Direct interaction between p47^{phox} and protein kinase C: evidence for targeting of protein kinase C by p47^{phox} in neutrophils

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p47^{phox} is an essential component of the NADPH oxidase, and phosphorylation of p47^{phox} is associated with activation of the enzyme. Here we have used p47^{phox} affinity chromatography to extract a p47^{phox} kinase from neutrophil cytosol. The kinase activity was purified by gel filtration and Mini Q chromatography and shown to be indistinguishable from the catalytic fragments of protein kinase C (PKC)- β_1 , - β_{11} and - δ . The C-terminus of p47^{phox} represented the site of interaction with PKC. Co-immunoprecipitation experiments revealed that the interaction between PKC isotypes and p47^{phox} takes place in intact cells. However PKC- β and - δ showed different time courses of co-immunoprecipitation, suggesting that the interactions may serve different functions for the various PKC isotypes. Using cells lacking p47^{phox}, we investigated the functional relevance of the interaction between PKC and p47^{phox}. Subcellular fractionation

revealed an abnormal recruitment of PKC- β_1 and - β_{11} , but not PKC- δ , to particulate fractions in p47^{phox}-deficient cells. Phosphorylation of cytosolic proteins was generally increased in stimulated p47^{phox}-deficient neutrophils as compared with normal neutrophils. Furthermore, the cytoskeletal protein coronin was not phosphorylated upon stimulation of p47^{phox}-deficient neutrophils. These findings were confirmed in an *in vitro*-reconstituted system using rat brain cytosol in which addition of p47^{phox} affected phosphorylation by PKC/PKM (PKM is the catalytic fragment of PKC). These results indicate that p47^{phox} can act as a regulator of PKC in neutrophils.

Key words: chronic granulomatous disease, coronin, NADPH oxidase, polymorphonuclear leucocytes.

INTRODUCTION

Phagocytic leucocytes, including neutrophils, monocytes, macrophages and eosinophils, undergo a burst of non-mitochondrial respiration that is required for the efficient killing of the ingested microbe. This oxygen consumption is produced by NADPH oxidase, which consists of a membrane-bound flavocytochrome *b* that transports electrons across the wall of the phagocytic vacuole, the cytosolic activating proteins p40^{phox}, p47^{phox} and p67^{phox} and the small GTP-binding protein p21rac [1,2]. Lack of, or a defect in, the flavocytochrome *b*, p47^{phox} or p67^{phox}, is associated with a profound predisposition to bacterial and fungal infections, a syndrome called chronic granulomatous disease (CGD) [3].

Activation of the NADPH oxidase is associated with phosphorylation of p47^{phox} [4], p67^{phox} [5], the subunits of the flavocytochrome *b* [6] and of p40^{phox} [7]. A number of kinases have been implicated in the activation of the oxidase, including protein kinase C (PKC), mitogen-activated kinases ('MAPK/ERK') [8], p21-activated kinase [9] and several as-yet-undefined kinases [10,11]. Pharmacological evidence suggests these are regulated by receptor systems. However, it is not clear how the various kinase responses are integrated to result in the phosphorylation of NADPH oxidase components [12].

In the present study we attempted to purify a kinase from activated neutrophil cytosol using affinity chromatography on a p47^{phox} matrix. We found that the major kinase activity capable of binding p47^{phox} is PKC. Here we report on the possible relevance of this interaction for the regulation of PKC in neutrophils. We provide evidence that, in addition to being a

PKC substrate, p47^{phox} also serves to control the localization/activity of PKC and that this has repercussions for the regulation of the PKC substrate coronin in neutrophils.

EXPERIMENTAL

Reagents

All chemicals and reagents were reagent grade and were from Sigma unless stated otherwise. HiTrap *N*-hydroxysuccinimide-activated Sepharose columns were from Amersham Pharmacia Biotech. Antibodies to PKC were obtained from Santa Cruz Biotechnology. Latex particles (0.81 μ m diameter) were purchased from Difco and the PKC inhibitor Gö6850 was purchased from Calbiochem.

Recombinant p47^{phox}, p67^{phox} and p40^{phox} were made as glutathione S-transferase (GST) fusion proteins in the pGEX system (Pharmacia). Transformed *Escherichia coli* was cultured in an Electrolab 300 fermenter. Proteins were purified from the bacterial lysates on GSH-Sepharose (Pharmacia) [13]. Purified fusion proteins remained attached to the Sepharose or were cleaved using thrombin and chromatographed by affinity chromatography using heparin-agarose. Coronin was expressed in insect (*Spodoptera frugiperda*) cells (Sf9) using the baculovirus system (Invitrogen). Coronin cDNA was subcloned into the baculovirus transfer vector pVL 1393 with a His-tag (a tag consisting of six histidine residues) at the 3' end and transferred into baculovirus using the Baculogold system [obtained from Pharmingen (San Diego, CA, U.S.A.) through Becton-Dickinson]. Infection of Sf9 cells was done at 10⁶ cells/ml for 72 h. The coronin was purified from insect cell lysate by nickel

Abbreviations used: PKC, protein kinase C; PKM, protein kinase M (the catalytic fragment of PKC); CGD, chronic granulomatous disease; GST, glutathione S-transferase; Sf9, *Spodoptera frugiperda* 9; ECL[®], enhanced chemiluminescence (Amersham); MARCKS, myristoylated alanine-rich C-kinase substrate; His-tag, a tag consisting of six histidine residues.

