Characterization and partial purification of a novel neutrophil membrane-associated kinase capable of phosphorylating the respiratory burst component p47^{phox}

Aroon S. LAL*, Peter J. PARKER† and Anthony W. SEGAL*¹

*Department of Medicine, University College London, 5 University Street, London WC1E 6JJ, U.K., and †Imperial Cancer Research Fund, 44, Lincoln's Inn Fields, London WC2A 3PX, U.K.

The phosphorylation of p47^{phox} is widely viewed as an important step in the activation of the neutrophil respiratory burst oxidase system. The exact nature of the kinase(s) responsible remains to be elucidated. We show here that such a kinase was detected on neutrophil membranes activated by either PMA or formyl-methionyl-leucyl-phenylalanine. This enzyme is not intrinsic to the neutrophil membrane and could be eluted with 0.5 M NaCl. The kinase activity was partially purified and was found not to be due to the presence of previously suggested kinases, including protein kinase C isotypes, mitogen-activated protein kinase and protein kinase B. Gel filtration and renaturation in substrate gels suggest a molecular mass of between 45 and 51 kDa. The kinase activity was independent of calcium and lipids but was

potently inhibited by staurosporine. Treatment with protein phosphatase 2Ac suggested that the kinase was activated by serine/threonine phosphorylation. Phosphopeptide maps indicated that the kinase phosphorylated p47^{phox} on similar sites to those found *in vivo*. These results indicate that activation of neutrophils by PMA results in the activation of a membrane-associated kinase that may play a part in the regulation of neutrophil NADPH oxidase through its ability to phosphorylate p47^{phox}.

Key words: NADPH oxidase, phosphorylation, Phox proteins, superoxide.

INTRODUCTION

One of the crucial stages in bacterial killing by the phagocytic cells of the blood (neutrophils and monocyte-macrophage cells) is the so called 'respiratory burst', in which phagocytes markedly increase their oxygen uptake, using it to generate the molecular oxygen ion, superoxide. NADPH oxidase is the enzyme complex responsible for the generation of superoxide (O₂⁻) in stimulated neutrophils. This process is vital for the efficient killing and digestion of phagocytosed organisms, since patients with the inherited condition chronic granulomatous disease (CGD), who lack a functional oxidase, suffer frequent infections and show defective killing of certain strains of bacteria.

NADPH oxidase has been extensively investigated and at the molecular level has been shown to consist of a membrane-bound flavocytochrome b₅₅₈ (consisting of a large, heavily glycosylated subunit, gp91^{phox} and a smaller subunit p22^{phox}) and several cytosolic component proteins (p47^{phox}, p67^{phox}, p40^{phox}) which translocate to the membrane upon activation of the cell (see [1] for review). In addition, the small GTP-binding protein p21rac (p21rac2 in humans) [2,3] has been shown to be required for activity in a cell-free model [3,4], in which it is required to be in the GTP bound form.

Stimuli capable of activating neutrophils have been shown to cause extensive phosphorylation of p47^{phox} [5–7]. This phosphorylation may be critical in initiating translocation in intact neutrophils, as it occurs with a similar time course to activation of the oxidase [5,6,8], and inhibition of activation by many strategies results in coincident inhibition of phosphorylation of the p47^{phox} component [9–11]. It is possible that this step

(perhaps together with the exchange of GDP for GTP on p21rac) represents the final common trigger for the activation of the oxidase system present at the convergence of a number of upstream signalling pathways.

The exact nature of the kinase(s) responsible for the phosphorylation of p47^{phox} remains the subject of much debate. Site-directed mutagenesis [12] has identified specific serine residues within the p47^{phox} which become phosphorylated upon neutrophil activation. Many of these lie within the consensus sequences of known kinases [13,14]. Several kinases have also been shown to be capable of phosphorylating recombinant p47^{phox} *in vitro*, including protein kinase C (PKC) [15,16], mitogen-activated protein kinases (MAPK/ERK) and p38ERK [13,17]. Most recently, protein kinase B (PKB) has been suggested as having a role [18]. Additionally Ding and Badwey [19,20] have demonstrated the formyl-methionyl-leucyl-phenylalanine (fMLP)-induced activation of a number of uncharacterized kinases capable of phosphorylating a peptide based on the C-terminal of p47^{phox}. We have chosen to purify a kinase that was activated by an agonist of the oxidase system and acted upon intact p47^{phox}. We report here the characterization of such an activity.

EXPERIMENTAL PROCEDURES

Materials

PMA, fMLP, tosyl-lysylchloromethane (TLCK), PMSF, leupeptin, pepstatin A, staurosporine, mixed brain lipids (methanol/chloroform bovine brain phospholipid extract; [21])

Abbreviations used: fMLP, formyl-methionyl-leucyl-phenylalanine; GST, glutaryl S-transferase; MAPK, mitogen-activated protein kinase; Phox, phagocyte oxidase; PKC, protein kinase C.

