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TPA-induced activation of MAP kinase

Peter D. Adams and Peter J. Parker

Protein Phosphorylation Laboratory, Imperial Cancer Research Fund, 44 Lincoln's Inn Fields, London WC2A 3PX, UK

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Threonine and tyrosine residue phosphorylation of a 42 kDa protein identified as mitogen-activated protein kinase (MAP kinase) was stimulated in extracts from TPA-pretreated cells. It is further shown that TPA pretreatment leads to the enhancement of an activity that will induce reactivation of dephosphorylated/inactivated MAP kinase. This TPA-induced activity induces the threonine and tyrosine phosphorylation of p42 in extracts from unstimulated cells.

MAP kinase; Protein kinase C; Phorbol ester

1. INTRODUCTION

Phorbol esters stimulate a variety of responses in cultured cells including mitogenesis and differentiation (reviewed in [1]). This broad spectrum of events has been attributed to the activation of members of the protein kinase C (PKC) family (reviewed in [2,3]), which appear to function as high affinity receptors for biologically active phorbol esters (see [4]). Whether individual PKC isoforms are responsible for particular elements of these complex responses remains a critical issue. The lack of understanding with respect to the function of individual PKC isoforms in vivo is largely due to the limited information available on the specificity of these enzymes and to the dearth of physiological substrates of known function. As a prelude to defining PKC substrates, we have investigated the TPA-induced tyrosine/threonine phosphorylation of a 42 kDa protein [5–7] in the U937 cell line. These human monocytic cells express a number of PKC isoforms (α, β, ε, ζ; D.K. Ways, personal communication) and as such provide a useful model system for an analysis of isoform specificity. We report here that acute TPA treatment of U937 cells stimulates an activity that induces the tyrosine and threonine phosphorylation of the 42 kDa MAP kinase.

Abbreviations: MAP-2, microtubule-associated protein 2; MAP kinase, mitogen-activated kinase; MBP, myelin basic protein; PBS, phosphate-buffered saline; PKC, protein kinase C; PP2A, protein phosphatase 2A; TPA, tetradecanoyl phorbol acetate.

Correspondence address: P.J. Parker, Protein Phosphorylation Laboratory, Imperial Cancer Research Fund, 44 Lincoln's Inn Fields, London WC2A 3PX, UK. Fax: (44) (71) 405 0190.

2. MATERIALS AND METHODS

2.1. *Materials*

Radioisotopes and the ECL Western blotting kit were obtained from Amersham, UK. Other chemicals and biochemicals were obtained from Sigma, UK. Microtubule-associated protein 2 (MAP2) was purified according to [8,9]. Protein phosphatase 2A (PP2A) was purified according to Ramachandran et al. [10]; PP2A was also generously provided by Professor P. Cohen, Dundee.

2.2. *Cell culture and treatment*

U937 cells were grown in RPMI 1640 medium containing 5% fetal calf serum in 5% CO_2. Subconfluent cells were harvested at a density between 0.5 and 1.0 × 10^6/ml. Cells were washed once in phosphate-buffered saline (PBS) and resuspended in PBS at a density of 18.8 × 10^6/ml. Cells were treated with 160 nM TPA (or the ethanol vehicle) for 8 min at 30°C; this time and dose of TPA were determined to be optimum for the subsequent in vitro phosphorylation of p42 (see below). The cells were then pelleted in a microcentrifuge, the supernatant discarded and the cell pellet frozen in liquid nitrogen.

2.3. *In vitro phosphorylation of p42*

Frozen cell pellets were extracted in extraction buffer (EB; 20 mM β-glycerophosphate (pH 7.5), 20 mM NaF, 2 mM EDTA, 0.2 mM Na_3VO_4, 10 mM benzamidine, 25 μg/ml leupeptin, 50 μg/ml PMSF, 0.3% v/v β-mercaptoethanol) using 1 ml/10^9 cells. Protein concentration was 10–15 mg/ml. Crude extracts were centrifuged at 12 000 rpm for 8 min at 4°C in a microcentrifuge.

