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MAP kinase kinase from rabbit skeletal muscle

A novel dual specificity enzyme showing homology to yeast protein kinases involved in pheromone-dependent signal transduction

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MAP kinase kinase (MAPKK) was purified 30,000-fold to homogeneity from extracts of rabbit skeletal muscle and shown to be a monomeric protein of apparent molecular mass 44 kDa. MAPKK activated the 42 kDa isoform of MAP kinase by phosphorylation of Thr-183 and Tyr-185, and phosphorylated itself slowly on tyrosine, threonine and serine residues, establishing that it is a 'dual specificity' protein kinase. Peptide sequences from MAPKK were homologous to other protein serine/threonine kinases, especially to the subfamily that includes yeast protein kinases that lie upstream of yeast MAP kinase homologues in the pheromone-dependent mating pathways.

Mitogen-activated protein (MAP) kinase; Pheromone; Protein kinase; Signal transduction; Yeast

1. INTRODUCTION

Mitogen-activated protein kinases (MAP kinases) represent a family of protein serine/threonine kinases that are activated rapidly in response to many extracellular signals [1,2]. Potential in vivo substrates for MAP kinase include the transcription factor c-*jun* and two protein kinases termed S6 kinase-II [4,5] (or insulin-stimulated protein kinase-1 [6]) and MAP kinase-activated protein kinase-2 [7], all of which are activated by MAP kinase in vitro. In mammalian skeletal muscle, S6 kinase-II activates the glycogen-associated form of protein phosphatase-1 [6], promoting dephosphorylation (activation) of glycogen synthase and dephosphorylation (inactivation) of phosphorylase kinase. These events appear to underlie the stimulation of glycogen synthesis and inhibition of glycogenolysis by insulin [6]. The physiological roles of MAP kinase-activated protein kinase-2 are not yet known.

MAP kinase is unique in requiring phosphorylation of a threonine and a tyrosine residue to become active [8], and these regulatory phosphorylations occur at Thr-183 and Tyr-185 in the 42 kDa isoform of MAP kinase [9]. MAP kinase activators which promote phosphorylation of these residues have been identified in extracts of 3T3 cells [10], PC12 cells [11] and rabbit skeletal muscle [12], and were found to co-purify through several steps of purification. Furthermore, the ability to phosphorylate Thr-183 and Tyr-185 was lost in parallel upon incubation with protein phosphatase 2A (PP2A), a protein serine/threonine phosphatase, but not by incubation with protein tyrosine phosphatases [11,12]. The partially purified MAP kinase activator from skeletal muscle phosphorylated a kinase-inactive mutant of MAP kinase on Thr-183 and Tyr-185 in an identical manner to 'wild-type' MAP kinase, indicating that it is a protein kinase (MAP kinase kinase) [12], and not a protein which stimulates autophosphorylation of MAP kinase [13,14].

Recently, Matsuda et al. [15] purified a MAP kinase activator to apparent homogeneity from mature *Xenopus laevis* oocytes which promoted phosphorylation of recombinant *Xenopus laevis* MAP kinase on tyrosine, threonine and serine residues. Here we purified MAP kinase kinase (MAPKK) to homogeneity from rabbit skeletal muscle and established that this enzyme is responsible for phosphorylation of both Thr-183 and Tyr-185. Peptide sequencing revealed that MAPKK is homologous to other protein kinases, especially to a subgroup that includes enzymes which lie on the pheromone-dependent mating pathways of yeasts.

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2. MATERIALS AND METHODS

2.1. *Materials*

Recombinant murine 42 kDa MAP kinase was expressed as a glutathione-*S*-transferase fusion protein in *E. coli*, purified by affinity chromatography on glutathione–Sepharose and cleaved from glutathione-*S*-transferase with thrombin, as described previously [7], and was a generous gift from Miss Sally Leevers and Dr. Chris Marshall (Chester Beatty Laboratories, Institute for Cancer Research, Lon-

don). Recombinant human protein phosphatase 1γ was provided by Drs. Alasdair Street, Dario Alessi and Tricia Cohen in this Unit. Heparin-Sepharose, Superose 12 and Mono Q were purchased from Pharmacia Ltd. (Milton Keynes, UK) and the sources of all other reagents are given in [12].

