RESEARCH COMMUNICATION

Renaturation and partial peptide sequencing of mitogen-activated protein kinase (MAP kinase) activator from rabbit skeletal muscle

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Mitogen-activated protein kinase (MAP kinase) activator was purified 2000-fold from skeletal muscle, and proteins which co-purified with the activator were analysed after SDS/PAGE by renaturation and partial sequencing. Activity for tyrosine and threonine phosphorylation of MAP kinase was present in two bands of approx. 48 and 46 kDa, which have sequence similarity to small GTP-binding protein p25 GDP dissociation inhibitor and protein kinases (PBS2, SPK1⁺, STE7, BYR1) respectively.

INTRODUCTION

A family of protein kinases has been identified which require dual phosphorylation, at the sequence motif Thr-Glu-Tyr, of tyrosine and threonine residues for activity (for reviews, see [1–3]). Two protein kinases in this family, p42^{mapk} and p44^{mapk} [mitogen-activated protein (MAP) kinases of 42 and 44 kDa respectively], were originally identified as principal targets for tyrosine phosphorylation in quiescent cells stimulated with mitogens. Other members of the family include the related yeast protein kinases KSS1 and FUS3, which function in pheromone-induced arrest and mating. A requirement for both phosphorylation sites in FUS3 has been demonstrated genetically in yeast [4].

However, the enzyme(s) that phosphorylate and activate MAP kinase or other members of this family have not been biochemically identified or cloned. Activators of MAP kinase have been most extensively purified from *Xenopus* eggs [5] and from insulin-stimulated skeletal muscle [6]. The *Xenopus* activator was purified 650-fold and contained a principal 45 kDa Coomassie Blue-stained band that correlated with activity during chromatography. The skeletal muscle activator was purified approx. 3000-fold, with principal 50 kDa, 45 kDa and 25 kDa bands. Using phosphorylation of kinase-defective mutants of MAP kinase as a criterion, the MAP kinase activator appears to be a protein kinase with dual specificity for phosphorylating tyrosine and threonine residues [5–8].

To identify which protein(s) in skeletal muscle have MAP kinase activator activity, we have used renaturation assays of individual Coomassie Blue-stained protein bands from SDS/PAGE. Activity was found in two bands of 48 kDa and 46 kDa. Comparison of sequences obtained for three major peptides in the 48 kDa protein suggest strongly that the major protein in this band is closely related to smg p25 GDI, which was originally isolated from bovine brain as an inhibitor of GDP dissociation from a small GTP-binding protein, smg p25 [9]. Sequences of two major peptides from the 46 kDa band have sequence similarity to protein kinases, particularly PBS2 [23], SPK1⁺ [10], STE7 [11] and BYR1 [12], but are distinct from any sequences in the databases.

MATERIALS AND METHODS

Sources and assays

Recombinant wild-type p42^{mapk} and the kinase-defective K52R mutant were prepared as described [13]. MAP kinase activator was assayed by its ability to stimulate the myelin basic protein (MBP) phosphotransferase activity of wild-type p42^{mapk} in a one-stage activation assay or by phosphorylation of the K52R mutant [7]. In each case appropriate control values, from assays omitting p42^{mapk} or K52R, were subtracted. Dilutions were made to confine assays to the linear range. Chromatography columns, instrumentation and media, except DE-52 (Whatman), were from Pharmacia-LKB. The sources of other reagents have been given [7].

Purification of muscle activator

Procedures for purification of the activator are summarized, as the steps used differ substantially from those described by Nakiely *et al.* [6], and were developed independently. Two fed female New Zealand white rabbits were killed by intravenous injection of sodium pentobarbital [6]; the muscle (approx. 1.5 kg) was removed from the hind limbs and back, and chilled on ice. All steps thereafter were performed at 4 °C, using buffer A [14] (50 mM-β-glycerol phosphate, 1.5 mM-EGTA, 1 mM-dithiothreitol, 0.5 mM-sodium orthovanadate, 1 mM-benzamidine, 0.5 mM-phenylmethanesulphonyl fluoride) at the indicated pH. The muscle was ground and homogenized in 2 litres of buffer A (pH 7.4) in a Waring blender. The homogenate was clarified by centrifugation (6000 g, 30 min). The supernatant was filtered through glass wool and batch-adsorbed to 1 litre of DE-52 equilibrated with buffer A (pH 7.4). The filtrate, containing unadsorbed proteins, was recovered and adjusted to pH 8.8 with NaOH. The filtrate was centrifuged (10000 g, 30 min), and batch-adsorbed to 600 ml of Q-Sepharose Fast Flow equilibrated with buffer A (pH 8.8). Buffer A is weakly buffered at pH 8.8, but its use avoided buffer exchanges. The Q-Sepharose was washed on a sintered glass funnel with 3 litres of buffer A (pH 8.8). Activator was eluted with 1.8 litres of buffer A plus 0.4 M-NaCl. The eluate from Q-Sepharose, adjusted to 0.5 M-NaCl (final), was then batch-adsorbed to 500 ml of phenyl-Sepharose, equili-

