Purification of the 47 kDa phosphoprotein associated with the NADPH oxidase of human neutrophils

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A 47 kDa protein in the cytosol of phagocytic cells becomes heavily phosphorylated and translocates to the cell membrane upon stimulation. This protein was isolated from the cytosol of human neutrophils by chromatography on ion-exchange and hydroxyapatite resins. Polyclonal antibodies to this protein demonstrated that it was present in the neutrophils of two patients with X-linked chronic granulomatous disease (CGD) but not in those of three patients with the autosomal recessive pattern of inheritance. A sequence of amino acids was determined from a tryptic peptide of this protein: Glu-Met-Phe-Pro-Ile-Glu-Ala-Gly-Ala-Ile-Asn-Xaa-Glu. This served to establish that the phosphoprotein isolated here is the same as a protein of a similar molecular mass identified by other workers. These studies confirm the involvement of this 47 kDa phosphoprotein in the molecular pathology of autosomal recessive CGD and describe a method for the purification of the native protein.

INTRODUCTION

A microbicidal NADPH-dependent electron transport chain is present in neutrophils and other phagocytic cells. Phagocytosis is accompanied by a large increase in O_2 consumption (the respiratory burst) which is important for microbial killing (Selvaraj & Sbarra, 1966). Identification of some of the components of the NADPH oxidase has been facilitated by the study of neutrophils from patients with chronic granulomatous disease (CGD), in which the respiratory burst fails to be activated upon stimulation, and recurrent infections occur (Holmes *et al.*, 1967). Cytochrome b_{-245} was the first component of the oxidase to be identified (Segal *et al.*, 1981; Roberts *et al.*, 1982). It is thought to be the terminal component of the chain, donating electrons to O_2 . Its involvement in the oxidase was confirmed by its absence from the cells of patients with X-linked CGD, who account for approximately two-thirds of all CGD patients (Segal *et al.*, 1983).

The majority of the remaining cases of CGD have an autosomal recessive form. Activation of the respiratory burst in normal cells is associated with the enhanced phosphorylation of several proteins, but autosomal recessive CGD is characterized by the absence of a prominent band of phosphorylation at 47 kDa when stimulated ^{32}P -labelled cells are electrophoresed on SDS/polyacrylamide gels (Segal *et al.*, 1985; Heyworth & Segal, 1986; Kramer *et al.*, 1988). Subsequently, by the method of Western blotting, it was discovered that about 90% of these patients demonstrate the absence of a protein of a similar molecular mass from their neutrophils (Nunoi *et al.*, 1988; Clark *et al.*, 1989).

We report the isolation of this 47 kDa phosphoprotein from human neutrophils and demonstrate that this is the same as the 47 kDa protein recently cloned by Lomax *et al.* (1989) and Volpp *et al.* (1989).

EXPERIMENTAL

Preparation, labelling and fractionation of cells

Neutrophils were prepared from the blood of healthy donors, from leukopheresis samples from patients with chronic granulo-

cytic leukaemia (CGL) or from buffy coat residues obtained from the South London Blood Transfusion Centre, Tooting, London, U.K. (Segal & Jones, 1980). Cells $[(5 \times 10^7) - (5 \times 10^8)/\text{ml}]$ were labelled with ^{32}P , stimulated with phorbol 12-myristate 13-acetate (PMA) (Sigma, Poole, Dorset, U.K.), disrupted and fractionated as described previously (Heyworth *et al.*, 1989). Cytosol and membranes were stored at -70°C .