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affinity chromatography (Invitrogen ProBond resin) followed by heparin-agarose.

Purification of p47^{phox}-interacting kinase activity

Neutrophils were purified from fresh human blood by dextran sedimentation and centrifuged through Ficoll/Hypaque [14]. Human neutrophil lysates were prepared as follows: 2×10^9 cells were suspended in 10 ml of buffer A (0.15 M NaCl/10 mM NaHPO₄/10 mM KCl, pH 7.3) containing 5 mM glucose, stimulated with PMA (1 µg/ml) for 3 min or human IgG-coated latex particles [5], for 10 min. Cells were then lysed in buffer B [10 mM KCl/3 mM NaCl/4 mM MgCl₂/10 mM Pipes (pH 7.0)/leupeptin (1 µg/ml)/pepstatin (1 µg/ml), tosyl-lysylchloromethane ('TLCK'; 1 µg/ml)/5 mM pyrophosphate/25 mM NaF] [16] and centrifuged at 250 000 g for 30 min.

For affinity chromatography, purified recombinant p67^{phox}, p47^{phox}, p40^{phox} or BSA as a control, were coupled to 1 ml of HiTrap N-hydroxysuccinimide-activated columns. Prepared columns were pre-equilibrated in buffer B. Via a Minipuls 2 Gibson pump, the neutrophil cytosol was first passed over a BSA column to decrease non-specific binding and subsequently on to the *phox* protein columns or a second BSA column as a control. The columns were sealed and incubated at 37 °C for 20 min, cooled to 4 °C and washed with 10 bed vol. of ice-cold buffer B. Bound proteins were eluted in 2 ml of buffer B containing 1 M NaCl and concentrated to 200 µl using a Centricon 10 filter unit (Amicon). The eluate from the p47^{phox} affinity column was further purified by sequential gel filtration and anion-exchange chromatography using the Pharmacia Biotech SMART system. For gel filtration, a Superdex 200 PC 3.2/30 column was equilibrated in buffer B supplemented with 500 mM NaCl at 40 µl/min. A 50 µl portion of p47^{phox} eluate was loaded on the column and 30 fractions (80 µl each) were collected at 40 µl/min. As chromatography markers, BSA (67 kDa), ovalbumin (43 kDa) and chymotrypsinogen A (25 kDa) were used. The peak of kinase activity was located and pooled, diluted 10-fold in buffer B, loaded on to a Mini Q PC 3.2/3 and eluted at 100 µl/min with a 3 ml gradient of 50–500 mM NaCl in buffer B; 30 fractions (100 µl each) were collected.

Subcellular fractionation

Subcellular fractionation of neutrophils lysates for the PKC distribution experiment was as follows. Neutrophils (5×10^7 cells) were sonicated in 200 µl of buffer B, and a postnuclear supernatant was prepared by centrifugation for 15 min at 1000 g at 4 °C. The supernatant was loaded on a 1 ml discontinuous sucrose gradient [0.5 ml of 17.5% and 0.5 ml of 34% sucrose (w/w)] and centrifuged for 30 min at 120 000 g. The top layer (cytosol, 200 µl) was collected, and 20 µl of 10× buffer C [62.5 mM Tris/HCl (pH 6.8)/2% SDS/2.5% glycerol/1 mM β-mercaptoethanol/0.025% (w/v) Bromophenol Blue] was added to it. Membranes floating between 17.5% and 34% sucrose were collected, diluted in 4 vol. of buffer B and centrifuged for 30 min at 200 000 g at 4 °C. The pellet was resuspended in 50 µl of buffer C (plasma membrane particulate fraction). The pellet under the 34% sucrose cushion was washed in buffer B and resuspended in 100 µl of buffer C (non-plasma membrane particulate fraction).

For the experiment in Figure 7 below, cytosol was collected from the sucrose gradient, centrifuged at 250 000 g and, to the supernatant, 0.1 vol. of 10× buffer C was added.

Recombinant proteins and binding studies

Further binding studies were performed using p47^{phox} constructs previously described [17]. These proteins remained as fusion

proteins attached to GSH-Sepharose (Amersham Pharmacia). As a control the GST protein itself was used. Purified kinase was incubated with the p47^{phox} domains at 37 °C for 15 min. Unbound material was removed and the beads were washed three times in buffer B. Beads were resuspended in buffer C and the proteins were resolved by SDS/10% PAGE [18].

In vitro phosphorylation of recombinant p47^{phox}, immunoprecipitations and phosphopeptide mapping

p47^{phox} kinase assays were performed as described in [10] using 20 µl of enzyme fraction in a final volume of 30 µl. Reactions were stopped by the addition of 0.1 vol. of 10× buffer C, and 20 µl of the resulting mixture was resolved by SDS/PAGE. Gels were then autoradiographed or, alternatively, the p47^{phox} band was excised and counted for radioactivity in a liquid-scintillation counter (channel fully open). One unit of kinase activity was defined as pmol of phosphate transferred on to 20 µg of p47^{phox}/min.

