The major phosphorylation site of the NADPH oxidase component p67^{phox} is Thr²³³

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Phosphorylation of p67^{phox} was shown to increase two- to three-fold upon stimulation by PMA, *N*-formylmethionyl-leucylphenylalanine or serum-opsonized zymosan. Phosphopeptide mapping showed one major tryptic peptide for p67^{phox} immunoprecipitated from resting or stimulated cells. *In vitro* phosphorylation of p67^{phox} by isolated cytosol or mitogen-activated protein kinase also generated the same phosphopeptide. Results of cyanogen bromide digestion and HPLC–MS suggested

that Thr²³³ was the phosphorylated residue. Mutagenesis of Thr²³³ to alanine resulted in loss of phosphorylation *in vitro*. In the present work, Thr²³³ has been identified as the major phosphorylation site of p67^{phox}, which is situated in a proline-rich domain.

Key words: MAP kinase, mutagenesis, neutrophil, phosphopeptide mapping.

INTRODUCTION

The multicomponent NADPH oxidase, which causes production of superoxide, is central to the defence mechanisms employed by phagocytes against invading micro-organisms. Upon activation of NADPH oxidase, many proteins become phosphorylated, including the cytosolic components p67^{phox} [1], p47^{phox} [2], p40^{phox} [3] and both subunits of the membrane-bound flavocytochrome b₅₅₈ [4]. The requirement of these components in the NADPH oxidase system is proved by the manifestation of chronic granulomatous disease (CGD) when they are absent, but the exact role for their phosphorylation has not been determined.

The interactions between p67^{phox} and the other components of NADPH oxidase have been widely studied. p67^{phox} has particular affinity for p40^{phox} [5], its activation-induced translocation to the membrane depends on the presence of p47^{phox} [6], and it appears to be an essential effector target for p21^{rac} [7]. It has been proposed that p67^{phox} regulates electron transport at the membrane by facilitating electron flow from NADPH to the flavin centre of the flavocytochrome b₅₅₈ [8]. Kinetic studies on a cell-free system suggest that activation of NADPH oxidase is dependent on an alteration of p67^{phox} [9]. It is feasible that phosphorylation could bring about a conformational change thereby altering the resting-state protein interactions, leading to an active configuration.

Phosphorylated p67^{phox} is found in cytosol and membrane fractions, and it appears that continuous translocation is required to maintain oxidase activity [1,10]. A causal relationship between p67^{phox} phosphorylation, translocation and oxidase activation has been suggested but not proved beyond a correlation being found. In fact, the phosphorylation of p67^{phox} has been little investigated, and doubt has been raised as to whether it is a real phenomenon [11]. In a recent publication it was reported that p67^{phox} phosphorylation may occur via protein kinase C (PKC)-

dependent and PKC-independent pathways [12]. In the present work, we further characterize the phosphorylation of p67^{phox}, determine the major phosphorylation site and identify models *in vitro* which mimic phosphorylation *in vivo*.

MATERIALS AND METHODS

Materials

All reagents were purchased from Sigma, except for BSA fraction V (BDH) and the trypsin preparations, tosylphenylalanylchloromethane (TPCK)-treated and modified (resistant to autolytic digestion) sequencing grade, which were from Promega. The radiochemicals used were [³²P]P_i (10 mCi/ml) and [³²P]ATP (3000 Ci/mmol) (Amersham International). Mitogen-activated protein kinase (MAPK) [recombinant extracellular signal-regulated kinase (ERK) 2] was from New England Biolabs, p21-activated kinase (PAK) [glutathione S-transferase (GST)-fusion of rat brain βPAK (65 kDa)] was generously given by Ms E. Prigmore (Institute of Neurology, London, U.K.) [13] and PKC was a rat brain isolate of mixed isoforms, prepared by a standard method [14].