The soluble fraction was incubated for 7 min at 30°C in 20 mM β-glycerophosphate (pH 7.5), 0.2 mM Na_3VO_4, 10 mM $MgCl_2$, 0.13 mM [γ-^{32}P]ATP (500 counts/pmol) at a protein concentration of 1.3–1.9 mg/ml. Reactions were stopped by addition of SDS sample buffer or by dilution (see below). Proteins were separated on 10% SDS polyacrylamide gels and radiolabelled proteins detected by autoradiography. The effects of incubation in vitro were time-dependent and 7 min proved to be optimum for these conditions (not shown).

2.4. *MAP kinase assays*

MAP kinase activity was assayed by incubation of the crude soluble fraction or column fractions in 20 mM β-glycerophosphate, 20 mM NaF, 2 mM EDTA, 0.2 mM Na_3VO_4, 10 mM $MgCl_2$, 0.13 mM [γ-^{32}P]ATP (1000 counts/pmol), 0.28 mg/ml myelin basic protein (MBP) or 0.56 mg/ml MAP-2 as indicated for 10 min at 30°C. Reactions were stopped by spotting 30 μl (out of a total volume of 45 μl) onto 3MM paper which was dropped into 20% TCA. Papers were

washed 3 × 10 min in 20% TCA and then counted by Cerenkov. A unit of MAP kinase activity is defined as that which incorporates 1 nmol/min into substrate under these standard assay conditions.

2.5. *Phenyl-Sepharose chromatography*

Cell pellets were extracted in EB and the soluble fraction incubated with Mg/ATP as described above. The reactions were stopped by dilution 1:1 with ice-cold EB containing 0.2 M NaCl, and loaded onto a phenyl-Sepharose at a flow rate of 0.4 ml/min. Up to 10 mg of protein was loaded onto a 1 ml column. The column was washed with 10 ml of EB containing 0.1 M NaCl followed by 10 ml of EB. MAP kinase was step-eluted with EB containing 50% ethanediol at a flow rate of 0.04 ml/min.

2.6. *Mono Q and Mono S chromatography*

Cell pellets were extracted in EB as described. The soluble fraction (20 mg) was loaded onto a 1 ml Mono Q (5/5) column, equilibrated in EB. The column was washed with 5 ml of EB and developed with a linear 5 ml gradient from 0–1.0 M NaCl. In some experiments the peak fractions of MAP kinase reactivating activity (see results) were pooled and loaded onto a Mono S (5/5) column. The column was washed and developed in the same way as the Mono Q column.

2.7. *MAP kinase inactivation*

100–200 μl of MAP kinase, eluted off the phenyl-Sepharose column, was dialysed in 2 × 1 litre of TE (20 mM Tris-HCl (pH 7.5), 2 mM EDTA, 10 mM benzamidine, 50 μg/ml PMSF, 0.3% β-mercaptoethanol) for 4.5 h at 4°C. After dialysis, leupeptin was added to a final concentration of 25 μg/ml. Dialysed MAP kinase was incubated for 20 min at 30°C with PP2A (10 U/ml) in 20 mM Tris-HCl (pH 7.5), 2 mM EDTA, 10 mM benzamidine, 25 μg/ml leupeptin, 50 μg/ml PMSF, 0.08 mM DTT, 0.25 mM β-mercaptoethanol, 0.17 mg/ml BSA. PP2A was added as a 60 U/ml stock in 20 mM Tris-HCl (pH 7.5), 1 mg/ml BSA, 0.5 mM DTT. The inactivation was stopped by addition of 20 mM NaF, 2 mM EDTA (pH 7.0). Inactivation was time-dependent and this routine 20 min treatment resulted in ~70% inactivation of MAP kinase.