2.2. *Assay of MAP kinase kinase*

Inactive recombinant MAP kinase was diluted in 50 mM Tris-HCl, pH 7.5 (20°C), 0.1 mM Na-EGTA, 0.1% (v/v) 2-mercaptoethanol (solution A) containing 0.04% (w/v) Brij 35 to give a concentration of 120 U/ml MAP kinase after maximal activation by MAPKK. A 5 μl aliquot of inactive MAP kinase was incubated for 3 min at 30°C with 5 μl of MAPKK in solution A containing 0.04% (w/v) Brij 35 and 5 μl of 100 mM Tris-HCl, pH 7.5 (20°C), 0.2 mM Na-EGTA, 0.5 mM sodium orthovanadate and 0.1% (v/v) 2-mercaptoethanol, and the activation of MAP kinase initiated with 5 μl of 40 mM magnesium acetate–0.8 mM unlabelled ATP. Control incubations were carried out in parallel in which solution A, containing 0.04% Brij 35, replaced MAPKK or recombinant MAP kinase. After 20 min, the incubation was diluted 5-fold in solution A containing 1.0 mg/ml bovine serum albumin and 0.5 mM sodium orthovanadate, and a 10μl aliquot was added to 40 μl of 0.4 mg/ml myelin basic protein in solution A containing the specific peptide inhibitor of cyclic AMP-dependent protein kinase at μM [16], 12.5 mM magnesium acetate and 0.125 mM [γ-^{32}P]ATP (6×10^5 cpm/nmol) prewarmed to 30°C. After incubation for 10 min at 30°C incorporation of phosphate into myelin basic protein was measured as described previously [17]. One unit of MAP kinase is that amount which catalyses the incorporation of 1.0 nmol of phosphate into myelin basic protein in 1 min. One unit of MAPKK is that amount which produces 50% reactivation of MAP kinase in 1 min. The activation of MAP kinase was linear with time up to 30% reactivation and fractions containing MAPKK were therefore diluted appropriately before assay to ensure that initial rate conditions were met.

2.3. *Purification of MAP kinase kinase from rabbit skeletal muscle*

All buffers contained 0.1% (v/v) 2-mercaptoethanol and 1 mM benzamidine. Extracts were prepared from 1,800 g of skeletal muscle (two rabbits) and subjected to batchwise chromatography on QAE-Sephadex, precipitation with ammonium sulphate and chromatography on Q-Sepharose, as described previously [12], but with the following modifications. Firstly, the amount of QAE-Sephadex resin was increased from 30 to 35 g (dry weight). Secondly, the enzyme was precipitated with 60% (rather than 50%) ammonium sulphate (by the addition of solid salt). Thirdly, the ammonium sulphate pellet was resuspended in buffer containing 5 mM sodium pyrophosphate to ensure complete inactivation of any remaining traces of protein phosphatase 2A. The peak of MAPKK from Q-Sepharose [12] was pooled, diluted 10-fold in 25 mM Tris-HCl, pH 7.4 (20°C), 1.0 mM Na-EDTA, 5% (v/v) glycerol, 0.02% (w/v) Brij 35 (solution B), and chromatographed on heparin-Sepharose as described in the legend to Fig. 1A. The fractions containing MAPKK were pooled, diluted 3.5-fold in 25 mM Tris-HCl, pH 8.9 (20°C), 1.0 mM Na-EDTA, 5% (v/v) glycerol and 0.02% (w/v) Brij 35 (solution C) and applied to a 5 × 0.5 cm column of Mono Q equilibrated in solution C. After washing with 5 ml of equilibration buffer, the column was developed with a 60 ml linear salt gradient to 1.0 M NaCl, and 0.5 ml fractions were collected at a flow rate of 1.0 ml/min. The active fractions, which eluted as a single peak at 0.19 M NaCl (data not shown) were pooled (2.5 ml), concentrated to 0.25 ml by ultrafiltration, diluted to 20 ml in solution B and chromatographed on Mono S, as described in the legend to Fig. 1B. The active fractions were pooled, concentrated to 0.1 ml by ultrafiltration and subjected to gel-filtration on Superose 12, as described in the legend to Fig. 1C. The fractions containing MAPKK were stored at −20°C.