¹ To whom correspondence should be addressed (e-mail t.segal@ucl.ac.uk).

and phosphatidic acid were from Sigma. Microcystin-LR was from Gibco. Antibodies to PKC isoenzymes and cJun-glutaryl S-transferase (cJun-GST) were as described previously [22]. Antibodies to ERK 1 and 2 were a gift from Dr. Chris Marshall (Chester-Beatty Research Laboratories, London). Antibodies to PKB were a gift from Dr. Jim Woodgett (Ontario Cancer Institute, Toronto, Canada). The phosphatases were a gift from Professor Phillip Cohen (University of Dundee, Dundee, Scotland, U.K.). Recombinant p47^{phox} was generated using the pGEX system and further purified using a heparin-agarose column (Pharmacia Biotech, Uppsala, Sweden). Antibodies to p47^{phox} were raised to the recombinant protein in rabbits and affinity purified on p47^{phox} bound to a HiTrap column (Pharmacia Biotech, Uppsala, Sweden). Chromatography was carried out using MonoS, MonoQ and heparin-agarose columns on an FPLC system, whereas gel filtration was performed using a Superdex-75 column and a SMART system (Pharmacia Biotech, Uppsala, Sweden).

Methods

Isolation of human neutrophils

Neutrophils were isolated from healthy human volunteers by dextran sedimentation followed by centrifugation on a Ficoll gradient (Lymphoprep, Nycomed, Robbins Scientific, Solihull, U.K.). Residual red cells were removed by brief hypotonic lysis before resuspending at 5×10^7 cells/ml in PBS (0.14 M NaCl/10 mM KCl/10 mM NaH₂PO₄, pH 7.4).

Isolation of neutrophil membranes

Neutrophils were sonicated on ice in break buffer (10 mM Pipes, pH 7.0/10 mM KCl/3 mM NaCl/4 mM MgCl₂/5 mM sodium pyrophosphate/25 mM sodium fluoride/1 µg/ml leupeptin/1 µg/ml pepstatin/0.5 mM PMSF/1 µM Microcystin-LR/1 µg/ml TLCK) for three bursts of 12 s, and unbroken cells and nuclei were removed by centrifugation at 1000 g in an MSE Mistral centrifuge, at 4 °C for 15 min. The supernatant (20 ml) was loaded into tubes containing discontinuous 5 ml cushions of 15 % and 34 % (w/w) sucrose in break buffer and then centrifuged at 120000 g in a Centrikon centrifuge using an AH627 centrifuge head at 4 °C for 45 min. Membranes were removed from the interface between the 15 % and 34 % sucrose cushions, diluted with 4 vol. of break buffer and then pelleted at 200000 g at 4 °C for 45 min in a Centrikon centrifuge using an A641 centrifuge head. This method has previously been shown to provide considerable enrichment for the plasma membrane [23]. Moreover, Golgi and endoplasmic reticulum are relatively poorly represented in neutrophils.

Assay of p47^{phox} kinase activity

An aliquot of sample (10 µl) was added to 3 µl of recombinant p47^{phox} (approx. 1 nmol) and 12 µl of buffer (50 mM Tris/HCl, pH 6.0). The reaction was initiated by the addition of 5 µl of assay buffer containing 20 mM Tris/HCl (pH 7.0), 120 µM ATP, 3 µCi [γ -³²P]ATP, 240 mM magnesium acetate and 0.6 mM calcium acetate. The reaction was incubated at 30 °C for 15 min (a time period that was found to be well within the linear phase of phosphorylation; results not shown) after which it was stopped by the addition of 3 µl of 10 × Laemmli sample buffer and boiled. Proteins were then separated by SDS/PAGE before being subjected either to autoradiography or excision and Cherenkov counting of the labelled substrate band. In the case of the latter,

the kinase activity was quantified in arbitrary units; 1 unit is defined as the number of pmol of phosphate transferred to the substrate/min.

Elution of activity from membranes

Membranes were resuspended in the buffer indicated by gentle pipetting and then incubated at 4 °C end-over-end on a rotary mixer for 60 min. The membranes were then re-pelleted by centrifugation in a Beckmann Optima TL ultracentrifuge at 200000 g for 30 min using a TLA 100.4 head.

Chromatography

Supernatant from the membrane elution preparation was diluted 1:5 in buffer Q [20 mM Tris/HCl (pH 8.0)/1 mM EDTA/5 mM sodium pyrophosphate/25 mM sodium fluoride/1 µg/ml TLCK/1 µg/ml pepstatin A/1 µM Microcystin-LR/1 µg/ml leupeptin/0.5 mM PMSF/0.025 % (v/v) Brij 35] before loading onto a 1 ml MonoQ column using a superloop. Elution was with a 25 ml linear gradient of 0–1 M NaCl in buffer Q and 1 ml fractions were collected. Active fractions from this stage were identified and diluted 1:10 in buffer S (20 mM Mes, pH 5.5/1 mM EDTA/5 mM sodium pyrophosphate/25 mM sodium fluoride/1 µg/ml TLCK/1 µg/ml pepstatin A/1 µM Microcystin-LR/1 µg/ml leupeptin/0.5 mM PMSF/0.025 % (v/v) Brij 35] before loading onto a 1 ml MonoS column, again using a superloop. Elution was as detailed for the MonoQ column, using a linear gradient of 0–1 M NaCl in buffer. Active fractions from the MonoS were then identified and desalted into buffer H [20 mM Tris/HCl (pH 7.0)/1 mM EDTA/1 µg/ml TLCK/1 µg/ml pepstatin A/1 µM Microcystin-LR/1 µg/ml leupeptin/0.5 mM PMSF/0.025 % (v/v) Brij 35] using a PD-10 column (Pharmacia Biotech, Uppsala, Sweden) before injecting onto a 1 ml heparin-agarose column. Elution was again with a linear 0–1 M NaCl gradient over 15 ml of buffer H, collecting 1 ml fractions. For gel filtration, active fractions from the MonoS were concentrated to a final volume of 100 µl using a Centricon-10 membrane (Amicon). A 2.4 ml Superdex-75 column was equilibrated with buffer H containing 250 mM NaCl, and 50 µl of the concentrated material was loaded onto the column and eluted at 60 µl/min, collecting 80 µl fractions using the SMART system (Pharmacia Biotech, Uppsala, Sweden).