2.8. *MAP kinase reactivation*

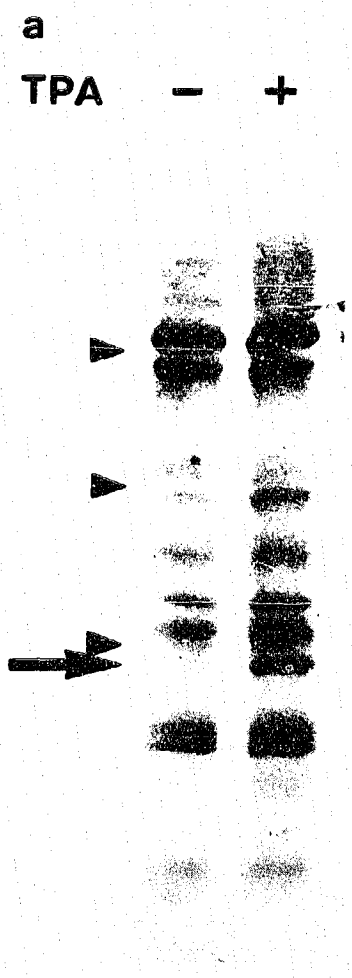
Fractions derived from Mono Q or Mono S column fractionation were incubated in the presence or absence of PP2A-inactivated MAP kinase. Inactivated MAP kinase (as indicated in the text or figure legend) was added with column fractions to tubes, which also contained 20 mM β-glycerophosphate (pH 7.5), 20 mM NaF, 2 mM EDTA, 0.2 mM Na_3VO_4, 10 mM $MgCl_2$, 0.13 mM [γ-^{32}P]ATP (1000 counts/ pmol) and 0.22 mg/ml MBP in a final volume of 45 μl. Incubations were carried out for 10 min and the phosphorylation of the MBP determined as above. A unit of MAP kinase activator is defined as that increasing the MAP kinase activity by one unit under the conditions described.

2.9. *Other methods*

Protein concentration was determined by the method of Bradford [11]. Phospho-amino acid analysis was carried out following transfer of proteins to PVDF membranes as described previously [12]. Antisera were raised to the peptide ITVEEALAHPYLEQYYDPTFDEPV (based on the ERK-1 sequence; [13,14] coupled to keyhole limpet hemocyanin. Western analyses were carried out using an ECL kit according to the manufacturer's recommendation at a serum dilution of 1/5000.

3. RESULTS

In order to determine whether the kinase(s) responsible for p42 phosphorylation was stable to extraction, U937 cells with or without a TPA pretreatment were lysed in buffers designed to inhibit serine/threonine and



(*Fig. 1 continued on the next page →*)

tyrosine phosphatases; extracts were subsequently incubated in vitro with Mg^{2+}-ATP. Fig. 1a shows that extracts from TPA-pretreated cells retain kinase activity that leads to the phosphorylation of p42; very little activity is detected in control extracts. Phospho-amino acid analysis indicates that both tyrosine and threonine residues of p42 become phosphorylated in TPA-treated extracts (Fig. 1b). The rate of dephosphorylation of ^{32}P-labelled p42 added to extracts from control or TPA-treated cells is indistinguishable (not shown). This indicates that TPA treatment leads to enhanced kinase activity and not decreased phosphatase activity.

In view of the apparent molecular weight and phospho-amino acid content of p42, the relationship between this protein and the mitogen-activated kinase (MAP kinase; [6,15,16]) was investigated. Chromatography of the phosphorylated extract on phenyl-Sepharose showed that the ^{32}P-labelled p42 and kinase activity co-eluted in the 50% ethanediol eluate (Fig. 2a and b). This kinase activity phosphorylated both MAP-2 (Fig. 2b) and myelin basic protein (MBP: not shown) consistent with the specificity of MAP kinase [17]. No ^{32}P-labelled p42 was obtained from fractionated control