For ³²P-labelling, neutrophils were incubated at 5×10^7 cells/ml in 1 ml of phosphate-free RPMI medium (Gibco BRL) for 45 min at 30 °C in the presence of 0.5 mCi of [³²P]_i (Amersham Pharmacia). Cells were then stimulated with 1 µg/ml PMA for 3 min at 37 °C (or not stimulated), after which they were lysed in buffer B. The resulting lysate was centrifuged for 10 min at 100 000 g at 4 °C, the supernatant was taken, set to buffer D (buffer B containing 1% Triton X-100, 300 mM NaCl, 2% sodium deoxycholate and 0.2% SDS) and used for immunoprecipitation. Immunoprecipitation of p47^{phox} or coronin with affinity-purified antibodies was performed as described in [19]. Two-dimensional phosphopeptide mapping was carried out as described in [5] either on p47^{phox} isolated from ³²P-labelled neutrophils or on recombinant p47^{phox} phosphorylated *in vitro* by the isolated kinase.

Preparation of rat brain cytosol

A 2 g portion of frozen (−80 °C) rat brains was homogenized in buffer B and centrifuged for 30 min at 100 000 g. The supernatant was supplemented with 20 mM magnesium acetate, 30 µM calcium acetate, 600 pmol of ATP and [³²P]ATP (3 µCi/100 pM ATP) and incubated for 15 min at 30 °C with various combinations of recombinant p47^{phox}, coronin and purified kinase as indicated in the Figure legends. The total reaction volume was 115 µl. Reactions were stopped by the addition of 11 µl of 10× buffer C and analysed by SDS/PAGE and autoradiography.

Electrophoresis and immunoblotting

Samples were run on SDS/PAGE minigels and transferred to nitrocellulose membrane using a semi-dry blotter for 1 h at 1.4 mA/cm². Antibodies to PKC were incubated for 16 h at 4 °C. Horseradish peroxidase-conjugated goat anti-rabbit antibody was used to detect reactive bands by the enhanced chemiluminescence (ECL[®]) system (Amersham). ECL bands were quantified by densitometry using an Alpha Imager 2000 (Alpha Innotech Corporation, San Leandro, CA, U.S.A.).

RESULTS

Immobilized p47^{phox} extracts a p47^{phox} kinase from activated neutrophil cytosol

Using the phosphorylation of p47^{phox} as an indicator, we sought to identify a kinase in neutrophil cytosol capable of phos-

Figure 1 Neutrophil cytosol contains a kinase that binds to and phosphorylates p47^{phox}

(a) Cytosols derived from 2×10^9 control cells, PMA-activated cells ($1 \mu\text{g}/\text{ml}$; 3 min) or phagocytosing cells (2×10^{11} IgG-opsonized particles, $0.81 \mu\text{m}$ diameter; 10 min), were passed over immobilized p47^{phox}. Bound proteins were eluted with 2 ml of buffer B containing 1 M NaCl and concentrated to 200 μl . A 20 μl portion was then used to phosphorylate recombinant p47^{phox} as described in the Experimental section. (b)–(d) Cytosol from PMA-activated neutrophils (lane 1) was passed over immobilized p67^{phox}, p47^{phox}, p40^{phox} (lanes 3, 4 and 5) or BSA as control (lane 2). Bound proteins were eluted as described above, and 20 μl was either analysed by SDS/PAGE and Coomassie Blue staining (b) or assayed for p47^{phox} kinase activity (c) or analysed for the presence of PKCs- β_1 , - β_{II} or - δ by SDS/PAGE and Western blotting (d). This result is representative of ten separate experiments.

phorylating p47^{phox}. Figure 1(a) shows that whole cytosol did not contain significant kinase activity but that, when it was passed over immobilized p47^{phox}, an active kinase was recovered. It was only present in cytosol from stimulated cells, either after exposure to PMA or after the phagocytosis of IgG-coated latex particles. In addition to this kinase activity other proteins were extracted with apparent molecular masses of 43, 75 and about 200 kDa (Figure 1b). The 75 kDa protein was identified as moesin by amino acid sequencing and the 43 kDa protein as actin by

Figure 2 Gel-filtration chromatography of the kinase and comparison with the elution profile of PKCs- β_1 , - β_{II} or - δ

A 50 μl portion of concentrated eluate from the p47^{phox} affinity column was chromatographed by gel filtration on a Superdex 200 PC3.2/30 column and 30 fractions (80 μl each) were collected. (a) Protein elution profile. (b) A 20 μl portion of fractions 9–15 was used to phosphorylate p47^{phox} as described in the Experimental section. The lane marked 'start' shows the phosphorylation of p47^{phox} by 20 μl of p47^{phox} eluate before gel filtration. (c) As under (b), but fractions were analysed by SDS/PAGE and silver staining (arrow indicates the major protein co-eluted with the kinase activity). (d) As under (b), but fractions were analysed for the presence of PKCs- β_1 , - β_{II} and - δ by SDS/PAGE and Western blotting. The lane marked 'C' shows PKC isotypes in 5 μl of neutrophil supernatant prior to p47^{phox} affinity chromatography. The lane marked 'start' shows PKC isotype content of 20 μl of p47^{phox} eluate prior to gel filtration. These elution profiles were highly reproducible and consistently observed in a large number of experiments.

immunoblotting. The 200 kDa protein may represent myosin. p47^{phox} kinase activity was selectively immobilized by p47^{phox} and not by p40^{phox} or p67^{phox} (Figure 1c).