Substrate gel analysis

Using a method modified from Gotoh et al. [24], the recombinant p47^{phox} (0.5 mg/ml) was fixed in a 10 % SDS/PAGE mini-gel by adding to the polyacrylamide gel mix immediately before polymerization. After separation of the proteins by SDS/PAGE, SDS was removed from the gel by washing in two changes of 100 ml of 20 % (v/v) propan-2-ol in 50 mM Tris/HCl (pH 8.0) for 30 min each. The gel was then washed once in 250 ml of 50 mM Tris/HCl (pH 8.0) containing 5 mM 2-mercaptoethanol for 1 h. The gel was then denatured in two changes of 100 ml of 6.0 M guanidinium chloride in 50 mM Tris/HCl, pH 8.0/5 mM mercaptoethanol for 30 min each. Renaturation was achieved by incubating at 4 °C in four changes of 250 ml of: 50 mM Tris/HCl, pH 8.0; 5 mM mercaptoethanol; 0.04 % (v/v) Tween 40, for 4 h each. The assay was performed at room temperature for 30 min using 25 ml of 20 mM Tris/HCl (pH 6.0)/2.0 mM dithiothreitol/0.1 mM EGTA/40 mM MgCl₂/0.1 mM CaCl₂/50 µCi [γ -³²P]ATP (1 µCi/ml)/50 µM ATP. The gel was then washed extensively in 5 % (w/v) trichloroacetic acid with 1 % (w/v)

sodium pyrophosphate until the radioactivity in the wash became negligible, dried and subjected to autoradiography.

Effect of protein phosphatases and staurosporine

Samples were exchanged into buffer H and concentrated using a Centrikon-10 membrane (Amikon). These were then incubated for 30 min at 30 °C in the presence of 10 units/ml protein phosphatase 1 γ or 2Ac before the addition of 2 μ M Microcystin-LR to halt further activity. Control samples were treated similarly, but the Microcystin was present from the start. The material was then used in a standard kinase assay, as previously described. The effect of staurosporine was studied by pre-incubation with the appropriate concentration (diluted from a 1 mM stock in DMSO) for 10 min at 30 °C before conducting a standard kinase assay. The control was incubated in the presence of an equivalent volume of DMSO alone.

Phosphopeptide and phospho amino acid analysis

Neutrophils were isolated from blood using the standard protocol, but were resuspended in phosphate-free Hepes-buffered saline [HBS; 20 mM Hepes/NaOH (pH 7.4)/136 mM NaCl/2.7 mM KCl/1 mM MgSO₄/1 mM CaCl₂/5.6 mM glucose/0.1 mg/ml BSA]. Cells were then gently pelleted at 500 *g* for 2 min, before being resuspended in HBS at a density of approx. 5 $\times$ 10⁷ cells/ml. ³²P (1 mCi/10⁸ cells) was added and the cells were incubated for 90 min at 30 °C. Free ³²P was removed by washing twice in HBS before resuspending in HBS at the original cell density. The cells were then stimulated with 0.1 μ g/ml PMA at 37 °C for 3 min. The cells were broken by the addition of one fifth volume of lysis buffer [100 mM Tris/acetate (pH 7.0)/1.35 M sucrose/5 mM EDTA/5 mM EGTA/5% (v/v) Triton X-100/50 mM sodium β -glycerophosphate/250 mM NaF/25 mM sodium vanadate/5 mM benzamidine/5 μ g/ml leupeptin/5 μ g/ml pepstatin/2.5 mM PMSF/5 μ M Microcystin-LR/5 μ g/ml TLCK). Unbroken cells and nuclei were pelleted by centrifugation at 2000 *g* for 10 min and p47^{phox} was immunoprecipitated from the supernatant using anti-p47^{phox} bound to Protein A-Sepharose beads (Pharmacia Biotech, Uppsala, Sweden). *In vitro* labelled p47^{phox} was obtained by incubation of recombinant protein with material from the heparin-agarose stage of purification. After separation of proteins using SDS/PAGE, the labelled protein was visualized by autoradiography, and the band excised.