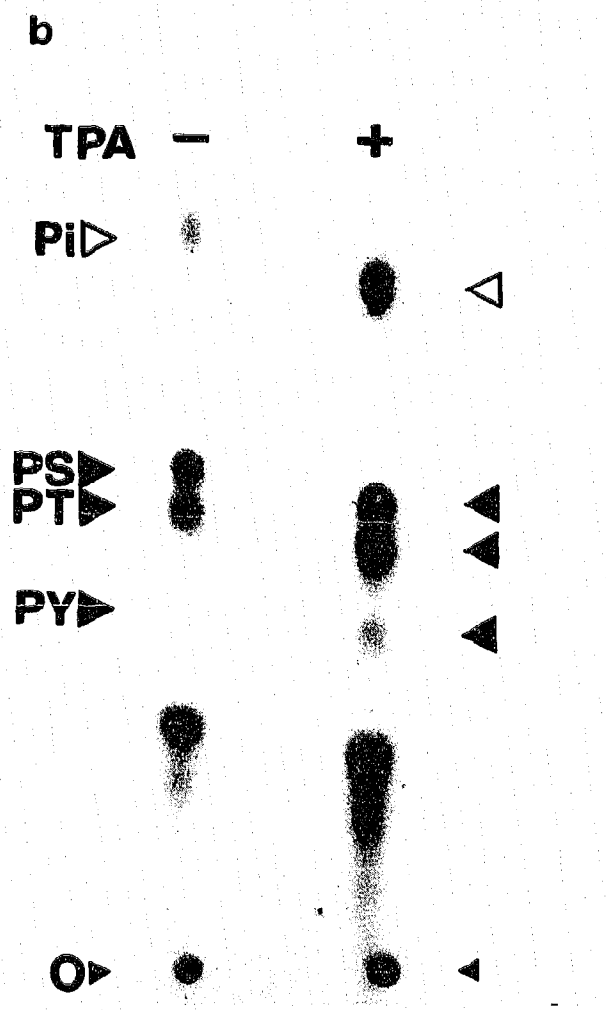
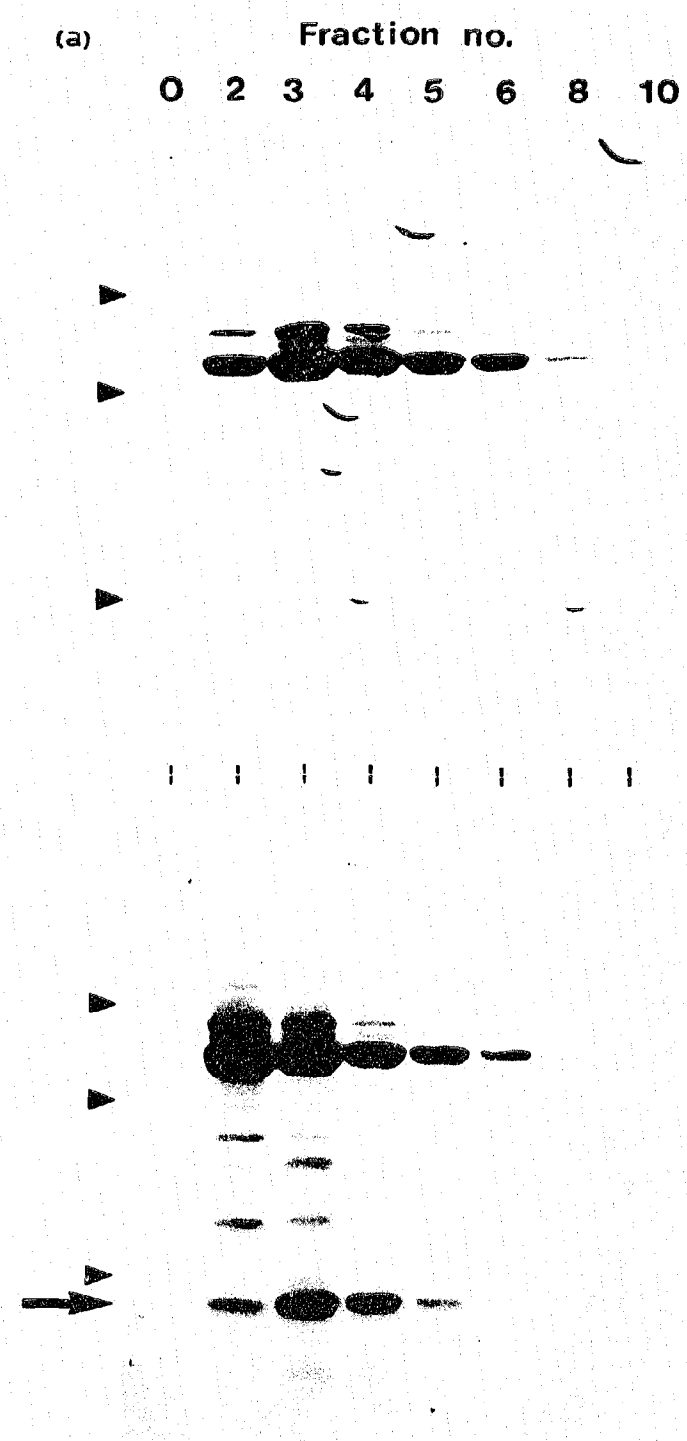