Preparation and fractionation of neutrophils

Neutrophils were prepared from freshly-drawn, heparinized blood from healthy volunteers [15] and resuspended at 5 × 10⁷/ml in phosphate-free Hepes-buffered saline (HBS) (20 mM Hepes/137 mM NaCl/2.7 mM KCl/1 mM MgSO₄/1 mM CaCl₂, pH 7.4) [16]. Cells were preincubated with cytochalasin B (5 μg/ml for 5 min), PMA (1 μg/ml for 5 min) or serum-opsonized zymosan (1 mg/ml for 7 min) and then stimulated in HBS/5.5 mM glucose at 37 °C with *N*-formylmethionyl-leucylphenylalanine (fMLP) (1 μM for 1 min). Stimulation of cells was

Abbreviations used: CGD, chronic granulomatous disease; CNBr, cyanogen bromide; EBV, Epstein-Barr virus; fMLP, *N*-formylmethionyl-leucylphenylalanine; GST, glutathione S-transferase; MALDI, matrix-assisted laser-desorption ionization; MAPK, mitogen-activated protein kinase; PAK, p21-activated kinase; PKC, protein kinase C; TOF, time-of-flight.

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quenched by the addition of a greater than five volume excess of ice-cold HBS. The cells were sedimented by centrifugation at 200 *g* for 3 min at 4 °C, then lysed in relaxation buffer (10 mM Pipes/3 mM NaCl/100 mM KCl/3.5 mM MgCl₂/1.25 EGTA, pH 7.3) [10] by sonication using three short bursts (2–4 s) at 10 μ m amplitude (MSE Soniprep). Protease and phosphatase inhibitors were present in relaxation buffer and in subsequent procedures [1 mM diisopropyl fluorophosphate, 0.2 mM PMSF, 10 μ g/ml tosyl-lysylchloromethane (TLCK), 10 μ g/ml leupeptin, 10 μ g/ml pepstatin, 1 mM benzamidine, 20 mM NaF, 5 mM sodium pyrophosphate, 200 μ M NaVO₄, 100 nM microcystin]. Cytosol was prepared by centrifuging the lysate at 500 *g* for 5 min at 4 °C, followed by 100 000 *g* for 12 min at 4 °C.

Metabolic labelling of cells

Neutrophils were resuspended at 5 $\times$ 10⁷/ml in HBS/5.5 mM glucose/0.025 % (w/v) BSA and incubated with [³²P]P_i (0.1–0.5 mCi/ml) for 60 min at 30 °C. Epstein-Barr virus (EBV)-transformed B cells were grown in culture using RPMI medium, resuspended at 1 $\times$ 10⁷/ml in phosphate-free RPMI containing 10 % (v/v) dialysed fetal-bovine serum and incubated with [³²P]P_i (0.1–0.5 mCi/ml) for 4 h at 37 °C.

Immunoprecipitation of p67^{phox}

The method and buffers were as described previously [10]. Immunoprecipitations were performed with cytosol from 5 $\times$ 10⁷ cells/500 μ l. Non-specific binding was reduced by preadsorbing the cytosol in 50 % (v/v) Protein A–Sepharose (40 μ l), with rotation, for 1 h at 4 °C. The Sepharose was sedimented by centrifugation at 1000 *g* for 2 min at 4 °C and the supernatant was incubated for 1 h, on ice, with 30 μ l of affinity-purified anti-p67^{phox} antibody, raised in rabbits, against full-length GST–p67^{phox} fusion protein. Protein A–Sepharose (40 μ l) was added and the mixture was incubated, with rotation, for 1 h at 4 °C. The supernatant was removed, the Protein A–Sepharose was washed with ice-cold 1 $\times$ solubilization buffer [20 mM Tris/HCl, pH 7.3/150 mM NaCl/1 % (v/v) Triton X-100/1 % (w/v) sodium deoxycholate/0.1 % (w/v) SDS] [10] and boiled in 20 μ l of 2 $\times$ Laemmli sample buffer for 5 min. The Protein A–Sepharose was sedimented by centrifugation at 10 000 *g* for 2 min at room temperature, and the supernatant (35 μ l) was subjected to SDS/PAGE (8 % gel) and the gel was autoradiographed (Hyperfilm; Amersham). The bands were quantified using a BAS1000 PhosphorImager (Fuji) or an AlphaImager 2000 (Alpha Innotech).