2.4. *Isolation and sequencing of tryptic peptides from MAP kinase kinase*

MAPKK (0.5 ml at 40 μg/ml, 0.5 nmol) in solution B containing 0.5 mM Na-EGTA and 0.2 M NaCl was allowed to autophosphorylate for 60 min at 30°C by incubation with 5 mM magnesium acetate, 0.2 mM [γ-^{32}P]ATP (10^6 cpm/nmol). Poly Glu/Tyr (50 μg) was added as a carrier, and the solution precipitated by the addition of 0.03 ml of 100% (w/v) trichloroacetic acid. After centrifugation for 2 min at 13,000 × g the supernatant was discarded and the pellet washed three times with trichloroacetic acid to remove ATP, and twice with water to remove trichloroacetic acid. The pellet was resuspended in 0.1 ml of 0.1 M ammonium bicarbonate, vortexed and placed in a sonicating water bath for 10 min. Trypsin (0.6 μg) was added and after digestion for 2 h at 37°C a further 0.6 μg of trypsin was added. After 16 h at 37°C, the solution was centrifuged for 2 min at 13,000 × g and the supernatant chromatographed on a Vydac C18 218TP54 reverse-phase column equilibrated in 0.1% trifluoroacetic acid. The column was developed with a linear gradient of acetonitrile containing 0.1% trifluoroacetic acid with an increase in acetonitrile concentration of 0.33% per min. The flow rate was 0.8 ml/min and fractions of 0.4 ml were collected. Peptides eluted from the column were sequenced on an Applied Biosystems 470A/120A gas-phase sequencer.

2.5. *Isolation of tryptic phosphopeptides from MAP kinase*

Recombinant MAP kinase (1.4 μg) was incubated for 30 min at 30°C with purified MAPKK (0.03 μg) in a volume of 30 μl as in section 2.2., but using [γ-^{32}P]ATP (10^6 cpm/nmol) instead of unlabelled ATP. Poly Glu/Tyr (50 μg) was added as carrier and MAPKK precipitated with 2 μl of 100% (w/v) trichloroacetic acid. After centrifugation for 2 min at 13,000 × g, the supernatant was discarded and the precipitate washed with trichloroacetic acid and water, as described for MAPKK in section 2.4. The precipitate was resuspended in 0.2 ml of 0.1 M ammonium bicarbonate and 10 μg trypsin added. After 2 h at 37°C a further 10 μg of trypsin was added and digestion continued for a further 16 h. The digest was chromatographed on a C18 column as described in section 3.

2.6. *Other analytical procedures*

SDS-PAGE was carried out according to Laemmli [18], protein concentrations were estimated by the method of Bradford [19] and phosphoamino acid analysis was performed as described in Gomez and Cohen [11].

3. RESULTS

3.1. *Purification and characterization of MAP kinase kinase*

MAPKK eluted as a single major peak of activity at each stage of its purification (Fig. 1) and the isolation procedure is summarized in Table I. The overall enrichment from muscle extracts was over 30,000-fold and about 50 μg of purified enzyme was isolated from 1,800 g muscle (2 rabbits) within 6 days, the overall recovery being 2%. At the final step, gel filtration on Superose 12, activity coincided with a peak of protein which eluted slightly earlier than ovalbumin (43 kDa) and corresponded to the apparent molecular weight of 45–50 kDa previously reported for the partially purified enzyme [12]. SDS-PAGE of the peak tubes from Superose 12 revealed a single 44 kDa protein (Fig. 2A), whose staining intensity correlated with the peak of activity when individual fractions across the gel filtration column were analysed (data not shown).

Two further pieces of evidence suggest that the 44 kDa protein is a protein kinase. Firstly, the 44 kDa protein is inactivated and ^{14}C-labelled by incubation for 60 min with *p*-fluorosulphonyl [^{14}C]benzoyl 5′-aden-

Table 1

Purification of MAP kinase kinase from rabbit skeletal muscle

Step	Volume (ml)	Activity (U)	Protein (mg)	Specific activity (U/mg)	Purification (-fold)	Recovery (%)
1. Extract	3,700	128,000	88,800	1.4	1.0	100
2. QAE-Sephadex	3,000	88,800	2,520	35	25	69
3. Ammonium sulphate precipitation	66	71,300	2,970	24	17	56
4. Q-Sepharose	21	51,500	305	169	121	40
5. Heparin-Sepharose	15	23,100	15	1,540	1,100	18
6. Mono Q	2.5	9,300	2.75	3,380	2,410	7.3
7. Mono S	2.0	3,500	0.13	26,900	19,200	2.7
8. Superose 12	0.75	2,100	0.047	44,800	32,000	1.6

1,100 g of muscle (two rabbits) were used in this preparation. Activity was measured as described in section 2, and protein was determined by the procedure of Bradford [19].