Fig. 1. Phosphorylation of p42 in vitro. (a) Extracts from control (−) or TPA- (+) treated cells were incubated as described in section 2. Labelled proteins were separated by SDS-PAGE and visualised by autoradiography. The arrow indicates the phosphorylated p42 protein. The arrowheads represent molecular weight markers which are in descending order: phosphorylase (92 kDa), bovine serum albumin (66.5 kDa) and ovalbumin (45 kDa). (b) The ^{32}P-labelled p42 protein derived from TPA treated cells (as in (a)) and an equivalent gel piece from a control sample were extracted, hydrolysed and analysed for phospho-amino acid content. Internal unlabelled standards were included to allow identification: Pi, inorganic phosphate; PS, phosphoserine; PT, phosphothreonine; PY, phosphotyrosine; O, origin.

extracts and similarly negligible kinase activity was eluted from the phenyl-Sepharose column (Fig. 2b). A further indication that p42 is the human equivalent of MAP kinase is provided by the observation that antisera raised to a synthetic peptide based on the published ERK- 1 sequence [13,14] specifically recognises a 42 kDa polypeptide eluting with the p42/MAP kinase activity of both phenyl-Sepharose and Mono Q columns (not shown). Thus it appears that p42 is indeed a form of MAP kinase and it is subsequently referred to as such.

As observed previously for purified MAP kinase [16], treatment of the phenyl-Sepharose eluate from the



(Fig. 2 continued on the next page)

TPA-treated U937 cells with PP2A inactivates the kinase; this parallels loss of [^{32}P]phosphate derived from in vitro labelling (data not shown). Since the extraction procedure employed here allows the retention in vitro of both tyrosine and threonine phosphorylating activity, the PP2A-inactivated MAP kinase was used to screen for a reactivating activity. Such an activity was

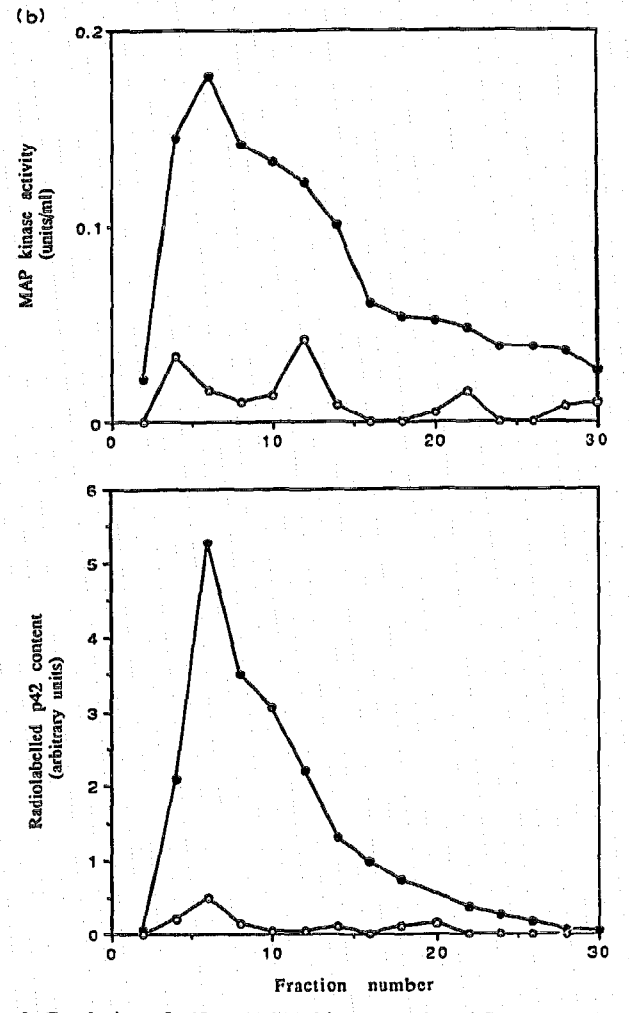
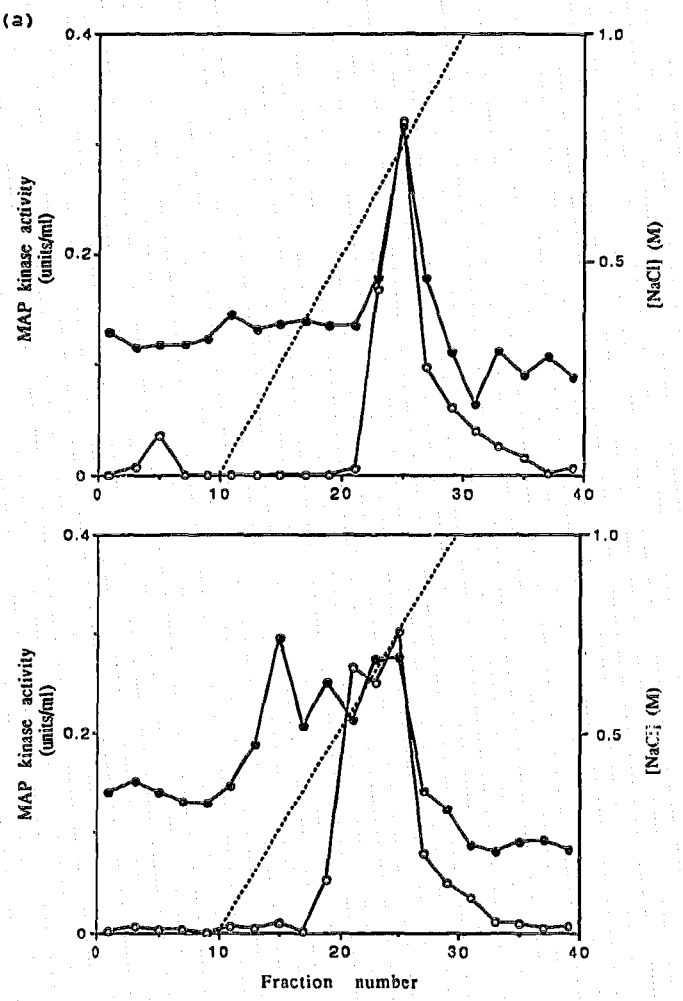