The p47^{phox} kinase is protein kinase M (PKM)

Material eluted from immobilized p47^{phox} was further purified by gel filtration as shown in Figure 2. Kinase activity eluted with an apparent molecular mass of about 45 kDa (Figure 2b). On SDS/PAGE a 45 kDa protein was co-eluted with the kinase activity (arrow in Figure 2c). Western blotting showed that this protein did not represent p21-activated kinase or mitogen-activated protein kinase ('MAP kinase'); however, it reacted

Figure 3 Anion-exchange chromatography of kinase and PKC isotypes

Fractions 13 and 14 from three separate gel-filtration runs (Figure 2) were pooled (total volume 480 µl), diluted 10-fold in buffer B and loaded on a Mini Q 3.2/30 anion-exchange column. A total of 30 fractions (100 µl each) were collected. (a) Protein elution profile. (b) A 20 µl portion of fractions 10–20 was used to phosphorylate p47^{phox} as described in the Experimental section. Abbreviations: st, 20 µl of pooled gel filtration eluate prior to dilution; un, 20 µl of MiniQ breakthrough fraction. (c) As (b), but fractions were analysed for the presence of PKC isotypes by SDS/PAGE and Western blotting. These elution profiles were highly reproducible and consistently observed in a large number of experiments.

with antibodies to PKC isotypes, suggesting it represents the free catalytic fragment of PKC (often referred to as PKM). Catalytic fragments of PKC-β_I, -β_{II} and -δ all co-purified with the kinase activity after gel filtration (Figure 2d). Only p47^{phox} and not p40^{phox} or p67^{phox} bound the PKMs (Figure 1d). MiniQ ion-exchange chromatography resulted in a partial separation of the PKMs and the distribution of kinase activity corresponded most closely to that of PKC-β_{II} (Figure 3).

Three lines of evidence supported the identification of the p47^{phox}-interacting kinase as PKC/PKM. The selective PKC inhibitor Gö6850 inhibited the kinase activity with a *K*_i of 10 nM, similar to that published for PKC [17]. A mixture of antibodies to PKC extracted the kinase activity from solution. And finally phosphopeptide maps prepared from recombinant p47^{phox} phosphorylated *in vitro* with the purified kinase showed that many of the same peptides were phosphorylated as occurred in p47^{phox} from PMA-stimulated cells (results not shown).

Purified PKM binds to the C-terminus of p47^{phox}

In the initial affinity step on immobilized p47^{phox}, PKM was extracted from the cytosol of activated cells, together with a number of other proteins, raising the question as to whether the binding of the kinase to p47^{phox} was direct or through an intermediate protein or protein complex. To demonstrate direct

Figure 4 Binding of purified kinase to p47^{phox}

Kinase activity purified as described in Figures 2 and 3 was re-applied to immobilized p47^{phox} or GST fusion proteins representing sections of p47^{phox}. (a) Phosphorylation of p47^{phox} by 20 µl of fraction 17 as obtained in Figure 3 (start). A 60 µl portion of fraction 17 (Figure 3) was diluted to 1 ml in buffer B and bound to the p47^{phox} affinity column, washed with 10 ml of buffer B and eluted with 1 ml of buffer B plus 1 M NaCl, after which unbound, wash and final eluate were all concentrated to 200 µl. A 20 µl portion of concentrated fraction was used to phosphorylate p47^{phox}. (b) A 20 µl portion of fraction 17 (Figure 3) was incubated with 200 pmol of immobilized GST-p47^{phox} fusion proteins. Unbound material was collected, after which the beads were washed in buffer B and resuspended in 20 µl of buffer C. Unbound and bound material was analysed by SDS/PAGE and Western blotting with a mixture of antibodies to PKC isotypes β_I, β_{II}, and δ. For reference, 20 µl of fraction 17 was loaded in the lane marked 'S'. The experiment shown is representative of three independent ones.

binding, the purified kinase was re-exposed to immobilized p47^{phox}. As shown in Figure 4(a), all the kinase activity re-bound to the p47^{phox}.

In order to determine the part of the p47^{phox} molecule to which PKM was attaching, binding was examined with a series of recombinant p47^{phox} constructs expressing different regions of the molecule. Figure 4(b) shows that PKM does not bind to the N-terminal half of the molecule or to the region of the two SH3 domains, but binds strongly to the C-terminus. The binding to the isolated C-terminus is stronger than to full-length p47^{phox} or to p47^{phox} lacking the last 44 amino acids. This may be attributed to the intramolecular binding that exists within p47^{phox} between the proline-rich domain that extends from residues 360–373 [20] and the SH3 domains. Binding sites on p47^{phox} that are normally less accessible in an inactive closed conformation

Figure 5 Co-immunoprecipitation of p47^{phox} and PKC β_{II}

Postnuclear supernatants were prepared from 5×10^7 cells stimulated with PMA ($1 \mu\text{g/ml}$) for the indicated length of time (min) and solubilized in buffer as described in the Experimental section. Immunoprecipitations were carried out with affinity-purified antibodies to p47^{phox}, resuspended in $80 \mu\text{l}$ of buffer C of which $20 \mu\text{l}$ was analysed by SDS/PAGE and Coomassie Blue staining (top panel) or Western blotting with a PKC- β_{II} (middle panel) or PKC- δ (lower panel) antibody. Two further experiments showed similar co-immunoprecipitation results.

become exposed upon activation [21,22] which may explain the greater kinase binding capacity.