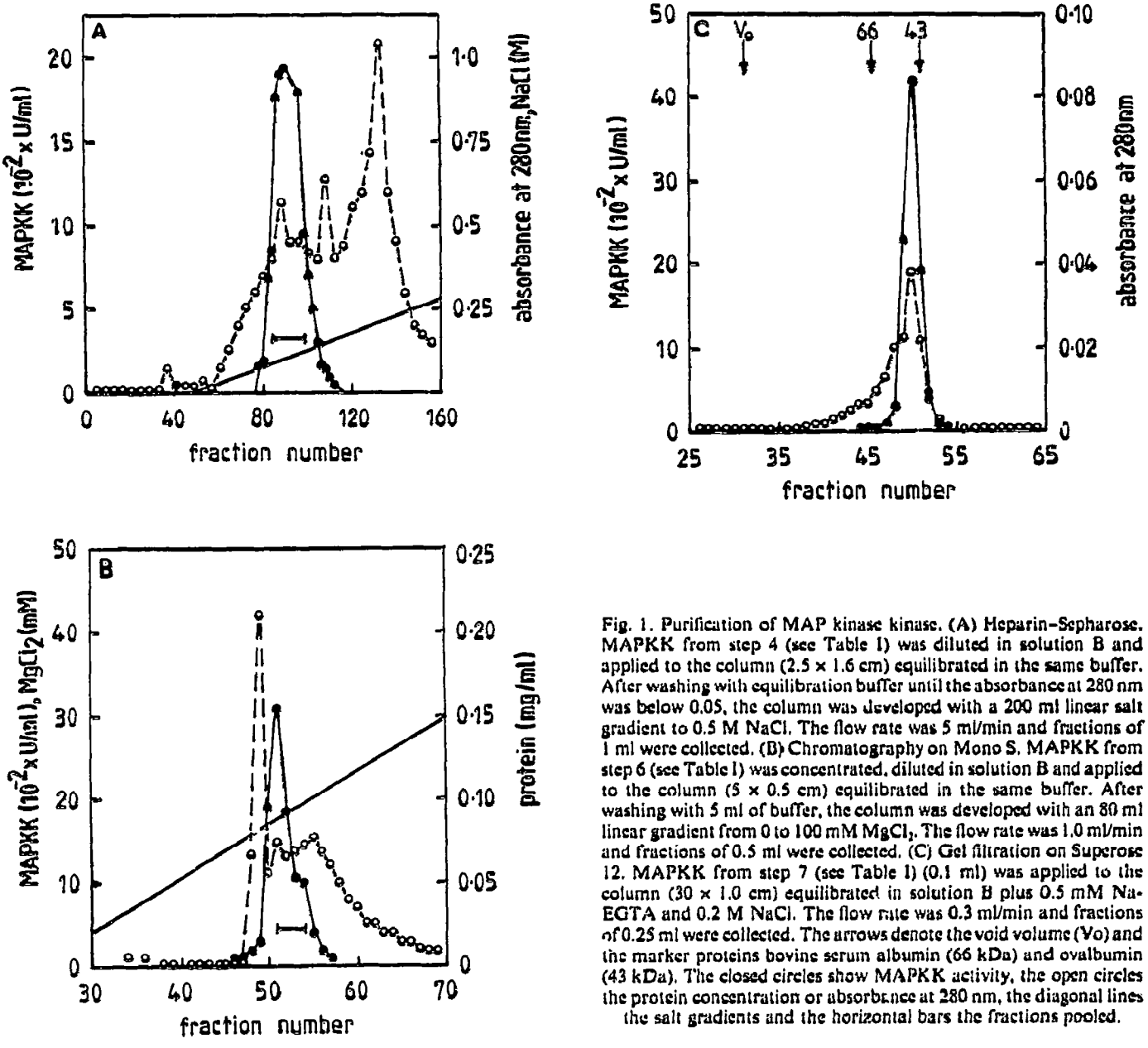

Fig. 1. Purification of MAP kinase kinase. (A) Heparin-Sepharose. MAPKK from step 4 (see Table 1) was diluted in solution B and applied to the column (2.5 × 1.6 cm) equilibrated in the same buffer. After washing with equilibration buffer until the absorbance at 280 nm was below 0.05, the column was developed with a 200 ml linear salt gradient to 0.5 M NaCl. The flow rate was 5 ml/min and fractions of 1 ml were collected. (B) Chromatography on Mono S. MAPKK from step 6 (see Table 1) was concentrated, diluted in solution B and applied to the column (5 × 0.5 cm) equilibrated in the same buffer. After washing with 5 ml of buffer, the column was developed with an 80 ml linear gradient from 0 to 100 mM $MgCl_2$. The flow rate was 1.0 ml/min and fractions of 0.5 ml were collected. (C) Gel filtration on Superose 12. MAPKK from step 7 (see Table 1) (0.1 ml) was applied to the column (30 × 1.0 cm) equilibrated in solution B plus 0.5 mM Na-EGTA and 0.2 M NaCl. The flow rate was 0.3 ml/min and fractions of 0.25 ml were collected. The arrows denote the void volume (Vo) and the marker proteins bovine serum albumin (66 kDa) and ovalbumin (43 kDa). The closed circles show MAPKK activity, the open circles the protein concentration or absorbance at 280 nm, the diagonal lines the salt gradients and the horizontal bars the fractions pooled.