Fig. 2. Co-elution of p42 and MAP kinase on phenyl-Sepharose chromatrography. (a) Phosphorylated extracts from control (upper panel) and TPA treated (lower panel) cells were chromatographed on phenyl-Sepharose as described in section 2. Samples from eluted fractions (as indicated) were analysed by SDS-PAGE for ^{32}P-labelled p42 content (arrow in lower panel). Arrowheads indicate molecular weight markers as in Fig. 1. (b) Phosphorylated extracts derived from control (○–○) or TPA treated cells (●–●) were chromatographed on phenyl-Sepharose and analysed for MAP kinase activity (upper panel) and for ^{32}P-labelled p42 content by SDS-PAGE, autoradiography and densitometry (lower panel).



(Fig. 3 continued on the next page)

detected in the initial gradient fractions from an extract separated on a Mono Q column (Fig. 3a). Consistent with the phosphorylation of p42 found in cell extracts (see Fig. 1) this MAP kinase activator was increased by TPA treatment of cells. The p42/MAP kinase itself elutes at a higher NaCl concentration and is largely separated from the MAP kinase activator.

In order to further fractionate and characterize the MAP kinase activator, peak fractions from a Mono Q column were applied to a Mono S column and the activity eluted with a NaCl gradient. With this relatively steep gradient the MAP kinase activator was found to elute at ~450 mM NaCl (Fig. 3b).

To assess the relationship between the MAP kinase activator and the extract threonine/tyrosine kinase activities stimulated by TPA treatment, the partially purified (Mono Q/Mono S) kinase activator was incubated with control or TPA-treated cell extracts. In TPA-treated cell extracts there was no increase in the extent of p42 phosphorylation above that induced by TPA treatment alone (Fig. 4). By contrast phosphorylation of p42 in the control extracts was only observed on addition of MAP kinase activator obtained from TPA-treated cells; significantly this phosphorylation was on both threonine and tyrosine residues (Fig. 4). Comparison of control extract p42 phosphorylation and MAP kinase reactivation showed that these activities co-eluted from the Mono S column (not shown).

4. DISCUSSION

TPA pretreatment of intact U937 cells stimulates phosphorylation of p42 on tyrosine and threonine residues in cell extracts. p42 is identified as MAP kinase on the basis of chromatographic properties, co-elution with MAP kinase activity and immunological evidence;