Co-immunoprecipitation of PKC isotypes and p47^{phox}

In order to assess the binding of PKC to p47^{phox} *in vivo*, we performed immunoprecipitation experiments. Figure 5 shows that PKC- β_{II} co-immunoprecipitates with p47^{phox} in cells stimulated with PMA. The association occurred rapidly and was maximal after 1 min. PKC- δ also co-immunoprecipitated with p47^{phox}; however, the time course of interaction was critically different. No interaction occurred at the early time points, although interaction was observed at the 5 min point. This suggests that, although all PKC isotypes are able to interact with p47^{phox}, the characteristics and perhaps the function of these interactions are different for each isotype.

Aberrant regulation of PKC- β_I and - β_{II} in cells lacking p47^{phox}

To investigate the function of the interactions in more detail, we employed cells from CGD patients lacking p47^{phox} and examined the regulation of PKC isotypes in these cells. No differences in overall PKC isotype levels were found between wild-type cells and cells lacking p47^{phox} (results not shown). Figure 6 shows the result of fractionation experiments of normal cells and cells lacking p47^{phox}. In normal cells PKC- β_I , - β_{II} and - δ are present in the cytoplasm and rapidly recruited to particulate fractions (Figures 6a, 6c and 6e) upon stimulation with PMA. At longer time points PKC isotype levels are reduced in the particulate fractions. The pattern of recruitment of PKC- δ in cells lacking p47^{phox} is the same as that in normal cells; however, PKC- β_I and - β_{II} show different patterns. Specifically, the decrease in PKC

Figure 6 Localization of PKC isotypes in normal and p47^{phox}-deficient cells

Wild-type cells (a, c and e) or cells lacking p47^{phox} (b, d and f) were stimulated with $1 \mu\text{g/ml}$ PMA for the indicated length of time (min) and fractionated as described in the Experimental section. A $5 \mu\text{l}$ portion of each subcellular fraction was analysed by SDS/PAGE and Western blotting for PKC- β_I (a and b), PKC- β_{II} (c and d) or PKC- δ (e and f). Squares, cytosol fraction; circles, plasma-membrane particulate fraction; triangles, non-plasma-membrane particulate fraction. Western signals were quantified and expressed as percentage of maximal. A repeat experiment showed the same patterns as the experiment shown.

Figure 7 Phosphorylation in p47^{phox}-deficient human neutrophils

(a) Wild-type cells (lanes 1, 2, 5 and 6) or cells lacking p47^{phox} (lanes 3, 4, 7 and 8) were labelled with ³²P and stimulated with PMA (1 µg/ml, 3 min). Cytosol fractions (200 µl) were isolated as described in the Experimental section, and 50 µl was analysed by SDS/PAGE and Coomassie Blue staining (lanes 5–8) or autoradiography (lanes 1–4). The same phosphorylation pattern was observed in four separate experiments. (b) Autoradiographs of coronin immunoprecipitations from the cytosol of 5 × 10⁸ ³²P-labelled neutrophils. A repeat experiment showed the same increase in phosphorylation. (c) Presence of coronin in cytosols from control and 10 µM Gö6850-treated cells incubated for 3 min in the presence (+) or absence (–) of 1 µg/ml PMA. The experiment shown was repeated with an identical result. (d) Presence of coronin in cytosols from control and p47^{phox}-deficient cells incubated for 3 min in the presence (+) or absence (–) of 1 µg/ml PMA. The experiment shown is representative of four separate experiments. For (c) and (d) cytosols were analysed by SDS/PAGE and Western blotting using a coronin antibody followed by quantification.

isotype content seen at the longer time points in the particulate fractions is no longer manifest (Figures 6b, 6d and 6f). Thus the presence of p47^{phox} affects PKC-β₁ and -β_{II} isotypes, but not PKC-δ in this subcellular compartment.