Electrophoresis, autoradiography and Western blotting

SDS/PAGE [using 10% (w/v) acrylamide] and subsequent autoradiography were performed as described by Heyworth & Segal (1986). Mini-gels (8 cm $\times$ 7 cm; Bio-Rad, Watford, Herts., U.K.) were used if gels were to be Western-blotted. Proteins were transferred from the gels on to 0.45 μm nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany) using an LKB 2117–250 NOVABLOT blotting apparatus (Pharmacia LKB Biotechnology, Milton Keynes, Bucks., U.K.). Electrophoretic transfer was performed at 0.8 mA/cm² for 60–90 min in 25 mM-Tris/192 mM-glycine/0.1% SDS/4% methanol, pH 8.3. Proteins were revealed by staining with 0.02% Ponceau S (Sigma) for 1–2 min. The blots were rinsed in water and incubated for 60 min in blocking buffer [3% (w/v) BSA, 10% (v/v) normal goat serum, 1% (w/v) gelatin, 0.1% (w/v) NaN_3 and 0.05% (v/v) Tween 20 in 50 mM-Tris/20 mM-NaCl (TBS), pH 7.4] and then in specific antibody at a dilution of 1:100 in blocking buffer overnight. The blots were rinsed for 60 min in five changes of TBS containing 0.05% (v/v) Tween 20 and then incubated for 60 min in alkaline phosphatase-labelled goat anti-rabbit IgG at a dilution of 1:7500 in blocking buffer as recommended by the suppliers (Stratagene, La Jolla, CA, U.S.A.). The blots were washed in three changes of TBS/Tween for 30 min and then in two changes of TBS alone for a further 30 min. Bound antibody was revealed by adding a mixture of Nitro Blue Tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate according to the supplier's instructions (Stratagene).

Purification of the 47 kDa phosphoprotein

All procedures were performed at 0–4 $^\circ\text{C}$. ^{32}P -labelled cytosol

Abbreviations used: CGD, chronic granulomatous disease; CGL, chronic granulocytic leukaemia; PMA, phorbol 12-myristate 13-acetate; Tos-Lys-CHCl₂, tosyl-lysylchloromethane ('TLCK'); TBS, 50 mM-Tris/20 mM-NaCl; PBS, phosphate-buffered saline (140 mM-NaCl/9.2 mM-Na₂HPO₄/1.3 mM-NaH₂PO₄, pH 7.4).

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prepared from 5×10^9 stimulated neutrophils (approx. 95 mg of protein) was diluted 1:4 in 20 mM-Tris/HCl/1 mM-EDTA/27 μ M-tosyl-lslylchloromethane (Tos-Lys-CH₂Cl, Sigma)/100 units of aprotinin (Trasylol; Bayer)/ml, pH 7.4, and passed through a column (1 cm $\times$ 10 cm) of Fast-Flow DEAE-Sepharose (Pharmacia) and a column (1 cm $\times$ 5 cm) of Fast-Flow CM-Sepharose connected in series and equilibrated in Tris buffer, pH 7.4, at a flow rate of 60 ml/h. Protein bound to the DEAE-Sepharose was eluted with a 2 M-NaCl wash. This should not contain any of the 47 kDa phosphoprotein. The CM gel was then equilibrated in 10 mM-Mes, 1 mM-EGTA, 27 μ M-Tos-Lys-CH₂Cl and 100 units of aprotinin/ml, pH 6.8. Protein was eluted from this column in two steps with six column vol. of 0.3 M-NaCl and six column vol. of 2 M-NaCl in Mes buffer. This particular buffer was used because it was found to result in a cleaner 47 kDa fraction eluting from the CM-Sepharose than if 20 mM-Tris/HCl, pH 7.4, was used. The 0.3 M-NaCl fraction was desalted on a PD-10 desalting column (Pharmacia) equilibrated in 10 mM-sodium phosphate, pH 6.8, including Tos-Lys-CH₂Cl and aprotinin at the concentrations given above, and then applied on to a column of hydroxyapatite (DNA grade Bio-Gel HTP; Bio-Rad) equilibrated in the same buffer (see Fig. 2 legend for running conditions). Protein was eluted with a gradient of sodium phosphate from 10 mM to 1 M. The 47 kDa protein eluted between 0.2 M- and 0.4 M-sodium phosphate. The entire purification was completed in 1 day where possible to minimize dephosphorylation of the 47 kDa protein.

For larger preparations of protein the procedure was scaled up 10-fold using cytosol from CGL cells. Under these circumstances, the hydroxyapatite fraction was contaminated by other proteins. Further purification was achieved on a Mono S column (an f.p.l.c. cation exchanger from Pharmacia) in 50 mM-Mes, pH 5.5, including EGTA, Tos-Lys-CH₂Cl and aprotinin, at a flow rate of 60 ml/h. Protein was eluted with a linear NaCl gradient (0–1 M-NaCl).