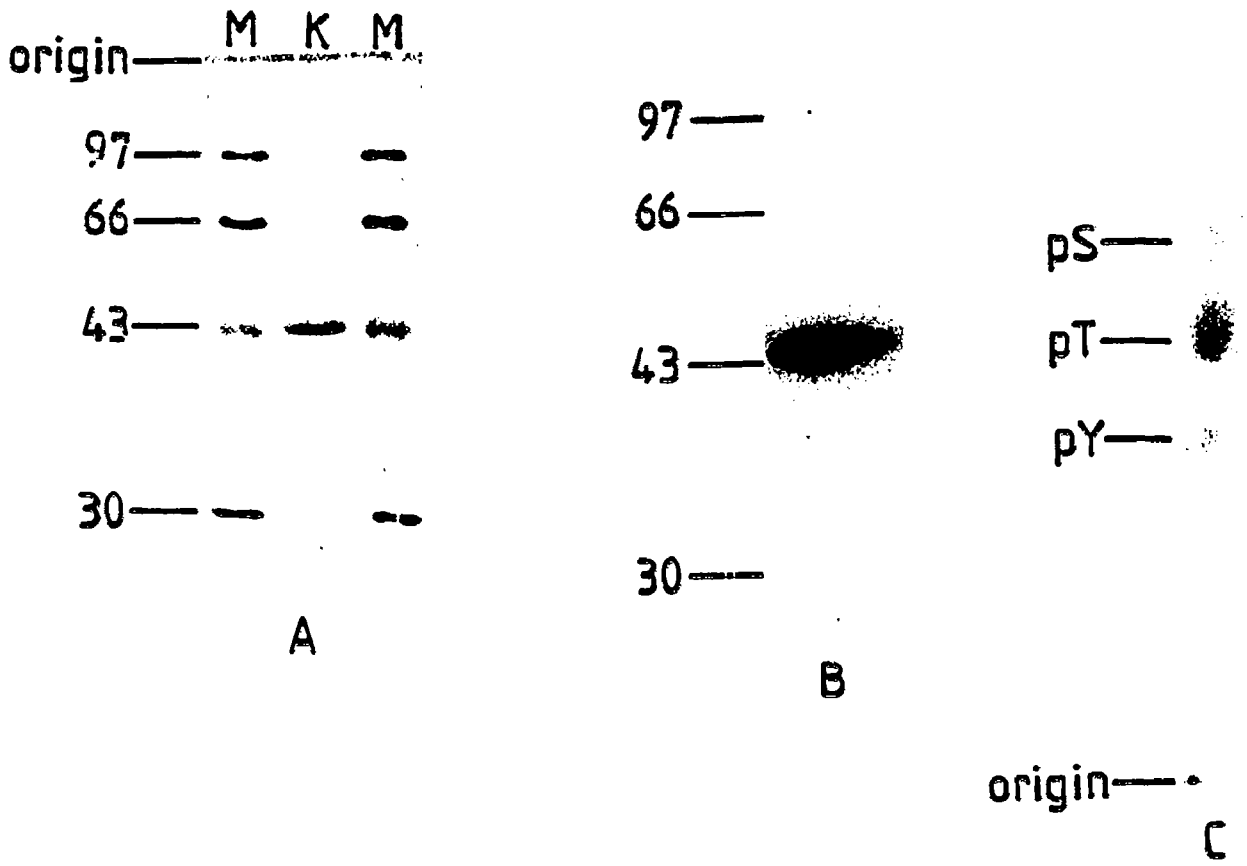

Fig. 2. SDS-PAGE and autophosphorylation of purified MAPKK. (A) 0.4 μg of the peak fraction of MAPKK activity from Superose 12 (Fig. 1C) was subjected to electrophoresis on a 15% polyacrylamide gel according to Laemmli [18] and stained with Coomassie blue (lane K). The molecular weight markers (lanes M) are phosphorylase (97 kDa), bovine serum albumin (66 kDa), ovalbumin (43 kDa) and carbonic anhydrase (30 kDa). Migration is from top to bottom. (B and C) Purified MAPKK (2.5 μg in 85 μl) was incubated for 60 min at 30°C in solution B containing 0.5 mM Na-EGTA, 0.2 M NaCl, 1 μM okadaic acid, 0.5 mM sodium orthovanadate, 5 mM magnesium acetate and 0.2 mM [γ^{32}P]ATP (10^6 cpm/nmol). An aliquot of the incubation mixture was subjected to electrophoresis as in (A) and an autoradiograph of the gel is shown in (B). A further aliquot was precipitated with trichloroacetic acid and subjected to phosphoamino acid analysis as described in section 2. Phosphorylated amino acids were located by autoradiography (C). The positions of phosphotyrosine (pY), phosphothreonine (pT), phosphoserine (pS) and the point of sample application (origin) are marked.