Cyanogen bromide (CNBr) digestion of p67^{phox}

A gel slice containing immunoprecipitated p67^{phox} was ground using a small Eppendorf tube fitted with a pestle (Kontes Scientific, Vineland, NJ, U.S.A.) and incubated with 66 μ l of CNBr [300 mg/ml, stored as aliquots in 70 % (v/v) formic acid at –70 °C] and 133 μ l of freshly-prepared 70 % (v/v) formic acid, on ice, overnight. The preparation was dried (Speedvac), resuspended in 50 μ l of water and dried twice more, and finally boiled in Laemmli sample buffer containing Coomassie Blue G-250. The samples (30 μ l) were subjected to Tricine gel electrophoresis [17], using a 16.7 % resolving gel. Bands on Western blots were detected using enhanced chemiluminescence (ECL[®]; Amersham). The peptides used to raise the p67^{phox} N-terminal and C-terminal antibodies were amino acids 1–192 and 507–526 respectively. As described previously, GST-fusion proteins of these peptides [19] were used to immunize rabbits, and the resultant antisera was affinity purified [5].

In vitro phosphorylation assay

Recombinant p67^{phox} protein was phosphorylated *in vitro* with neutrophil cytosol, MAPK, PKC or PAK preparations as the sources of kinase activity. Reaction mixtures (25 μ l), on ice, consisted of 2.5 μ l of 10 $\times$ or 5 μ l of 5 $\times$ kinase buffer (see below), 5 μ l of 0.5 mM ATP (approx. 1000 c.p.m./pmol), 0.5–5 μ g of recombinant protein substrate (p67^{phox} or myelin basic protein) and cytosol [from (1–5) $\times$ 10⁴ cells], MAPK (5 units), PAK (0.7 μ g) or PKC (5 ng). After incubation for 20 min at 30 °C, the reactions were stopped by the addition of 3 μ l of 10 $\times$ Laemmli sample buffer, boiled and subjected to SDS/PAGE. A buffer of slightly different composition was used for each kinase, 10 $\times$ MAPK buffer: 500 mM Tris/HCl, pH 7.5/100 mM MgCl₂/10 mM EGTA/20 mM dithiothreitol/0.1 % (v/v) Brij-35; 5 $\times$ PAK buffer: 250 mM Hepes, pH 7.0/25 mM MgCl₂/25 mM MnCl₂/5 mM dithiothreitol/0.25 % (v/v) Triton-X 100; 5 $\times$ PKC buffer: 100 mM Hepes, pH 7.4/50 mM MgCl₂/2.5 mM CaCl₂. Phosphorylations in cytosol were carried out using either the MAPK or PKC buffers.

Two-dimensional phosphopeptide mapping and phosphoamino acid analysis

Samples were prepared by trypsin digestion, as described previously [18]. Briefly, phosphorylated p67^{phox} was eluted from an SDS-polyacrylamide gel slice, precipitated, oxidized by performic acid and incubated with TPCK-treated trypsin. Two-dimensional maps on cellulose-coated plates were obtained by electrophoresis in pH 1.9 buffer [2.5 % (v/v) formic acid/7.8 % (v/v) acetic acid] for 35 min at 1000 V (Hunter Thin Layer Electrophoresis 7000 system; CBS Scientific), followed by TLC in acetic acid/pyridine/butanol/water (3:10:15:12, by vol.) for 6–8 h. For phosphoamino acid analysis, electrophoresis was in pH 1.9 buffer for 20 min at 1500 V, followed by pH 3.5 buffer [0.5 % (v/v) pyridine/5 % (v/v) acetic acid] for 16 min at 1300 V. Phosphoamino acid markers were detected by ninhydrin spray [0.25 % (w/v) in acetone] and their positions outlined on the phosphorimage (PhosphorImager).