Amino acid sequencing

Amino acid sequence analysis was performed using an Applied Biosystems model 477A amino acid sequencer equipped with a 120A on-line phenylthiohydantoin amino acid analyser. Approx. 20 μ g of 47 kDa protein was electroblotted and digested *in situ* with trypsin (Boehringer Mannheim, Lewes, E. Sussex, U.K.) using the method of Abersold *et al.* (1987). Resultant peptides were separated using reverse-phase h.p.l.c. on a Brownlee OD-300 column (1 mm $\times$ 100 mm).

Production of antibodies to the 47 kDa protein

The protein (about 200 μ g) was eluted from 10% (w/v) SDS/polyacrylamide gels in 0.4% NH₄HCO₃/0.2% SDS/1 mM-dithiothreitol at 25 V overnight. The eluate was mixed with 4 vol. of ice-cold acetone and spun at 1400 g, 2 °C for 10 min. The supernatant was aspirated and the precipitate washed with ice-cold acetone and centrifuged as before. The precipitate was then dissolved in phosphate-buffered saline (PBS) and mixed with an equal volume of Freund's complete adjuvant (Difco Laboratories, Detroit, MI, U.S.A.) for the first injection (approx. 25 μ g of protein) and with Freund's incomplete adjuvant for the two booster injections (approx. 50 μ g each) at six subcutaneous sites in two New Zealand White rabbits.

Protein estimation

Protein was estimated by the BCA method as described by Pierce, Cambridge, U.K.

Library screening

A λ gt11 cDNA expression library constructed from poly(A)⁺

RNA isolated from the HL60 promyelocytic leukaemia cell line, induced to differentiate with retinoic acid (kindly donated by Dr D. Bentley, Imperial Cancer Research Fund Laboratories, London, U.K.), was screened using the rabbit antiserum described above, at a dilution of 1:100, essentially as described by Huynh *et al.* (1985). Colonies binding specific antibody were revealed with an alkaline-phosphatase conjugated goat anti-rabbit second antibody, also as described previously. Positively reacting plaques were isolated and replated at a density of approx. 500 plaques per 90 mm Petri dish. The primary screening of about 200 000 plaques yielded 14 original positives, of which ten re-screened as positive in the second cycle. These ten clones were purified to homogeneity and three were shown to contain specific sequences of the 47 kDa phosphoprotein by hybridization in buffer containing 50% formamide (Wahl *et al.*, 1979), for 16 h at 42 °C, to a ³²P-labelled, gel-purified, insert from a 47 kDa phosphoprotein cDNA clone whose identity had been established by DNA sequence analysis (kindly donated by Dr. D. Bentley; see the Results and discussion section).

RESULTS AND DISCUSSION

The only method available for the identification of this protein was the characteristic mobility of the radiolabelled phosphoprotein on SDS/PAGE, and thus the purification was constrained by conditions that minimized dephosphorylation. These included the addition of fluoride to the cell-disruption buffer and the strict maintenance of the temperature below 4 °C.

In early experiments the DEAE-Sepharose was equilibrated with buffer containing 20 mM-NaF. However, it was found that this competed with protein for binding sites on the gel. When fluoride was omitted from the buffer, approx. 50% of the total protein was bound (Table 1), but the 47 kDa protein did not bind. The inclusion of this step improved the resolution from the CM-Sepharose column to which the 47 kDa protein bound at pH 6.8. It eluted from the CM between 0.15 and 0.3 M-NaCl. To obtain maximum recovery and minimum dilution, a single 0.3 M-NaCl elution step was employed routinely. At this stage the 47 kDa band was the only clearly visible phosphorylated protein in the mixture (Fig. 1), with major non-phosphorylated bands at molecular masses of approx. 68 kDa [possibly the same as that identified by Volpp *et al.* (1988) and Nunoi *et al.* (1988)] and 37 kDa. These contaminants were removed by chromatography on hydroxyapatite (Fig. 2). All the protein bound to the gel, with the 47 kDa protein eluting earliest.