Aberrant protein phosphorylation in cells lacking p47^{phox}

We next measured phosphorylation events in cells lacking p47^{phox} obtained from CGD patients. Overall phosphorylation was measured in normal neutrophil cytosol and in cytosol from p47^{phox}-deficient cells before and after stimulation with PMA. PMA stimulated protein phosphorylation twofold in wild-type cells (from 100 ± 63 to 189 ± 20%; *n* = 4, mean ± S.D.). Basal phosphorylation in neutrophils lacking p47^{phox} was higher than in wild-type cells, but the difference was not statistically different. PMA-stimulated phosphorylation was significantly higher in cells lacking p47^{phox} than in wild-type cells (189 ± 20 versus 251 ± 34%; *n* = 4, mean ± S.D.; *P* < 0.05). These differences were not observed in CGD cells deficient in the flavocytochrome or in those lacking p67^{phox} and are therefore specifically related to the lack of p47^{phox} (results not shown). In addition to the

Figure 8 Phosphorylation of rat brain cytosol and the effect of the addition of p47^{phox}, coronin and the purified kinase

(a) Rat brain cytosol incubated with [³²P]ATP, and the effect of the addition of p47^{phox}, coronin and the purified kinase (0.5 unit, 25 µl). A 15 µl portion of the kinase reaction mixture was analysed by SDS/PAGE and autoradiography. A representative experiment of three independent ones is shown. (b) Coronin immunoprecipitates from the mixtures in (a). The upper panel depicts the autoradiograph and the lower panel the Coomassie Blue-stained gel, illustrating coronin loadings. (c) Recombinant coronin was incubated in a total volume of 41 µl for 15 min at 30 °C as described [10] with purified kinase (0.5 unit, 25 µl), in the presence (lane 2) or absence (lane 1) of p47^{phox} (lane 1). The reaction was stopped by addition of 4 µl of 10 × buffer C, and 25 µl was analysed by SDS/PAGE and autoradiography. The experiment shown is representative of three independent ones. All experiments were done with 10 µg of p47^{phox} and 10 µg of coronin with or without 280 µg of rat brain cytosol.

general increase in phosphorylation observed in the p47^{phox}-deficient cells, other differences from normal were observed (Figure 7a). A heavily phosphorylated protein at 60 kDa in the normal cytosol was missing in the p47^{phox}-deficient cells. This protein was excised from the gel and identified by MS analysis as coronin. Immunoprecipitation using a specific coronin antiserum confirmed that coronin became phosphorylated on activation by PMA (Figure 7b). The reduced phosphorylation of coronin in cytosols from cells lacking p47^{phox} is due to the absence of coronin in these cytosols [16], and it thus appears that p47^{phox} regulates the solubility of coronin (Figure 7d). The same decreased solubilization of coronin was observed when cells were stimulated in the presence of a PKC inhibitor (Figure 7c). Thus the absence of p47^{phox} or the inhibition of PKC affects the solubilization of coronin in the same way.

In order to provide direct evidence for the regulatory function of the interaction of PKC with p47^{phox} we designed an *in vitro* reconstitution experiment using cells devoid of p47^{phox} but rich in PKC. It was confirmed that cytosol from rat brain did not

contain p47^{phox}, but contained PKM (results not shown). Addition of p47^{phox} to rat brain cytosol resulted in a decrease of endogenous brain phosphorylation (Figure 8, lanes 8 and 9), similar to the phenomenon observed in neutrophils containing or lacking p47^{phox} (see Figure 7). Thus p47^{phox} appears to affect the level of phosphorylation exerted by endogenous kinases in the cytosol.

The effect of p47^{phox} on phosphorylation of coronin by PKC/PKM was also apparent in this system. Addition of p47^{phox} in the mixture increases phosphorylation of coronin both by endogenous kinase and by added purified PKM (Figure 8a). The effect on coronin was confirmed by immunoprecipitation and did not appear due to small differences in the amount of protein present (Figure 8b). Furthermore, addition of pure p47^{phox} increases the phosphorylation of pure coronin by PKM (Figure 8c).

DISCUSSION

In the present paper we describe the interaction between PKC isotypes and p47^{phox}. This interaction occurs at the catalytic site of PKC and the C-terminus of p47^{phox} and is specific for p47^{phox}, since it was not observed for the other *phox* proteins. The interaction occurred *in vivo* and has several consequences for the cell. Cells lacking p47^{phox} show a difference in the recruitment and processing of PKC isotypes to particulate fractions, in the level of phosphorylation in neutrophil cytosol fractions and the specific phosphorylation and solubilization of coronin. This suggests that p47^{phox} is not just a downstream effector of PKC but a regulator of PKC at the same time.

There are several lines of evidence supporting a role for PKC in the phosphorylation of p47^{phox}. p47^{phox} is a good substrate for PKC, and the potent PKC inhibitor staurosporin blocks PMA-induced superoxide production and inhibits phosphorylation of p47^{phox} [23–25]. Phosphopeptide maps of p47^{phox} isolated from PMA-activated neutrophils and Epstein–Barr-virus-transformed B-lymphocytes are identical with the phosphopeptide map of p47^{phox} phosphorylated *in vitro* by PKC, except for the absence of the Ser^{345,348} peptide from the latter map [26]. Finally PKC is capable of phosphorylating p47^{phox} at various sites which are absolutely essential for activation and translocation of the protein [27].

Whilst the phosphorylation of p47^{phox} by PKC is well established, a direct interaction between this substrate and the kinase has not been observed before. The possibility that such interactions took place had been suggested previously [28]. It was observed that PKC- β recruitment to Triton-insoluble fractions was different in cells lacking p47^{phox}. The present observations extend these conclusions, since we show that PKC binds directly to p47^{phox} and this may underlie the aberrant fractionation pattern. Furthermore, our data suggest that the regulation of PKC by p47^{phox} occurs for the PKC- β isotypes but not for PKC- δ .