HPLC–MS

Tryptic digestion was performed on gel slices containing phosphorylated p67^{phox}. In the in-gel digestion method, trypsin modified by reductive alkylation to limit contamination by trypsin peptides, and the chromophore-free detergent (Thesit; Sigma), to avoid interference with the HPLC spectrum, were used. The resultant peptide mix was dried (Speedvac), dissolved in water (50 μ l) and applied to a C₁₈ column (7 μ m, 220 mm $\times$ 2.1-mm i.d.; Aquapore OD-300, Brownlee) with AX-300 and C₁₈ precolumns (Brownlee) for HPLC (Hewlett Packard 1090) with solutions A [0.06 % (v/v) trifluoroacetic acid] and B [0.052 % (v/v) trifluoroacetic acid/80 % (v/v) acetonitrile]. A gradient of 0–60 % of solution B at 0.15 ml/min for 60 min was used to elute the peptides and fractions were collected at 1 min intervals. A portion of each fraction (15 μ l) was monitored for radioactivity by scintillation spectroscopy. Selected fractions were dried (Speedvac) for matrix-assisted laser-desorption ionization (MALDI) MS with a reflectron time-of-flight (TOF) apparatus equipped with a nitrogen laser (337 nm, 3 ns pulse) and delayed extraction (PerSeptive Biosystems Voyager Elite XL).

Mutagenesis of p67^{phox} and recombinant protein purification

Wild-type p67^{phox} protein was prepared from full-length cDNA cloned into plasmid pGEX.2T and overexpressed in *Escherichia*

coli as previously described [19]. A PCR overlap extension method was used to introduce a point mutation into wild-type p67^{phox}, so that Thr²³³ was changed to alanine. The final construct was checked by DNA sequencing. The N-terminal GST-fusion protein of the Ala²³³ mutant was purified as for the wild-type. Briefly, the GST was cleaved from p67^{phox} by thrombin followed by anion-exchange chromatography (Mono Q Sepharose; Pharmacia).

p67^{phox}-p40^{phox}-binding assay

p40^{phox} cDNA was cloned into the pGEX.2tK vector (Pharmacia) to introduce an N-terminal protein kinase A site, so allowing the expressed protein to be radiolabelled. Purified GST-p40^{phox} fusion protein (100 µg) was labelled with [γ -³²P]ATP (25 µCi) by three incubations of 15 min at 30 °C, each time with a fresh addition of protein kinase A (5 units; catalytic subunit, Sigma). The fusion protein was purified from the labelling mixture by elution from glutathione-Sepharose beads and was used, in solution, to probe dot-blots. p67^{phox} or control proteins (30 pmol) were applied to a nitrocellulose membrane, the membrane was then blocked with 3% (w/v) dried-milk powder and incubated with radiolabelled p40^{phox} (2 nmol/5 ml) for 1 h at room temperature. The membrane was then washed and exposed to film (Hyperfilm; Amersham) for 1 h.

RESULTS

Specificity tests for p67^{phox} antibody

The antibody used for immunoprecipitation of p67^{phox} was raised in rabbits against recombinant p67^{phox} and affinity-purified against recombinant p67^{phox}. To ensure specificity for p67^{phox}, the antibody was tested against a Western blot of trichloroacetic

Figure 1 Western blots and autoradiograms of p67^{phox} from whole cells and immunoprecipitates from cytosol

(A) Trichloroacetic acid precipitates of 1×10^6 neutrophils from a normal subject and from a CGD patient lacking p67^{phox} were subjected to SDS/PAGE, blotted on to nitrocellulose and probed with affinity-purified antibodies to p67^{phox}, p47^{phox} and p40^{phox}. The bands were detected using the enhanced chemiluminescence system. Molecular-mass markers (kDa) are shown on the left. (B) EBV-B cells were labelled with [32 P]P_i and the cytosol immunoprecipitated with the p67^{phox} antibody raised in rabbits. The Western blot was carried out with a goat antibody to p67^{phox} and developed by enhanced chemiluminescence. Molecular-mass markers (kDa) are shown on the left. (C) Autoradiogram (autorad) of radiolabelled immunoprecipitates exposed to X-ray film for 16 h at -70 °C.