Phosphopeptide and phospho amino acid analysis was carried out according to the method detailed previously [25]. Briefly, proteins were eluted from the gel and all particulate matter was removed. Proteins were precipitated using trichloroacetic acid and methionine and cysteine residues were oxidized with performic acid. Part of the protein (10%) was subjected to acid hydrolysis with 6 M HCl and used in phospho amino acid analysis by thin-layer electrophoresis [first dimension pH 1.9, using a buffer consisting of 50 ml of formic acid (88% w/v), 156 ml of glacial acetic acid, 1794 ml of deionized water; second dimension pH 3.5, using a buffer consisting of 50 ml of glacial acetic acid, 5 ml of pyridine, 945 ml of deionized water]. The remaining protein was subjected to digestion with trypsin (10 μ g per 50 μ l of sample) before separating the resulting peptides by thin-layer electrophoresis at pH 1.9 followed by separation in the second dimension using TLC (using 750 ml of n-butanol/500 ml of pyridine/945 ml of deionized water). Areas of radioactivity were visualized using a FujiBas1000 PhosphorImager (Fuji Co., Tokyo, Japan).

Sequencing of proteins was carried out using nano-electrospray MS [26] of material excised directly from PAGE gels.

RESULTS

p47^{phox} kinase activity on neutrophil membranes increases after stimulation

The most extensively phosphorylated forms of p47^{phox} are found exclusively on the membranes of stimulated neutrophils and are not seen when translocation is prevented by the absence of wild-type cytochrome [5,27]. We reasoned that the membranes may either possess a kinase capable of phosphorylating p47^{phox} or that such a kinase may translocate to the membranes upon stimulation. We therefore examined the ability of membranes isolated from neutrophils stimulated for various times with either 0.1 μ g/ml PMA or 1 μ M fMLP for their ability to phosphorylate recombinant p47^{phox} *in vitro*. Figure 1 shows that the ability of a crude membrane preparation to phosphorylate the p47^{phox} substrate increased after activation with either stimulant. For PMA, increased phosphorylation was maximal by 2–3 min and then began to decline, whereas for fMLP, increased phosphorylation was maximal by 40 s and declined to resting levels by 120 s. These time courses closely parallel the kinetics of superoxide production and p47^{phox} phosphorylation induced in intact neutrophils by these activators. Activity in cytosolic fractions was barely detectable and did not appear to change significantly with stimulation (results not shown).

Figure 1 Activation of neutrophils leads to an increase in p47^{phox} kinase activity on the membranes

Neutrophils were stimulated with either 1 μ M fMLP (A) or 0.1 μ g/ml PMA (B) before halting the reaction with iced PBS at the intervals shown. Membranes were subsequently prepared and used in *in vitro* kinase assays to assess their ability to phosphorylate recombinant p47^{phox} substrate. Approximately similar quantities of proteins derived from 10⁷ cell equivalents (membrane) were separated using SDS/PAGE and then subjected to autoradiography. The arrows indicate the positions of the recombinant p47^{phox}. After stimulation with fMLP, kinase activity appears to increase by 20 s and is maximal at 40 s, before returning to baseline by 90 s. PMA activates the kinase in a slower and more sustained fashion.

Figure 2 Purification of p47^{phox} kinase activity using sequential chromatography

Material eluted from PMA-activated membranes was diluted into MonoQ buffer and separated on a MonoQ column (A). Active fractions from each subsequent purification were pooled, diluted into the appropriate buffer, and then separated on MonoS (B) and then heparin-agarose (C) columns. Activity (shown by the histograms) was assayed by Cherenkov counting of *in vitro* phosphorylated recombinant p47^{phox} excised from 10% SDS/PAGE gels, autoradiographs of which are shown above each graph. The A_{280} (shown as a trace) is given as a guide to protein concentration. The results shown are representative of typical purifications. (D) Shows a 12.5% Coomassie Blue-stained gel of the heparin-agarose-derived material. No obvious co-purifying band is apparent.

Purification of p47^{phox} kinase activity from activated neutrophil membranes

As shown in Figure 1, PMA activation of neutrophils caused the highest level of p47^{phox} kinase activity and so membranes stimulated for 3 min with 0.1 μ g/ml PMA were employed as a starting point for further purification. Induced kinase activity could be eluted most effectively from membranes with 0.5 M NaCl. This is highly suggestive of the fact that the protein is not intrinsic to the membrane, since such proteins would typically require solubilization in detergents. Subsequent purification was achieved by successive chromatographic separation of the eluted material on MonoQ, MonoS and finally heparin-agarose columns using the FPLC system (Pharmacia, Sweden). Figure 2 shows elution profiles for these steps, and details of the purification are shown in Table 1.

Starting with approx. 770 units of activity, derived from 10^{11} cell equivalents, overall purification was approx. 250-fold. The protein was not pure, as judged by SDS/PAGE and silver

staining (Figure 2D). Attempts at further purification led to substantial losses of activity.

Determination of the molecular mass of the kinase activity

Two techniques were employed to estimate the size of the protein responsible for the observed p47^{phox} kinase activity. In the first, concentrated kinase active material was separated on SDS/PAGE gels containing recombinant p47^{phox} fixed within the gel matrix. After renaturation and incubation with [γ -³²P]ATP, autoradiography detected phosphorylation of the fixed p47^{phox} in a single band representing a molecular mass of approx. 51 kDa (± 0.9 , $n = 4$). The renaturable activation was not present in gels devoid of p47^{phox} substrate or using kinase-inactive fractions. In the second technique, concentrated kinase-active fractions from the MonoS stage of purification were pooled and separated on a Superdex-75 gel-filtration column using the SMART system (Pharmacia, Sweden). Activity eluted as a single peak corresponding to a size of approx. 45 kDa (results not shown).