The fact that p47^{phox} and PKC interact is in keeping with a growing body of evidence that PKC and its substrates show direct interactions [29,30]. PKC has been shown to interact with GAP-43 [31], myristoylated alanine-rich C-kinase substrate (MARCKS) [32] and a number of substrates collectively known as STICKS ('substrate that interacts with C-kinase') [30]. The sites of interaction are dependent on the substrate involved. For instance GAP-43 interacts with the C2-like domain of novel PKCs, whereas MARCKS interacts with the catalytic as well as the regulatory domain of various different PKC isotypes. For p47^{phox} it appears from our data that the interaction occurs at the catalytic domain of PKC. This is evident from the affinity-

purification experiments in which PKM binding was detected from cytosols of activated neutrophils. Although it is possible that PKM is the relevant entity *in vivo*, the immunoprecipitation results suggest that full-length PKC is capable of binding, provided the cells have been exposed to PKC-activating cofactors such as PMA. Thus the most likely site of interaction appears to be a cryptic domain in the catalytic domain of PKC which is exposed upon activation of the kinase by PMA.

Attempts have been made to assess the function of the association between PKC and its substrate. It is likely that interactions are necessary for the subsequent phosphorylation event, but in addition it has been suggested that they may participate in the targeting of PKC to subcellular sites, and a targeting function has been proposed for some PKC substrates [33]. To establish a functional role for the binding of PKC to p47^{phox} we expanded our studies into cells lacking p47^{phox}. In these cells PKC- β_1 and - β_{II} were retained in the particulate fractions, suggesting that p47^{phox} plays a role in the regulation of the normal processing of PKC- β_1 and - β_{II} upon activation of the cells. It is thus likely that the interaction between p47^{phox} and PKC- β_1 and - β_{II} results in a conformation that allows the proper clearance of PKC- β_1 and β_{II} from subcellular compartments to which it is recruited upon activation. In the absence of an interaction this process is disturbed and PKC- β_1 and - β_{II} accumulate in these fractions. It is of interest that the aberrant processing phenomenon was not observed for PKC- δ , providing at the same time a control for the observations on PKC- β_1 and β_{II} , as well as suggesting specificity of regulation of the isotypes.

Parallel with the accumulation of PKC- β_1 and β_{II} in the particulate fractions, differences in the endogenous phosphorylation patterns became apparent in cells lacking p47^{phox}. We found that activated cytosols from p47^{phox}-deficient patients all expressed a level of hyperphosphorylation above the normal. These differences did not occur in non-stimulated cells, suggesting that this phenomenon may be related to the aberrant clearing of PKC- β_1 and - β_{II} after stimulation. Interestingly the same phenomenon was observed in a reconstitution experiment using rat brain cytosol (which lacks p47^{phox}) to which we added p47^{phox}. At present we do not know the consequences of the hyperphosphorylation.

Most importantly we observed the lack of phosphorylation of coronin in the cytosol of p47^{phox}-deficient cells. This is due to an absence of coronin in this fraction, as shown by Western blotting [16]. Phosphorylation by PKC is necessary for normal appearance of coronin in the soluble fraction, since a PKC inhibitor inhibits its solubilization. We hypothesize that p47^{phox} allows appropriate targeting of PKC to coronin so that phosphorylation and solubilization can occur. Interestingly, the same phenomenon was observed in an *in vitro* reconstitution experiment, providing direct evidence for the targeting function of p47^{phox}. This is compatible with the idea that the cytosolic *phox* proteins exist in a multiprotein complex together with coronin and may regulate coronin, as suggested in [16]. The present data suggest that the likely way in which coronin is regulated by the *phox* proteins is through phosphorylation by PKC. The exact mechanism by which phosphorylation affects coronin function is not clear. We consistently observe an appearance of coronin in cytosol fractions upon activation of neutrophils and have taken this as a reflection of its activation state [16]. The consequences of coronin activation appear related to processes in which cytoskeletal rearrangements occur, including cell locomotion and phagocytosis [34,35]. As such, regulation of coronin by NADPH oxidase components may provide an explanation for the spatial restriction of superoxide generation to the site of phagocytosis in neutrophils.

In summary we have shown that p47^{phox} can act as a regulator

of PKC as well as being a direct target. In the case of p47^{phox}-deficient cells this may result in deregulation of at least a fraction of PKC (in particular the PKC- β isotypes) which correlates with the hyperphosphorylation seen in these cells. However, binding between p47^{phox} and PKC may have a dual function, as p47^{phox} directs kinase activity to at least one target here defined as the cytoskeletal protein coronin.