Figure 2 Increased phosphorylation of p67^{phox} by stimulation of neutrophils

(A) [32 P]P_i-labelled neutrophils were untreated (a), treated with serum-opsonized zymosan (b), fMLP (c) or PMA (d), and then homogenized and cytosol (from 2×10^5 cells) was prepared as described in the Materials and methods section. A Coomassie-Blue stained gel [molecular-mass markers (kDa) are shown on the left] and an autoradiogram (autorads) is shown. (B) p67^{phox} immunoprecipitated from cytosol (5×10^7 cells). The gels were exposed to film for 5 days at -70 °C. The autoradiogram is shown on the right.

acid precipitates of whole neutrophils from a CGD patient deficient in p67^{phox} and normal control neutrophils (Figure 1A). Both neutrophil samples gave positive signals with antibodies to p47^{phox} and p40^{phox} but no signal was obtained in the p67^{phox}-deficient CGD neutrophils with the p67^{phox} antibody. In order to check the antibody specificity under immunoprecipitation conditions, normal and p67^{phox}-deficient CGD EBV-transformed B cells were labelled with [32 P]P_i and subjected to immunoprecipitation with rabbit antibodies. The immunoprecipitates were blotted and probed with an affinity-purified antibody against recombinant p67^{phox} raised in goats. p67^{phox} was detected in normal cells only (Figure 1B). In a corresponding autoradiogram, a 67 kDa band was present in normal cells, whereas no 67 kDa phosphoprotein was immunoprecipitated from the p67^{phox}-deficient cells (Figure 1C). The immunoprecipitated 67 kDa phosphoprotein was also recognized by two other antibodies raised against p67^{phox} peptides (see Figure 5).

In vivo phosphorylation of p67^{phox} in neutrophils and B cells

The results of immunoprecipitations of p67^{phox} from the cytosol of metabolically-labelled neutrophils, which had been stimulated before lysis, are shown in Figure 2. Repeated experiments showed a consistent increase in the incorporation of radiolabel into p67^{phox} upon stimulation, although unstimulated cells also showed a low level of incorporation. Stimulation of the cells gave increases above basal phosphorylation of 1.4 ± 0.2 ($n = 3$), 2.2 ± 0.5 ($n = 5$) and 3.2 ± 1.1 ($n = 4$) for fMLP, serum-opsonized zymosan and PMA respectively.

In order to determine whether the increase upon stimulation was caused by the phosphorylation of multiple sites on p67^{phox}, tryptic phosphopeptide maps of resting and stimulated cells were compared (Figure 3). The p67^{phox} phosphopeptide pattern was the same for resting and stimulated cells, suggesting that no new sites were phosphorylated. Neutrophil and B lymphocyte p67^{phox}

Figure 3 Phosphoamino acid and phosphopeptide maps of p67^{phox} before and after stimulation

p67^{phox} was immunoprecipitated from [³²P]P_i-labelled cells. Phosphorimages of two-dimensional phosphopeptide maps are shown. (A) Neutrophils before stimulation, (B) neutrophils after stimulation with serum-opsonized zymosan, (C) unstimulated EBV-transformed B cells, (D) PMA-stimulated EBV-transformed B cells. The insets a, b, c, d show the results of phosphoamino acid analyses. The positions of phosphoserine (S), phosphothreonine (T) and phosphotyrosine (Y) are indicated on inset a.

phosphopeptide maps were similar, both having one major tryptic peptide signal. Phosphopeptide mapping of neutrophil p67^{phox} was performed several times and showed the same pattern with the three stimuli, PMA, serum-opsonized zymosan or fMLP. PMA alone was used to stimulate EBV-B cells because these cells do not respond to fMLP or phagocytosed particles. Analysis of the phosphoamino acid content showed that p67^{phox} was phosphorylated on threonine and serine residues in neutrophils and B cells (Figure 3, insets a,b,c,d). The predominant signal for neutrophil p67^{phox} was phosphothreonine.

***In vitro* models for phosphorylation of p67^{phox}**

Recombinant p67^{phox} protein was phosphorylated *in vitro* with neutrophil cytosol, MAPK, PKC or PAK preparations as the sources of kinase activity. Cytosol of unstimulated and stimulated neutrophils phosphorylated recombinant p67^{phox} equally. The resultant phosphorylated p67^{phox} was subjected to two-dimensional phosphopeptide mapping to determine if any of the kinase reactions *in vitro* was able to mimic the native phosphorylation of p67^{phox}. To allow comparison of similar maps, it was important that the two-dimensional mapping was carried out simultaneously because of slight variations in resolution between experiments. For example, the major phosphopeptide signal (see Figure 3) sometimes appeared as two closely resolved dots (Figures 4A and 4B), which was probably due to partial tryptic digestion. Figure 4 shows that the *in vitro* phosphopeptide maps of cytosol- or MAPK-phosphorylated p67^{phox} were similar to the *in vivo* map, but the PKC- or PAK-phosphorylated p67^{phox} were markedly different. Both PKC and