Table 1 Purification of p47^{phox} kinase activity from activated neutrophil membranes

Activity was isolated from approximately 10¹¹ cell equivalents as described in the Experimental procedures section. At each stage, kinase activity was determined using the *in vitro* phosphorylation of recombinant p47^{phox} determined by Cherenkov counting of substrate excised from SDS/PAGE gels. At the later stages, too little protein was present to allow formal protein quantification, so the A₂₈₀ from flow-through detection was used to calculate protein concentration, taking 1 absorption unit as approx. 1 mg/ml protein.

Fraction	Total activity (units)	Total protein (A ₂₈₀)	Recovery of activity (%)	Specific activity (units/A ₂₈₀)	Purification (fold)
Membranes	773	320.00	100	2	1
Wash	101	20.00	13	5	3
MonoQ	119	3.20	15	37	19
MonoS	36	0.47	5	77	39
Heparin	9	0.02	> 1	450	225

Figure 3 p47^{phox} kinase activity does not appear to be due to PKC

Concentrated material from the MonoQ stage of purification (Q) and whole neutrophil lysate (+) were separated using SDS/PAGE before Western blotting for the PKC isoforms, PKC α , PKC β , PKC ϵ , PKC η , PKC ζ and PAN100 (PAN) with an antiserum raised against a conserved region of the PKC family of kinases. Immunoreactive protein was not identified in the kinase-active fractions.

Several known kinases have been suggested to be involved in the phosphorylation of p47^{phox}, or have been shown to be capable of utilizing p47^{phox} as a substrate *in vitro*, and the possibility that one of these may be responsible for the kinase activity being isolated was investigated by Western blotting of purified material after concentration by means of trichloroacetic acid precipitation. Blotting with antibodies to PKC isoforms α , β , γ , η , ζ and a conserved region of the PKC family of kinases was negative (Figure 3), as was PKB, p21-activated kinase (PAK), ERK 1 and ERK 2 (results not shown). In addition GST-cJun affinity precipitation from the purified material failed to identify any stress-activated protein kinase activity (results not shown).

Characterization of the purified p47^{phox} kinase activity

The kinase activity was characterized further. Material obtained after the heparin–agarose stage of purification was considered sufficiently pure for this purpose.

The optimal pH was found to be in the range pH 5.5–6.0 (results not shown). The values for K_m and V_{max} using the kinase substrates ATP and p47^{phox} compare favourably with other protein kinases (Table 2). Determination of the optimum concentration of magnesium (in the presence of 20 μ M ATP) is shown in Figure 4 (top). Surprisingly, maximal activity was found in the presence of relatively high concentrations (half-maximal activation occurred at between 20 and 40 mM Mg²⁺), suggesting that its effects are not confined to the binding of ATP.

As the PKC family have been suggested as being responsible for p47^{phox} phosphorylation, and this activity was derived ultimately from neutrophils stimulated with PMA, a direct activator of PKC, the possibility that the p47^{phox} kinase activity isolated was due to a member of this family of kinases was

Table 2 Enzyme characteristics of the material from the heparin–agarose stage of purification

Values for ATP were calculated using a p47^{phox} concentration of approx. 2 μ M, while those for p47^{phox} were obtained in the presence of 20 μ M ATP. Each was measured in the presence of 0.1 mM calcium and 40 mM magnesium using triplicate assays at each concentration of substrate. Values were calculated using linear regression.

Substrate	K_m (μ M)	V_{max} (nmol/min per A ₂₈₀)
ATP	80	5
p47 ^{phox}	1.4	2.5

investigated further. The classical PKCs require both calcium and phospholipid for activity, whereas the novel PKCs are calcium independent [28]. PMA acts via the diacylglycerol-binding domain and is a potent activator of the classical family [28]. Its effect on the novel PKCs is less well characterized. Figure 4 (bottom) shows that calcium does indeed increase p47^{phox} kinase activity, but that its effect is markedly reduced in the presence of optimal magnesium levels, suggesting that the kinase is not specifically activated by calcium. Mixed phospholipids and two other ‘second messenger’ substances, PtdIns(3,4,5)P₃ and phosphatidic acid, in the presence or absence of PMA, had no effect on activity (results not shown). In the light of these results, it seems unlikely that the kinase is a member of the classical or novel PKC families. The third group, the so-called ‘atypical’ PKC family, show no dependency on either lipids, PMA or calcium [28,29]. It is not possible from our results to exclude the kinase from this group, although all its identified

Figure 4 Requirement of the p47^{phox} kinase activity for Ca²⁺ and Mg²⁺

Material from the heparin-agarose stage of purification was incubated with recombinant p47^{phox} substrate in the presence of varying concentrations of magnesium or calcium. Proteins were then separated using SDS/PAGE and phosphorylation was determined using Cherenkov counting of the excised p47^{phox} band. Zero concentration was achieved with 2 mM EGTA (Ca²⁺) or 2 mM EDTA (Mg²⁺). The p47^{phox} kinase requires relatively high concentrations of Mg²⁺ (top). At low concentrations of Mg²⁺ (5 mM), Ca²⁺ has a marked augmentary effect, but this is lost at high Mg²⁺ concentrations [40 mM (bottom)]. The results shown represent the means $\pm$ S.E.M. for three separate assays.