osine (data not shown), which modifies the ATP binding sites of other protein kinases covalently [20]. Secondly, the 44 kDa band is ^{32}P-labelled upon incubation at 30°C with 5 mM magnesium acetate and 0.2 mM [γ^{32}P]ATP (Fig. 2B), the level of phosphorylation reaching about 0.2 mol per mol protein after 60 min. This suggests that MAPKK, in common with most protein kinases, is capable of phosphorylating itself at a low rate. Phosphoamino acid analysis of the autophosphorylated MAPKK revealed the presence of phosphotyrosine, phosphothreonine and phosphoserine (Fig. 2C).

Homogeneous preparations of MAPKK phosphorylated recombinant 42 kDa MAP kinase in an identical manner to the partially purified enzyme [12]. Phosphorylation carried out as described in section 2.5 reached a plateau at 1.9 ± 0.4 mol phosphate per mol MAP kinase (average of two experiments), at which point the specific activity of the enzyme reached 1,250 U/mg. Stoichiometric phosphorylation of MAP kinase was attained by incubation with a 50-fold lower concentration of MAPKK, establishing that the latter enzyme does indeed act catalytically. Nearly all the ^{32}P radioactivity was contained within the single tryptic peptide that contains Thr-183 and Tyr-185 (Fig. 3A), and this peptide contained phosphothreonine and phosphotyrosine (Fig. 3B).

Homogeneous MAPKK, like the partially purified enzyme, was inactivated by PP2A and also by much higher levels of protein phosphatase 1 (PP1) activity (Fig. 4). The effects of PP1 and PP2A were blocked by okadaic acid (Fig. 4), a potent inhibitor of these enzymes [21].