Figure 4 *In vitro* phosphorylation of p67^{phox}

Two-dimensional phosphopeptide maps of (A) *in vivo* phosphorylated p67^{phox} immunoprecipitated from serum-opsonized zymosan-stimulated neutrophils, (B) *in vitro* phosphorylated recombinant p67^{phox} by neutrophil cytosol, (C) *in vitro* phosphorylated recombinant p67^{phox} by recombinant MAPK, (D) *in vitro* phosphorylated recombinant p67^{phox} by recombinant β PAK, (E) *in vitro* phosphorylated recombinant p67^{phox} by rat brain PKC (mixed isoforms). The insets a–e show phosphoamino acid analyses of the corresponding samples.

PAK phosphorylated p67^{phox} on serine residues only, whereas phosphoamino acid analysis of the cytosol- and MAPK-treated samples gave similar results to the *in vivo* phosphorylated p67^{phox}.

Identification of p67^{phox} phosphorylation site and mutagenesis of Thr²³³

CNBr digestion of phosphorylated p67^{phox} was used to locate the region of phosphorylation (Figure 5). Tricine gel electrophoresis followed by Western blotting enabled identification of the N-terminal and C-terminal peptides using antibodies raised against p67^{phox} peptides. Only partial digestion was achieved, as the few large peptides visible upon staining were not representative of all 13 cleavage sites. A band of approx. 13 kDa, which contained the phosphorylation site (detected by phosphorimaging), was also detected by the antibody raised against the N-terminal 192 amino acids, whereas the antibody raised against the C-terminal

Figure 5 Phosphorylation occurs on the N-terminal half of p67^{phox}

CNBr digestions of immunoprecipitated p67^{phox} were subjected to Tricine gel electrophoresis (gel), and Western blots were probed with peptide antibodies against the N-terminal 192 amino acids (αN) and C-terminal 20 amino acids (αC). p67^{phox}, immunoprecipitated from [³²P]_i-labelled PMA-stimulated neutrophils, was digested with CNBr, subjected to Tricine gel electrophoresis and the gel was analysed using a PhosphorImager (PI).

20 amino acids detected a different band. This information, taken together with the phosphopeptide and amino acid analyses, suggests that the major site of phosphorylation is on a threonine residue within the N-terminal portion of the protein.

Using the cytosol and MAPK *in vitro* models of phosphorylation, tryptic digestion products of radiolabelled p67^{phox} were resolved by reverse-phase HPLC and the fraction containing radiolabelled peptides was detected by scintillation spectroscopy. MALDI-TOF MS was used to determine the masses of the peptides in the fraction. Figure 6 shows the results of the MAPK *in vitro* system, and identical results were obtained when recombinant p67^{phox} was phosphorylated using cytosol. A major peak, equivalent to 762 Da, was found in fraction 20 and this was confirmed, by post-source decay spectra, to be the p67^{phox} peptide of amino acids 233–238. Although this peptide was not phosphorylated, the result suggested that Thr²³³ may be the phosphorylation site and was consistent with the two-dimensional mapping and CNBr data. An alternative matrix 2,6-dihydroxyacetophenone/di-ammonium hydrogen citrate (DHAP/DAHC), which is often used for phosphopeptide analyses, failed to detect any phosphorylated peptides. It seems likely that the low stoichiometry of p67^{phox} phosphorylation (estimated at 0.5% for these *in vitro* systems) is the reason for the lack of a phosphorylated peptide signal, despite detection by scintillation counting. It is possible that, with the relatively steep HPLC gradient used, the phosphorylated and non-phosphorylated forms of the same peptide co-elute. Also, the phosphorylated residues of the peptide may have become dephosphorylated during handling of the fraction after scintillation counting.

To test the theory that Thr²³³ was phosphorylated, mutagenesis of p67^{phox} was performed by a single amino acid substitution to Ala²³³. The Ala²³³-substituted p67^{phox} was subjected to *in vitro* phosphorylation, HPLC of tryptic digestion products and MS analysis as described above. No peak of radioactivity was eluted from the column (Figure 6), despite sequence data for the equivalent 233–238 amino acid peptide in fraction 20, and this confirmed that the phosphorylation detected in wild-type p67^{phox} was on Thr²³³.