Figure 5 The *in vitro* activity of p47^{phox} kinase is inhibited by staurosporine

Activity from the heparin-agarose stage of purification was incubated with recombinant p47^{phox} substrate in the presence of varying concentrations of staurosporine. Kinase activity was assessed by Cherenkov counting of the phosphorylated recombinant p47^{phox} excised after separation of proteins using SDS/PAGE. The results shown are the means $\pm$ S.E.M. for three independent incubations.

members have a molecular mass in excess of that estimated for the p47^{phox} kinase activity purified here [28].

The specificity of the kinase activity was investigated using three other substrates. At equimolar concentrations myelin basic protein was the most readily phosphorylated substrate, p47^{phox} and histone IIIs were intermediate, whereas phosphorylation of p67^{phox} was negligible (results not shown). The broad-specificity kinase inhibitor staurosporine was found to be a potent inhibitor of kinase activity *in vitro* (Figure 5), with an estimated IC₅₀ of

Figure 6 The *in vitro* activity of p47^{phox} kinase is inhibited on pre-treatment with protein phosphatase 2Ac

Material from the heparin-agarose stage of purification was buffer exchanged to remove salt and then incubated with protein phosphatase (10 units/ml) 1γ or 2Ac for 30 min at 30 °C in the presence (■) or absence (□) of the phosphatase inhibitor Microcystin-LR (2 μM). Microcystin-LR (2 μM) was then added to halt further phosphatase activity. Kinase activity was assessed by Cherenkov counting of the phosphorylated recombinant p47^{phox} excised after separation of proteins using SDS/PAGE. The results shown are the means $\pm$ S.E.M. for three independent measurements.

Figure 7 Purified p47^{phox} kinase activity phosphorylates recombinant p47^{phox} on serine residues only

Recombinant p47^{phox} was phosphorylated *in vitro* using material from the heparin-agarose stage of kinase purification before being subjected to phospho amino acid analysis by TLC. The plate was then exposed to a phospho-imager plate. (X marks the point of sample application).

0.15 nM (compared with PKC which has an IC₅₀ for staurosporine of approx. 6 nM).

Purified p47^{phox} kinase activity phosphorylates p47^{phox} on *in vivo* occupied sites and is itself subject to control by phosphorylation

To investigate whether the activation of p47^{phox} kinase observed in our membrane preparation was due to phosphorylation of the kinase itself we examined the effect of pre-incubation of the activity with two distinct protein serine/threonine phosphatases. Figure 6 shows that protein phosphatase 2Ac was capable of markedly reducing p47^{phox} kinase activity, presumably by the dephosphorylation of serine/threonine residues.

In order to determine the biological significance of the p47^{phox} kinase activity being isolated, phospho amino acid analysis and phosphopeptide mapping of p47^{phox}, phosphorylated either *in vivo* or *in vitro*, were compared. Phospho amino acid analysis revealed that the partially purified kinase phosphorylated recombinant p47^{phox} on serine residues only (Figure 7), in agreement

Figure 8 Phosphopeptide mapping of phosphorylated p47^{phox}

(A) Recombinant p47^{phox} phosphorylated *in vitro* by the kinase activity from the heparin–agarose stage of purification. (B) p47^{phox} immunoprecipitated from PMA-stimulated ³²P-labelled cells. (C) Co-application of labelled p47^{phox} from both sources showing the similarity of the major phosphorylated species. As an aid to orientation, the arrows indicate the major phosphorylated species. On co-application, the major spots from both sources co-migrate.

with previous findings in whole cells [7,12]. Comparison of phosphopeptide maps of tryptic digests derived from the two sources revealed marked similarity for the main phosphate-containing species (Figure 8).

DISCUSSION

Phosphorylation of p47^{phox} is a prominent event after activation of neutrophils with substances that activate NADPH oxidase and may be of prime importance in initiating translocation of this component to the membrane and assembly of the active oxidase complex. Although many protein kinases have been suggested as being responsible for the phosphorylation of this

protein [13,16,17,30,31], the evidence for any one in particular is not conclusive. We reasoned that as the most phosphorylated forms of p47^{phox} were found exclusively on the neutrophil membrane [5,27], a kinase may either be present at this site or translocated to it upon activation. We show here that an increase in kinase activity in fractions derived primarily from the plasma membrane can be observed after stimulation of the cells with both PMA and fMLP and closely follow that of superoxide generation and hence p47^{phox} phosphorylation. In contrast, little activity was detected in the cytosolic fractions and this appeared unaffected by stimulation of the neutrophils. We were able to elute the activity from the membranes with 0.5 M NaCl, suggesting that the activity resides in an extrinsic membrane protein, and to purify it further using successive chromatographic columns. Although the protein yield was small, it should be noted that membranes represent only approx. 10% of total neutrophil protein and hence overall purification is some 2000-fold.

We estimated the molecular mass of the kinase by two separate techniques which suggested a size of between 45 and 51 kDa. Protein bands of an appropriate molecular mass were excised from a PAGE gel of the heparin–agarose-purified material and identified by nano-electrospray MS using the method of Wilm et al. [26]. Proteins of 53, 48, 47, 45 and 43 kDa were identified as Vimentin and those of 53 and 48 kDa as a probable disulphide isomerase. All peptides were accounted for and none corresponded to a protein kinase.

