly up to 3 min.

To further demonstrate that p67phox becomes phosphorylated in neutrophils activated by PMA, we studied a case of an autosomal CGD form lacking p67phox. Spectral analysis and immunoblot experiments performed with anti-gp91phox and anti-p22phox specific antibodies demonstrated that both the cytochrome b_{558} subunits were normally present in CGD neutrophils (Figure 3). In contrast, an immunoblot performed with anti-p47phox and anti-p67phox specific antibodies showed a complete and selective lack of p67phox in CGD neutrophils. Figure 4 shows immunoprecipitation of phosphorylated p67phox

Figure 3 Analysis of cytochrome b_{558} spectra and immunoblotting of gp91phox, p22phox, p67phox and p47phox in normal and CGD neutrophils

The difference spectrum (oxidized minus reduced) of cytochrome b_{558} (a) was measured from normal (A) and CGD (B) neutrophils as described in the Materials and methods section. For the immunoblot screening of gp91phox and p22phox (b) and p47phox and p67phox (c), normal (lanes 1) and CGD (lanes 2) whole neutrophils were pelleted and resuspended directly in boiling sample buffer, warmed at 100 °C for 5 min and then subjected to electrophoresis (150 μ g of protein was charged on each well) and immunoblotting with specific antibodies (for details see the Materials and methods section).

and p47phox from normal and CGD neutrophils. No 67 kDa bands are phosphorylated in the cytosol or membranes of these defective PMA-stimulated neutrophils. However, p47phox was normally phosphorylated in both the cytosol and membrane fractions.

DISCUSSION

While the phosphorylation and translocation of p47phox have been demonstrated in many laboratories [8,10,11,13–15,21–23] to be involved in the activation of NADPH oxidase, the enzymic system responsible for the respiratory burst in leucocytes, no clear demonstration has been presented to date that p67phox, as well as being translocated, is also phosphorylated.

It has been reported that a protein in the 59–69 kDa range is markedly phosphorylated when neutrophils are stimulated with known activators of NADPH oxidase [21–23], and that the kinetics of this phosphorylation are consistent with those of the respiratory burst [24–26]. Nevertheless, no data are available to demonstrate that this 59–69 kDa phosphorylated protein is the p67phox cytosolic factor. In a recent paper [15], we observed that the stimulation of neutrophils with different NADPH oxidase agonists, such as PMA, FMLP and concanavalin A, is accompanied by a large enhancement of the phosphorylation of two proteins of 47 kDa and 67 kDa molecular mass, and that this phosphorylation correlated with the appearance of the respiratory burst. Moreover, with all the above agonists, the appearance of the phosphorylated 67 kDa protein at the plasma membrane was associated with the translocation of p67phox to the membrane, as identified using specific antibodies. Further-

Figure 4 Autoradiography of phosphorylated p67phox and p47phox immunoprecipitated from the cytosol (C) and membranes (M) of neutrophils from an autosomal CGD form lacking p67phox

(a) The protocol was the same as in Figure 1(b), except that the immunoprecipitation was performed with either anti-p67phox or anti-p47phox antibodies. (b) Oxygen consumption traces registered in parallel assays. Numbers indicate the time of sampling in min. UN, unstimulated neutrophils; Control Ab, rabbit irrelevant antibodies.

more, with PMA, the inhibition by staurosporine of phosphorylation of the 67 kDa protein was accompanied by the inhibition of p67phox translocation. These studies indicated that the 67 kDa phosphorylated protein could be p67phox. Thus we have performed experiments to investigate the identity of the 67 kDa phosphorylated protein. The results reported in this paper show that the 67 kDa protein previously observed by us is indeed p67phox and, therefore, provide the first direct evidence that p67phox is phosphorylated after stimulation of neutrophils with phorbol esters or receptor-mediated agonists.

The time course of p67phox phosphorylation was similar to that of p47phox phosphorylation. In fact, after challenging neutrophils with phorbol esters, phosphorylation of p67phox increased with time in both the cytosol and membrane fractions, suggesting that p67phox was first phosphorylated in the cytosol and then translocated to the membranes, as has been demonstrated for p47phox [10,11]. Furthermore, the amount of phosphorylated p67phox increased continuously with time in both the cytosol and membrane fractions, whereas oxygen consumption reached a maximal rate within 1 min, and then continued linearly. This indicates that continuous phosphorylation (and translocation) of p67phox is required in order to maintain the linearity of the respiratory burst.

The above data that p67phox is phosphorylated are reinforced by the finding that no 67 kDa phosphorylated proteins were immunoprecipitated by anti-p67phox antibodies in neutrophils from a patient with a rare form of autosomal CGD in which the

cytosolic p67phox is absent (for details see the Materials and methods section). In this patient, phosphorylation and translocation of p47phox observed after neutrophil stimulation was normal, thus confirming that p67phox is not necessary for the phosphorylation or the translocation of p47phox [11,16].

From the data reported in Figure 4 one could conclude that the extent of p67phox phosphorylation is smaller than that of p47phox. Further investigations on the specific ³²P activity of p67phox and p47phox are needed for such a conclusion. However, other explanations of the different extents of phosphorylation between the two proteins can be advanced. It is possible that p67phox appears to be less phosphorylated because it is less abundant in neutrophils than is p47phox [27]. Alternatively, the anti-p67phox antibody, that is directed against C-terminal peptide of the protein, may be less effective in immunoprecipitation than the antibody raised against the entire recombinant p47phox protein.

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