Figure 6 Peptide-sequence analysis of HPLC fractions from *in vitro* phosphorylated p67^{phox}

(A) Tryptic peptides of MAPK-phosphorylated p67^{phox} were subjected HPLC and the radioactivity in one-tenth of each fraction collected was monitored by scintillation spectroscopy. It was found that the radiolabel for wild-type (MAPK/wt, ●) p67^{phox} was contained in fraction 20. (○) Radioactivity in fractions of the Thr²³³ → Ala mutant (MAPK/mut), treated similarly. (B) HPLC fraction 20 from p67^{phox} wild-type (MAPK/wt) and from Thr²³³ → Ala mutant (MAPK/mut) peptides was analysed by MALDI-TOF MS using α-cyano-4-hydroxycinnamic acid for molecular-mass measurements. The peptide sequences were confirmed by post-source decay mass spectra.

Only traces of phosphorylation of recombinant p67^{phox} by cytosol or MAPK were observed after the mutation of Thr²³³ → Ala (Figure 7A). Phosphopeptide mapping, using the p67^{phox} Ala²³³ mutant as substrate in both of these *in vitro* models of phosphorylation, showed a lack of the major phosphopeptide band, and non-specific phosphorylation only was seen (Figure 7B, using cytosol as the source of kinase activity). To ensure that the loss of phosphorylation was due to the absence of Thr²³³ and not because of an artefact of the mutated protein, wild-type and mutant p67^{phox} were compared in a p40^{phox}-binding experiment (Figure 8). p40^{phox} bound equally to wild-type and Ala²³³-substituted p67^{phox}. A positive control (C-terminal p67^{phox}) and negative controls (N-terminal p67^{phox} and GST) were included to verify the specificity of the p67^{phox}–p40^{phox} interaction, which has been characterized previously [20].

Figure 7 Phosphorylation of p67^{phox} on Thr²³³ *in vitro*

(A) Coomassie-Blue stained gel (gel) and autoradiogram (autorad) showing p67^{phox} from *in vitro* phosphorylation reactions using wild-type p67^{phox} (wt) or p67^{phox} with Thr²³³ → Ala substitution (mut) with no added kinase activity (buffer), neutrophil cytosol or MAPK. Mr, 67 kDa albumin molecular-mass marker; C, cytosol alone; M, MAPK alone. (B) Two-dimensional phosphopeptide maps of wild-type p67^{phox} background phosphorylation without added kinase (wt bg), wild-type p67^{phox} phosphorylated by cytosol (cyt/wt) and Ala²³³-substituted p67^{phox} phosphorylated by cytosol (cyt/mut).

Figure 8 Ala²³³-substituted p67^{phox} exhibits normal binding to p40^{phox}

Autoradiogram of overlay experiment using radiolabelled p40^{phox} on a dotblot with equimolar applications of wild-type p67^{phox} (wt), Ala²³³-substituted p67^{phox} (mut), GST-fusion protein of the C-terminal (amino acids 300–526) of wild-type p67^{phox} (wtC), GST-fusion protein of the N-terminal (amino acids 1–199) wild-type p67^{phox} (wtN) and GST alone.

DISCUSSION

The results of the present work confirm previous reports that the NADPH oxidase component p67^{phox} is phosphorylated [1,10]. This had been contested by other workers because phosphorylated p67^{phox} was not found on immunoprecipitation and they suggested that the 67 kDa phosphoprotein was an artefact [11]. In the present work, the specificity of the antibody used for the immunoprecipitations has been rigorously tested and we have shown that there is no interference from any other 67 kDa phosphoprotein. It should be noted, however, that the magnitude and stoichiometry of p67^{phox} phosphorylation are in strong contrast to the increase in p47^{phox} phosphorylation which is much more obvious (L. V. Forbes, unpublished work), and represents multiple sites and isoforms [21,22].