3.2. *Structural analysis of MAP kinase kinase*

Tryptic peptides were isolated from MAPKK and

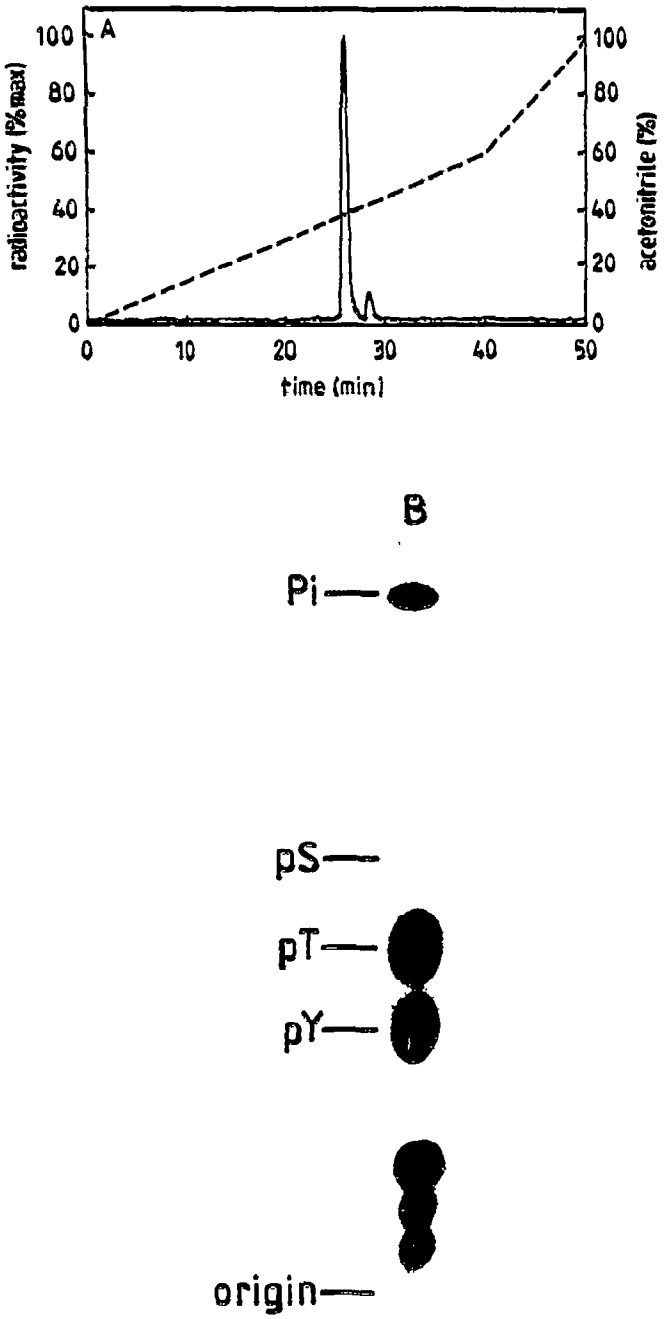

Fig. 3. Isolation and phosphoamino acid analysis of the tryptic phosphopeptide from MAP kinase phosphorylated by MAPKK. (A) Recombinant MAP kinase (1.4 μg) was phosphorylated with purified MAPKK, precipitated with trichloroacetic acid and digested with trypsin as described in section 2. The digest was chromatographed on a Vydac 218TP54 C18 column equilibrated in 0.1% (v/v) trifluoroacetic acid. The column was developed for 40 min with a linear acetonitrile gradient from 0–60% and ^{32}P radioactivity recorded with an on-line monitor. 37,000 cpm were applied to the column and radioactivity was recovered quantitatively. (B) Fractions containing the major tryptic phosphopeptide in A were pooled, dried and subjected to phosphoamino acid analysis as described in section 2. The positions of phosphotyrosine (pY), phosphothreonine (pT), phosphoserine (pS), inorganic phosphate (Pi) and the point of sample application (origin) are marked. Other ^{32}P-labelled spots represent phosphopeptides resulting from incomplete hydrolysis.

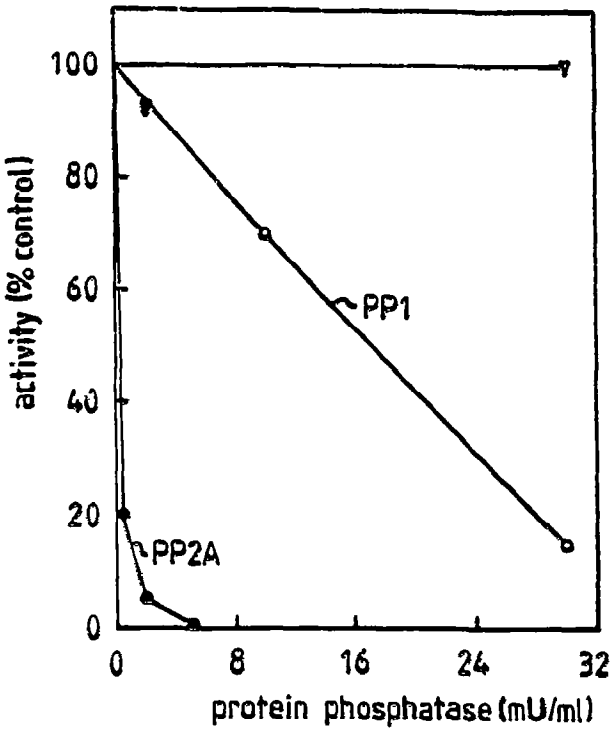

Fig. 4. Inactivation of MAPKK by protein Ser/Thr phosphatases. MAPKK (48 U/ml) was incubated at 30°C in a final volume of 4 μl with the indicated concentrations of PP1 (○) or PP2A (●) in 35 mM Tris-HCl, pH 7.4 (20°C), 0.1 mM Na-EGTA, 0.3 mg/ml bovine serum albumin, 0.02% (w/v) Brij 35 and 0.1% (v/v) 2-mercaptoethanol. The phosphatase reactions were terminated after 30 min by the addition of 1 μl of 40 μM okadaic acid, and each incubation assayed for MAPKK activity. The open and closed triangles show control incubations in which PP1 and PP2A, respectively, were treated with okadaic acid prior to incubation with MAPKK. One unit of protein phosphatase activity is that amount which catalyses the release of 1 μmol of phosphate from glycogen phosphorylase in 1 min [29]. Similar results were obtained in several different experiments.

sequenced. One peptide (peptide 1 in Table II) commenced with a proline residue. Since trypsin does not normally cleave Arg–Pro or Lys–Pro peptide bonds, this suggested that this peptide might correspond to the N-terminus of the protein, an idea confirmed by sequence analysis of the intact protein (data not shown).

The sequences of two further peptides (peptides 2 and 3 in Table II) were homologous to those found in other protein serine/threonine kinases. Peptide 2 corresponded to the end of Domain I and the beginning of Domain II, while peptide 3 corresponded to Domain VIb [22]. Table II also shows the homologous sequences of other protein kinases that these peptides resemble most closely, and these similarities are considered further in section 4.