Polyclonal antibodies to the 47 kDa band (see below) were used to determine whether the purification of the phosphorylated protein was also extracting the protein in its unphosphorylated state. Western blots of the material which bound to the DEAE-

Table 1. Purification of the 47 kDa phosphoprotein

Fraction	Protein	
	(mg/10 ⁹ cells)	(% of total cytosolic protein)
Cytosol	19.03 $\pm$ 1.62	100
Unbound from DEAE- and CM-Sepharose	8.56 $\pm$ 1.38	44.5 $\pm$ 7.2
2 M-NaCl eluate from DEAE-Sepharose	9.35 $\pm$ 1.03	46.5 $\pm$ 4.1
0.3 M-NaCl eluate from CM-Sepharose	0.71 $\pm$ 0.03	3.6 $\pm$ 0.1
2 M-NaCl eluate from CM-Sepharose	0.05 $\pm$ 0.01	0.27 $\pm$ 0.05

Fig. 1. Purification of 47 kDa protein and identification on SDS/PAGE

(a) SDS/PAGE (10% gels) of low molecular mass markers (lane 1), whole cytosol (lane 2), 2 M-NaCl eluate from DEAE-Sephacel (lane 3), unbound protein from DEAE- and CM-Sephacel (lane 4) and 0.3 M-NaCl eluate from CM-Sephacel (lane 5) (b) Corresponding autoradiograph of dried gel. The 47 kDa band is indicated by the arrow.

Sephacel or passed through the CM-Sephacel column did not reveal protein which reacted with the anti-47 kDa serum, indicating that all the immunoreactive material co-purified with the phosphoprotein. In a small proportion of our preparations it was noted that a doublet, detected by both autoradiography and Western blotting, was present at the 47 kDa position. This could result from proteolysis or from different degrees of phosphorylation.

A range of other chromatographic methods were investigated in the course of the work, and a summary of our findings is given in Table 2. The 47 kDa protein, together with many other proteins, bound to both 2'-5'- and 3'-5'-ADP-agarose. NaCl could be used for elution in both cases without loss of resolution. However, these substances were impractical for use on a large scale, and ion exchange was chosen as the first step in the purification because of the capacity of the gels and the high flow rates which could be used. Incorporation of ADP-agarose gels later on in the isolation procedure using gradient elution did not result in significant purification. Heparin-agarose was comparable with cation exchange in its resolving power, the latter being chosen because it gave somewhat more favourable results.

The results obtained with chromatofocusing were unsatisfactory, since it often proved difficult to detect the phosphoprotein in eluted fractions, and the 47 kDa protein frequently trailed off the column. This observation is consistent with the wide spectrum of isoelectric points observed on two-dimensional gels (Kramer *et al.*, 1988; Okamura *et al.*, 1988a,b).

Hydrophobic interaction chromatography initially appeared promising. However, this method gave highly variable results, demonstrating extreme sensitivity to differences in the amount of protein loaded and the running temperature. In some experiments the 47 kDa protein failed to elute from the gels. GTP-agarose did not bind the 47 kDa protein when cytosol, diluted 1:4 in PBS, was applied. However, other workers have successfully used this affinity matrix to extract the protein using a different buffer system and eluting at 25 °C, at which temperature we observed rapid dephosphorylation (Volpp *et al.*, 1988).

Antibody was detected in rabbit serum 3 weeks after the first booster injection. This antiserum was used to probe the protein on Western blots of cytosol from the cells of normal subjects and patients with X-linked and autosomal recessive CGD. The protein was detected in the cytosol from both normal subjects and the patients with X-linked CGD but not from these with autosomal recessive CGD (Fig. 3). This confirmed that the 47 kDa phosphoprotein is defective in these autosomal recessive CGD (Segal *et al.*, 1985). It was also consistent with the observation of Volpp *et al.* (1988) that a protein of a similar

Fig. 2. Purification of 47 kDa protein by chromatography on hydroxyapatite

(a) Elution of protein from hydroxyapatite gel. The 0.3 M-NaCl eluate (18 ml; approx. 3.5 mg of protein) from CM-Sephacel was desalted as described in the Experimental section and a total volume of 25 ml was loaded on to a column of hydroxyapatite (0.5 cm × 1.0 cm) equilibrated in 10 mM-sodium phosphate/27 μM-Tos-Lys-CH₂Cl/100 units of aprotinin/ml, pH 6.8, at a flow rate of 60 ml/h at 0 °C. Protein (—) was eluted with a linear gradient (---) of sodium phosphate, pH 6.8, from 10 mM to 1 M. Fractions of 1 ml were collected. (b) (i) SDS/PAGE (10% slab gel) of column fractions. Fractions (200 μl) were precipitated in 10% trichloroacetic acid and run. Lanes: a, low-molecular-mass markers; b, column sample; c, fractions 1-3; d, fractions 4-7; e, fractions 8+9; f, fractions 10+11; g, fractions 12+13; h, fractions 14-17; i, fractions 18-20; j, fractions 21+22; k, fractions 23+24; l, fractions 25-32; m, fractions 33-38; n, whole cytosol. (ii) Corresponding autoradiograph of dried gel. The 47 kDa band is indicated by the arrows.