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REFERENCES

- Morel, F., Doussiere, J. and Vignais, P. V. (1991) *Eur. J. Biochem.* **201**, 523–546
- Segal, A. W. and Abo, A. (1993) *Trends Biochem. Sci.* **18**, 43–47
- Thrasher, A. J., Keep, N. H., Wientjes, F. and Segal, A. W. (1994) *Biochim. Biophys. Acta* **1227**, 1–24
- Segal, A. W., Heyworth, P. G., Cockcroft, S. and Barrowman, M. M. (1985) *Nature (London)* **316**, 547–549
- Forbes, L. V., Truong, O., Wientjes, F. B., Moss, S. J. and Segal, A. W. (1999) *Biochem. J.* **338**, 99–105
- Garcia, R. C. and Segal, A. W. (1988) *Biochem. J.* **252**, 901–904
- Fuchs, A., Bouin, A. P., Rabilloud, T. and Vignais, P. V. (1997) *Eur. J. Biochem.* **249**, 531–539
- El Benna, J., Han, J., Park, J. W., Schmid, E., Ulevitch, R. J. and Babior, B. M. (1996) *Arch. Biochem. Biophys.* **334**, 395–400
- Knaus, U. G., Morris, S., Dong, H. J., Chernoff, J. and Bokoch, G. M. (1995) *Science* **269**, 221–223
- Lal, A. S., Parker, P. J. and Segal, A. W. (1999) *Biochem. J.* **338**, 359–366
- McPhail, L. C., Qualliotine Mann, D. and Waite, K. A. (1995) *Proc. Natl. Acad. Sci. U.S.A.* **92**, 7931–7935
- Segal, A. W., Wientjes, F. and Stockley, R. W. and Dekker, L. V. (1999) in *Advances in Cell and Molecular Biology of Membranes and Organelles*, vol. 5, Phagocytosis: The Host (Gordon, S., ed.), pp. 441–483, JAI Press Inc., Stamford, CT
- Suzuki, K., Nishihata, J., Arai, Y., Honma, N., Yamamoto, K., Irimura, T. and Toyoshima, S. (1995) *FEBS Lett.* **364**, 283–288
- Segal, A. W. and Jones, O. T. G. (1980) *FEBS Lett.* **110**, 111–114
- Segal, A. W. and Jones, O. T. G. (1978) *Nature (London)* **276**, 515–517
- Grogan, A., Reeves, E., Keep, N., Wientjes, F., Totty, N. F., Burlingame, A. L., Hsuan, J. J. and Segal, A. W. (1997) *J. Cell Sci.* **110**, 3071–3081
- Wientjes, F. B., Panayotou, G., Reeves, E. and Segal, A. W. (1996) *Biochem. J.* **317**, 919–924
- Cleveland, D. W., Fischer, S. G., Kirschner, M. W. and Laemmli, U. K. (1977) *J. Biol. Chem.* **252**, 1102–1106
- Dusi, S. and Rossi, F. (1993) *Biochem. J.* **296**, 367–371
- Finan, P., Shimizu, Y., Gout, I., Hsuan, J., Truong, O., Butcher, C., Bennett, P., Waterfield, M. D. and Kellie, S. (1994) *J. Biol. Chem.* **269**, 13752–13755
- Sumimoto, H., Kage, Y., Nunoi, H., Sasaki, H., Nose, T., Fukumaki, Y., Ohno, M., Minakami, S. and Takeshige, K. (1994) *Proc. Natl. Acad. Sci. U.S.A.* **91**, 5345–5349
- DeLeo, F. R., Ullman, K. V., Davis, A. R., Jutila, K. L. and Quinn, M. T. (1996) *J. Biol. Chem.* **271**, 17013–17020
- Kramer, I. M., Verhoeven, A. J., van der Bend, R. L., Weening, R. S. and Roos, D. (1988) *J. Biol. Chem.* **263**, 2352–2357
- Combadiere, C., Hakim, J., Giroud, J. P. and Perianin, A. (1990) *Biochem. Biophys. Res. Commun.* **168**, 65–70
- Nauseef, W. M., Volpp, B. D., McCormick, S., Leidal, K. G. and Clark, R. A. (1991) *J. Biol. Chem.* **266**, 5911–5917
- El Benna, J., Faust, L. P., Johnson, J. L. and Babior, B. M. (1996) *J. Biol. Chem.* **271**, 6374–6378
- Johnson, J. L., Park, J. W., Benna, J. E., Faust, L. P., Inanami, O. and Babior, B. M. (1998) *J. Biol. Chem.* **273**, 35147–35152
- Curnutte, J. T., Erickson, R. W., Ding, J. and Badwey, J. A. (1994) *J. Biol. Chem.* **269**, 10813–10819
- Mochly-Rosen, D. and Gordon, A. S. (1998) *FASEB J.* **12**, 35–42
- Jaken, S. (1996) *Curr. Opin. Cell Biol.* **8**, 168–173
- Dekker, L. V. and Parker, P. J. (1997) *J. Biol. Chem.* **272**, 12747–12753
- Fujise, A., Mizuno, K., Ueda, Y., Osada, S., Hirai, S., Takayanagi, A., Shimizu, N., Owada, M. K., Nakajima, H. and Ohno, S. (1994) *J. Biol. Chem.* **269**, 31642–31648
- Hyatt, S. L., Liao, L., Aderem, A., Nairn, A. C. and Jaken, S. (1994) *Cell Growth Differ.* **5**, 495–502
- Gerisch, G., Albrecht, R., Heizer, C., Hodgkinson, S. and Maniak, M. (1995) *Curr. Biol.* **5**, 1280–1285
- Maniak, M., Rauchenberger, R., Albrecht, R., Murphy, J. and Gerisch, G. (1995) *Cell* **83**, 915–924

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