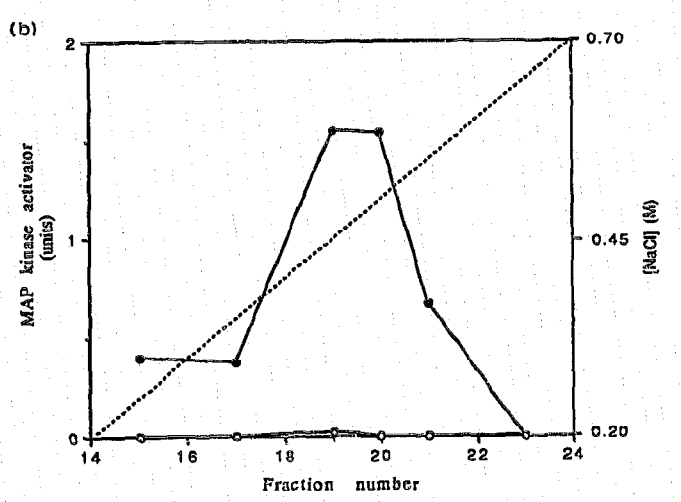


Fig. 3. Mono Q and Mono S chromatography of MAP kinase activator. (a) Extracts from control (upper panel) or TPA-treted cells (lower panel) were chromatographed on Mono Q (5/5) columns, washed and eluted on a linear gradient (see section 2). MAP kinase activity was assayed in the absence (○–○) or presence (●–●) of PP2A-inactivated phenyl-Sepharose-purified MAP kinase (1.3 milliunits). (b) Mono Q-fractionated MAP kinase activator from TPA-treated (lower panel) or the equivalent fractions from control (upper panel) cells, were re-chromatographed on a Mono S (5/5) column (section 2). MAP kinase activator was determined by assaying the increase in MAP kinase activity on incubation of PP2A-treated MAP kinase (1.3 milliunits) in the presence of column fractions.

the p42 protein reacts with an antiserum raised to an oligopeptide based upon the sequence of the MAP kinase homologue ERK1 [14] (It is possible that this serum would also recognise the related kinase ERK2 [13]).

A TPA-stimulated activity that reactivates PP2A inactivated/dephosphorylated MAP kinase has been identified (termed MAP kinase activator). This MAP kinase activator stimulates threonine and tyrosine phosphorylation of p42/MAP kinase, when added back to control cell extracts. The MAP kinase activator and p42/MAP kinase phosphorylating activity co-elute on Mono S chromatography.

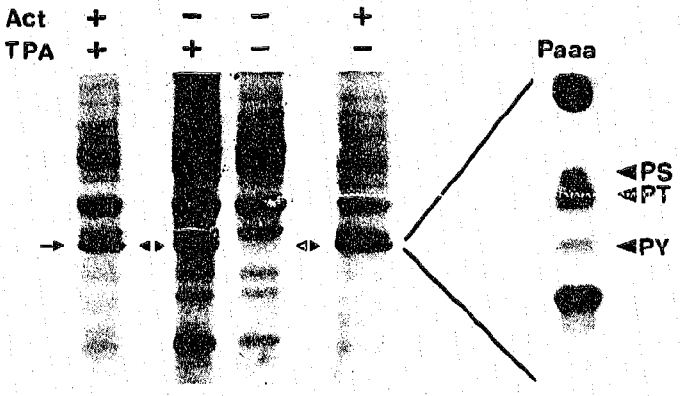


Fig. 4. Phosphorylation of p42 in control cell extracts. Extracts from control (−) or TPA- (+) pretreated cells were incubated as described in Fig. 1. Incubations also included the peak of MAP kinase activator (Act) as indicated. The ^{32}P-labelled p42 from control cells incubated with MAP kinase activator, was subjected to phospho-amino acid analysis (Paaa) and autoradiography. The migration of the internal phosphoamino acid standards are shown: PS, phosphoserine; PT, phosphothreonine; PY, phosphotyrosine.

The results suggest that a single activity is responsible for inducing activation of MAP kinase through phosphorylation on tyrosine and threonine residues. This conclusion is entirely consistent with those drawn from studies on PC12 cells following NGF treatment (P. Cohen personal communication) and also in Swiss 3T3 cells following EGF treatment [18]. MAP kinase is activated by a variety of mitogenic agents [19–26] and there is evidence for the existence of PKC-dependent and independent pathways of activation [27,28].

It remains to be firmly established whether, and if so, at what point PKC directly regulates an activity within this kinase cascade. It will be of particular interest if such a defined substrate for PKC can be identified since U937 cells express a number of PKC isoforms (α, β, ε and ζ) which could be investigated with respect to their specificity in the regulation of this cascade.

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