Although PKC isotypes (α , β , η , ϵ and ζ), p65^{PAK} and PKB are of a molecular mass consistent with the identified proteins, antisera to them failed to detect their presence in the purified fractions by Western blotting. Although ERK 1, 2 and stress-activated protein kinase are also of a suitable size, are activated after stimulation with fMLP or PMA and can phosphorylate p47^{phox} *in vitro* [17] they were not detected either. Krump et al. [32] have recently shown that the related kinase, p38 MAPK, is markedly activated after stimulation with either PMA or fMLP. We did not probe for this kinase, but its size is considerably lower than that of the kinase isolated here and its inhibition does not significantly inhibit PMA-induced activation of the NADPH oxidase or phosphorylation of the p47^{phox} component (A. S. Lal, A. D. Clifton, J. Rouse, A. W. Segal and P. Cohen, unpublished work).

Characterization of the kinase revealed certain unusual properties. It has a surprisingly high optimum concentration of magnesium and, unlike the classical PKC family, lipids, calcium and PMA have no specific effect upon activity. These results do not eliminate the possibility that the kinase is a member of the atypical or novel PKC family, although members of this family so far described have molecular masses considerably in excess of that calculated for our kinase [28]. The absence of an effect by lipids also distinguishes this kinase from the cytosol-derived activity recently described by Waite et al. [33] which was activated by incubation with phosphatidic acid. Uhlinger and Perry [15] identified a kinase in neutrophil cytosol that was capable of phosphorylating p47^{phox} and which appeared to be calcium and phosphatidyl serine independent. They speculated that it might be a PKC as it was inhibited by staurosporine and by PKC pseudosubstrate. We did not test the latter, but staurosporine was particularly effective in inhibiting the p47^{phox} kinase activity isolated here.

The substrate specificity of the kinase activity showed that myelin basic protein was the most effectively phosphorylated. Utilization of p47^{phox} as a substrate was similar to the utilization of histone IIIs. Importantly, another neutrophil *phox* protein, p67^{phox}, was not significantly phosphorylated.

The deactivation of the kinase by incubation with protein phosphatase 2Ac confirms that the kinase is activated itself by serine/threonine phosphorylation, although the nature of the upstream activating protein kinase is uncertain. PKC is a candidate, particularly as the kinase is activated after PMA stimulation of the cells, but pre-incubation of the partially purified p47^{phox} kinase with PKC β did not cause further activation (results not shown).

Finally, we compared p47^{phox} phosphorylated either *in vivo* (in PMA-stimulated neutrophils) or *in vitro* (by the kinase isolated from neutrophil membranes) by means of both phospho amino acid and phosphopeptide analysis. All studies on p47^{phox} *in vivo* have revealed extensive phosphorylation on serine residues only [12,13], and this was the case for p47^{phox} phosphorylated by the activity isolated in our experiments. This is in contrast with the phosphatidic acid activated kinase described by Waite et al. [33], which also caused phosphorylation of tyrosine residues. Importantly, the phosphopeptide maps for phosphorylated p47^{phox} derived from p47^{phox} phosphorylated *in vivo* or by the purified membrane-associated p47^{phox} kinase were similar for the major species. This is supportive of the fact that the kinase plays a role in p47^{phox} phosphorylation *in vivo*. We did not compare the *in vitro* map with p47^{phox} derived from fMLP-stimulated neutrophils, but previous studies have shown close similarities to that from PMA-stimulated neutrophils [7]. Others have shown that p47^{phox} recovered from membranes contains phosphorylated residues not present on p47^{phox} recovered from cytosol, and in situations where p47^{phox} translocation is absent (in neutrophils devoid of the cytochrome) certain of the phosphorylated species are not found [5,27]. This may imply that different protein kinases may be involved in the cytosolic as opposed to the membrane phosphorylation process, and may explain the differences in the phosphopeptide map with respect to the relative stoichiometry of phosphorylation of the major peptides and in the phosphorylation of several of the minor species.

In conclusion, we have shown that neutrophils possess a novel, inducible membrane-associated kinase capable of phosphorylating p47^{phox} on biologically relevant sites. The molecular mass for the kinase appears to lie between 45 and 51 kDa, and it is not dependent on lipids or calcium for its activation. It is subject to activation by phosphorylation, as indicated by its sensitivity to protein phosphatase 2Ac. It is likely that PMA acts via activation of PKC, leading to phosphorylation of this p47^{phox} kinase, which in turn leads to phosphorylation of p47^{phox}. Future work will focus on the identity of this kinase, in order to define the regulatory process involved.

We thank the Wellcome Trust for support. We also express our extreme gratitude to Malcolm Ward and Dr. Walter Blackstock of Glaxo-Wellcome, Stevenage, U.K. for the microsequencing of protein bands, and Richard Marais, Sohail Ahmed, Chris Marshall and Jim Woodgett for anti-sera to the various protein kinases. Finally, grateful thanks to Dr. Gordon Stewart for support and help in the preparation of the manuscript.