Table 2. Chromatographic methods investigated in the isolation of the 47 kDa protein but not incorporated in the final procedure

HaAC, acetic acid; TFA, trifluoroacetic acid.

Chromatographic method	Buffer	Binding	Elution	Comment
Hydrophobic interaction				
Phenyl Superose	1.2 M-(NH ₄) ₂ SO ₄ /50 mM-PO ₄ , pH 6.8	+	Decreasing [(NH ₄) ₂ SO ₄]	Elution very variable
Alkyl Superose	1.2 M-(NH ₄) ₂ SO ₄ /50 mM-PO ₄ , pH 6.8	+	Decreasing [(NH ₄) ₂ SO ₄]	Elution very variable
Affinity				
3'-ADP-agarose	PBS/10 mM-Mes, pH 6.8	+	10 mM-NADP/0.1 M-NaCl	Selectively removed 47 kDa
2'-ADP-agarose	PBS/10 mM-Mes, pH 6.8	+	10 mM-NADP/0.1 M-NaCl	More selective than 3'-ADP
GTP-agarose	PBS	-	1 M-NaCl/0.15 M-NaCl/1 mM-GTP	Most protein did not bind, therefore little resolution
Heparin-agarose	10 mM-PO ₄ , pH 6.8	+	0.1 M-NaCl	Comparable with cation exchange
Iminodiacetic acid-agarose	100 mM-HaAC/NaOH, pH 5.0	-	20 mM-NaH ₂ PO ₄	Most protein did not bind, therefore little resolution
Chromatofocusing				
Mono-P	75 mM-Tris acetate, pH 9.0	+	Polybuffer 6-9	47 kDa protein trailed off
	75 mM-Tris/HCl, pH 9.0	+	Polybuffer 6-9	As above
	25 mM-Triethanolamine, pH 8.3	+	Polybuffer 6-9	As above
Reverse-phase				
C ₈	0.1% TFA in water	+	Acetonitrile gradient	47 kDa eluted cleanly but solvent not compatible with functional studies

Fig. 3. Detection of 47 kDa protein in cytosol from normal subjects and from CGD patients

Western blot of cytosol (20 µg of protein in all cases except for 10 µg in lane 1) from two patients with X-linked CGD (lanes 1 and 2), two patients with autosomal recessive CGD (lanes 3 and 4), and from two normal subjects (lanes 5 and 6) probed with antiserum to the 47 kDa protein.

molecular mass was absent from these patients, but did not agree with results of experiments by Kramer *et al.* (1988), which suggested that the protein was in fact present but failed to become phosphorylated. After we had screened a differentiation-induced HL60 cell λgt11 library with our antibodies, Lomax *et al.* (1989) and Volpp *et al.* (1989) reported the cDNA cloning and derived sequence of their 47 kDa protein. To prove that the 47 kDa phosphoprotein and the 47 kDa protein whose gene was cloned were identical, we determined the amino acid sequence of a peptide purified from a tryptic digest of approx. 5 µg of protein. The sequence: Glu-Met-Phe-Pro-Ile-Glu-Ala-Gly-Ala-Ile-Asn-Xaa-Glu corresponded to residues 56-68 of the protein sequence predicted from both cDNA sequences. The ten clones picked from the λgt11 library were hybridized against a probe prepared from a 47 kDa phosphoprotein cDNA, independently cloned from a λgt11 cDNA library and shown by its nucleotide sequence to contain a full-length cDNA insert (D. Bentley, Imperial Cancer Research Fund Laboratories, London). Three of these clones cross-hybridized with this probe at high stringency.

These studies confirm that the 47 kDa protein initially described as abnormal in autosomal recessive CGD and that identified and cloned by Volpp *et al.* (1988, 1989) and Lomax *et al.* (1989) through the production of antibodies to GTP-binding proteins are in fact the same. A method is described for the purification of this protein in its native state.

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