4. DISCUSSION

In this paper we have purified MAPKK to homogeneity from rabbit skeletal muscle and established that

it is a monomeric protein with an apparent molecular weight of 44 kDa. Its slightly slower migration than ovalbumin on SDS-PAGE is identical to a MAP kinase activator isolated recently from mature *Xenopus laevis* oocytes [15]. However, whereas only 900-fold purification was apparently required to obtain homogeneous MAP kinase activator from oocytes, over 30,000-fold purification was needed to purify the skeletal muscle enzyme (Table I). This difference could either be explained by a 30-fold higher concentration of MAPKK in oocytes and/or by a low level of activation of MAPKK in skeletal muscle, despite prior stimulation with insulin. The latter explanation would imply that the phosphorylated and dephosphorylated forms of MAPKK were separated during the purification.

The finding that homogeneous preparations of MAPKK phosphorylate Thr-183 and Tyr-185 on MAP kinase (Fig. 3A,B) and autophosphorylate on Tyr, Thr and Ser residues (Fig. 2C) establishes that MAPKK is indeed a 'dual specificity' protein kinase that is capable of phosphorylating all three hydroxyamino acids.

We have reported that partially purified MAPKK is inactivated by pre-incubation with PP2A, but not by protein tyrosine phosphatases, indicating that MAPKK is likely to be activated by Ser/Thr phosphorylation [11], and this result was confirmed with the *Xenopus* oocyte enzyme [15]. Homogeneous MAPKK was not only inactivated by PP2A, but also by PP1, albeit at a much higher concentration (Fig. 4). This provides further evidence that MAPKK is regulated by Ser/Thr and not Tyr phosphorylation, because PP1 is devoid of activity towards any phosphotyrosine residue so far tested [23].

The sequencing of tryptic peptides from MAPKK established its homology to other protein kinases, the sequence corresponding to Domain VIb being characteristic of classical protein Ser/Thr kinases, rather than protein Tyr kinases [22]. Thus MAPKK is a further member of the new class of protein kinases which resemble classical protein Ser/Thr kinases most closely in structure, but are capable of phosphorylating Tyr as well as Ser/Thr residues [24].

Tryptic peptide 3 was very similar to sequences found in one particular subclass of protein Ser/Thr kinases that comprises four yeast enzymes termed STE7, byr1, PBS2 and wis1 [25,26]. The homology with STE7 of *S. cerevisiae* (and its homologue byr1 in *S. pombe* [27]) is of considerable interest, because this protein kinase is known from genetic studies to be involved in the activation of the protein kinases FUS3 and KSS1, which are required for induction of mating-specific genes [28]. FUS3 and KSS1 show about 55% amino acid sequence identity to one another, as well as to mammalian MAP kinases, and contain the Thr–Glu–Tyr sequence phosphorylated by MAPKK. Furthermore, it has been shown that FUS3 becomes phosphorylated on these two residues in response to stimulation by pheromones which coordinate the physiological changes that allow yeast cells to mate [28]. Tryptic peptide 2 is located in a region that is not as well conserved as Domain VIb between different protein kinases. Nevertheless, the most similar sequence to peptide 2 in the OWL composite data base was the homologous peptide from PBS2. Furthermore, the N-terminal peptide 1 of MAPKK showed significant homology to the N-terminus of byr1 (Table II). These observations suggest that at least two components of the protein kinase cascade which is triggered by insulin and growth factors, and which leads to the activation of MAP kinase in mammalian cells, are analogous to protein kinases in the cascade which is triggered by mating pheromones in yeast.

The finding that MAPKK from rabbit skeletal muscle has a free N-terminus may facilitate comparison with other forms of this enzyme that are currently being isolated from different cells and tissues, and hence provide a rapid answer to the question of whether tissue-specific isoforms exist.

Table II

Homology between MAPKK and protein kinases of the byr1 family

Peptide	Protein kinase	Peptide sequence	Domain
1	MAPKK	PKKK PTPIQLNPAPD	N-terminus
	byr1	MPKRRRNPKGLVLNPNAS	N-terminus
2	MAPKK	VSHKPSGLVMAR	I/II
	PBS2	VLHKPTNVIMAT	I/II
	STE7	ALHVPDSKIVAK	I/II
	byr1	VKHR--NIFMAR	I/II
3	MAPKK	DVKPSNILVNSR	VIb
	STE7	DIKPSNVLINSK	VIb
	byr1	DLKPSNVVVNSR	VIb

Sequences were obtained by searching the OWL composite database V.16.1 and from [26]. Peptides 1, 2 and 3 were eluted from the C18 column at 23.5, 17 and 19.5% acetonitrile, respectively. Protein kinase domains are as defined in [22].

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