REFERENCES

- Segal, A. W. and Abo, A. (1993) Trends Biochem. Sci. **18**, 43–47
- El Benna, J., Ruedi, J. M. and Babior, B. M. (1994) J. Biol. Chem. **269**, 6729–6734
- Kreck, M. L., Uhlinger, D. J., Tyagi, S. R., Inge, K. L. and Lambeth, J. D. (1994) J. Biol. Chem. **269**, 4161–4168
- Abo, A., Boyhan, A., West, I., Thrasher, A. J. and Segal, A. W. (1992) J. Biol. Chem. **267**, 16767–16770
- Rotrosen, D. and Leto, T. L. (1990) J. Biol. Chem. **265**, 19910–19915
- Heyworth, P. G., Shrimpton, C. F. and Segal, A. W. (1989) Biochem. J. **260**, 243–248
- El Benna, J., Faust, L. P. and Babior, B. M. (1994) J. Biol. Chem. **269**, 23431–23436
- Heyworth, P. G. and Segal, A. W. (1986) Biochem. J. **239**, 723–731
- Curnutte, J. T., Erickson, R. W., Ding, J. and Badwey, J. A. (1994) J. Biol. Chem. **269**, 10813–10819
- Vlahos, C. J., Matter, W. F., Brown, R. F., Traynor-Kaplan, A. E., Heyworth, P. G., Prossnitz, E. R., Ye, R. D., Marder, P., Schelm, J. A., Rothfuss, K. J. et al. (1995) J. Immunol. **154**, 2413–2422
- Ding, J., Vlahos, C. J., Liu, R., Brown, R. F. and Badwey, J. A. (1995) J. Biol. Chem. **270**, 11684–11691
- Faust, L. R., El Benna, J., Babior, B. M. and Chanock, S. J. (1995) J. Clin. Invest. **96**, 1499–1505
- El Benna, J., Faust, L. R. P., Johnson, J. L. and Babior, B. M. (1996) J. Biol. Chem. **271**, 6374–6378
- Lomax, K. J., Leto, T. L., Nunoi, H., Gallin, J. I. and Malech, H. L. (1989) Science **245**, 409–412
- Uhlinger, D. J. and Perry, D. K. (1992) Biochem. Biophys. Res. Commun. **187**, 940–948
- Ding, J., Badwey, J. A., Erickson, R. W., Balazovich, K. J. and Curnutte, J. T. (1993) Blood **82**, 940–947
- El Benna, J., Han, J., Park, J.-W., Schmid, E., Ulevitch, R. J. and Babior, B. M. (1996) Arch. Biochem. Biophys. **334**, 395–400
- Didichenko, S. A., Tilton, B., Hemmings, B. A., Ballmer-Hofer, K. and Thelen, M. (1996) Curr. Biol. **6**, 1271–1278
- Ding, J. and Badwey, A. (1993) J. Biol. Chem. **268**, 17326–17333
- Ding, J. and Badwey, J. A. (1993) J. Biol. Chem. **268**, 5234–5240
- Palmer, R. H., Dekker, L. V., Woscholski, R., Le Good, J. A., Gigg, R. and Parker, P. J. (1995) J. Biol. Chem. **270**, 22412–22416
- Bogoyevitch, M. A., Parker, P. J. and Sugden, P. H. (1993) Circ. Res. **72**, 757–767
- Segal, A. W. and Peters, T. J. (1977) Clin. Sci. Mol. Med. **52**, 429–442
- Gotoh, Y., Nishida, E., Yamashita, T., Hoshi, M., Kawakami, M. and Sakai, H. (1990) Eur. J. Biochem. **193**, 661–669
- Van der Geer, P., Luo, K., Sefton, B. M. and Hunter, T. (1993) in Protein Phosphorylation: A Practical Approach (Hardie, D. G., ed.), pp. 31–59, Oxford University Press, Oxford
- Wilm, M., Shevchenko, A., Houthaeve, T., Breit, S., Schweigerer, L., Fotsis, T. and Mann, M. (1996) Nature (London) **379**, 466–469
- Heyworth, P. G., Curnutte, J. T., Nauseef, W. M., Volpp, B. D., Pearson, D. W., Rosen, H. and Clark, R. A. (1991) J. Clin. Invest. **87**, 352–356
- Parker, P. J. (1994) in Protein Kinases (Woodgett, J. R., ed.), pp. 68–84, Oxford University Press, Oxford
- Dekker, L. V. and Parker, P. J. (1994) Trends Biochem. Sci. **19**, 73–77
- Edwards, S. J. (1994) in Biochemistry and Physiology of the Neutrophil, pp. 188–234, Cambridge University Press, Cambridge
- Majumdar, S., Kane, L. H., Rossi, M. W., Volpp, B. D., Nauseef, W. M. and Korchak, H. M. (1993) Biochim. Biophys. Acta **1176**, 276–286
- Krump, E., Sanghera, J. S., Pelech, S. L., Furuya, W. and Grinstein, S. (1997) J. Biol. Chem. **272**, 937–944
- Waite, K. A., Wallin, R., Qualliotine-Mann, D. and McPhail, L. C. (1997) J. Biol. Chem. **272**, 15569–15578

Received 10 August 1998/26 October 1998; accepted 9 December 1998