The phosphopeptide maps indicate that, for p67^{phox}, the

Figure 9 Amino acid Thr²³³ is in a proline-rich region of p67^{phox}

A schematic diagram of p67^{phox} showing the positions of the two SH3 domains (amino acids 245–295 and 462–512) and the polyproline region (amino acids 227–234; shaded grey).

increase in phosphorylation upon stimulation occurs without additional sites becoming phosphorylated. The basal phosphorylation of p67^{phox} may be a result of low level phosphorylation in resting cells, but might be due to partial stimulation of kinase activity by the cell handling procedures. An increase in the level of phosphorylation, with the same phosphopeptide map, occurred with NADPH oxidase activation regardless of agent used; phorbol ester, chemotactic peptide or phagocytic stimuli. This suggests a common downstream signalling pathway.

The investigation of *in vitro* phosphorylation models for p67^{phox} was pursued for two reasons: first, to provide a practical method for identifying the phosphorylation site and secondly, to obtain an insight into the cellular mechanisms involved. A combination of two-dimensional data, CNBr digestion, HPLC-MS and mutagenesis allowed the identification of Thr²³³ as the major phosphorylation site. The same site was phosphorylated on recombinant protein by unstimulated cytosol or MAPK *in vitro*. This suggests that the major phosphorylation event involving p67^{phox} might occur in the cytosol before translocation to the membrane, and that MAPK is the candidate kinase.

The results of the phosphorylation in intact cells represents only p67^{phox} in the cytosol. Immunoprecipitation of p67^{phox} from membrane fractions has been carried out, which confirmed the presence of phosphorylated p67^{phox} and the translocation of a fraction of cytosolic p67^{phox}, as previously established [10]. However, it was not possible to isolate sufficient p67^{phox} from immunoprecipitates of membranes for two-dimensional mapping (L. V. Forbes, unpublished work). Also, the low stoichiometry of *in vitro* phosphorylation by cytosol or MAPK (only 0.5%) rendered these systems unsuitable for cell-free determination of an effect of phosphorylation on translocation. Therefore it has not been possible, so far, to examine the relationship between p67^{phox} phosphorylation at Thr²³³ and translocation.

The results of the present work differ from those of a recent publication [12], which suggested that p67^{phox} is phosphorylated only on serine residues. Our finding that the major signal was phosphothreonine is supported by the loss of the corresponding *in vitro*-phosphorylated tryptic peptide band by mutagenesis of Thr²³³. Although some phosphoserine was detected, the majority of our experiments showed the stronger signal was for phosphothreonine. It may be possible that the weaker phosphoserine signal can be enhanced by subtle differences in cell handling or two-dimensional mapping procedures. Our results suggest that many serines residues may be phosphorylated with low stoichiometry, since, on two-dimensional mapping, there did not appear to be a distinct signal corresponding to a specific serine phosphopeptide.

The Thr²³³ phosphorylation site is in a consensus sequence for the proline-directed MAPKs (Pro-X-Ser/Thr-Pro, where X is not a proline residue) [23] as it is situated in a proline-rich region of p67^{phox} (Figure 9). Such polyproline regions are putative

binding sites for SH3 (Src-3 homology) domains [24] but, despite extensive studies on region-specific interactions between the components of NADPH oxidase, the polyproline region of p67^{phox} has yet to be assigned a binding partner [20,25]. However, this region of p67^{phox} is flanked by the N-terminal p21^{rac}-binding region [7] and the inter-SH3 region that binds p40^{phox} [20]. Recently it was proposed that p67^{phox} could be a substrate for PAK [26], but the mismatched two-dimensional map of the *in vitro* PAK phosphorylation of p67^{phox} has shown this to be unlikely. This is in agreement with the conclusions drawn from a phosphorylation study using immunoprecipitated neutrophil PAK [27].

The molecular structure of p67^{phox} has attracted increased interest recently. New studies of different domains within the protein have confirmed the importance of N-terminal p67^{phox} for protein-protein interactions [28], implicated a novel C-terminal p21^{rac}-interaction site [29] and identified a central region necessary for cell-free oxidase activity [30]. The identification of phosphorylation at the centrally located Thr²³³ site, adds to the growing analysis of p67^{phox} structure. The role of this modification is to be assessed by development of transfected cell models of NADPH oxidase.

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