Abbreviations used: MAP kinase, mitogen-activated protein kinase; p42^{mapk}, 42 kDa MAP kinase; smg p25, small GTP-binding protein p25; GDI, GDP dissociation inhibitor; MBP, myelin basic protein.

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brated with buffer A (pH 8.8) plus 0.5 M-NaCl. This resin was washed successively with buffer A (pH 8.8) with (1 litre) and without (2.5 litres) 0.5 M-NaCl. Activator was eluted with 1 litre of buffer A (pH 8.8) containing 60% (v/v) ethylene glycol.

The eluate from phenyl-Sepharose was applied to a Q-Sepharose column (5 cm internal diam. $\times$ 5 cm), equilibrated in buffer A (pH 8.8). The column was washed (500 ml) and eluted with a 2.4 litre gradient of 0–0.5 M-NaCl in buffer A (pH 8.8). The major peak of MAP kinase activator activity eluting between 75 and 100 mM was pooled, concentrated to 100 ml and dialysed against two changes of 5 litres of buffer A (pH 6.5) overnight. The dialysate was applied to an S-Sepharose column (HR 16/10). The column was washed (100 ml) and activator activity was eluted with a 400 ml gradient of 0–0.3 M-NaCl in buffer A (pH 6.5). Activator eluted as a single major peak between 50 and 110 mM. Pooled activity was concentrated, adjusted to 0.3 M-NaCl and pH 7.4, and applied to an HR5/10 phenyl-Superose column (equilibrated with buffer A, pH 7.4, containing 0.3 M-NaCl), and the flow-through and wash were collected. Active fractions were concentrated in a Centricon-10 (Amicon) to 300 μ l, and applied in 30–40 μ l aliquots to a PC 3.2/30 Superose 12 column in multiple runs. The active fractions were pooled, concentrated to 80 μ l, and rechromatographed as described in Fig. 1 and the text.

Renaturation of MAP kinase activator from SDS/PAGE

Renaturation after SDS/PAGE was as described by Hager & Burgess [15], except for the following modifications. (a) Samples were denatured by heating in SDS sample buffer at 55 °C for 10 min. (b) SDS/10%–PAGE was performed in a conventional slab gel apparatus (Hoeffer), using gels made with ultra-pure reagents, and polymerized for 1–2 days. (c) After electrophoresis, the gel was stained with Coomassie Blue [0.12% (w/v) dye/40% methanol/5% acetic acid] for 15 min, and destained with 40% methanol/5% acetic acid for 10 min, followed by 5% methanol/5% acetic acid for 30 min. (d) Renaturation buffer was buffer A (see above).

Aliquots were assayed for phosphotransferase activity towards the K52R mutant, and for activator activity using wild-type p42^{mapk}. An aliquot of renatured protein was mixed with kinase assay buffer [25 mM-Hepes, pH 7.5, 10 mM-MgCl₂, 1 mM-dithiothreitol, 50 μ M-[γ -³²P]ATP (5000 c.p.m./pmol)] plus K52R (0.55 μ g) or the unphosphorylated form (MKB) of p42^{mapk} (0.3 μ g) in 40 μ l. For phosphorylation of the K52R mutant, incubations were for 60 min (30 °C). For phosphorylation and activation of MKB, the reaction mixtures were first incubated at 30 °C for 45 min, followed by addition of 10 μ l of mix (8 μ g of MBP in kinase assay buffer) and incubation for an additional 30 min. Reactions were stopped with sample buffer and subjected to SDS/PAGE and autoradiography.

Tryptic digestion and mass spectrometry

Excised SDS/PAGE gel bands, containing protein fixed by Coomassie Blue staining in methanol, were washed three times in ultrapure water over 24 h. Each washed gel piece was partially dried in a Speedvac concentrator and rehydrated in a minimum volume of 0.1 M-NH₄HCO₃/1 mM-CaCl₂ containing 10 ng of trypsin/ μ l, followed by digestion at 37 °C for 24 h. The gel piece was cut into small pieces and extracted several times with 150 μ l of 2% acetic acid, twice with 20% acetonitrile, and twice with 30% acetonitrile over 3 days. The combined eluates were dried, resuspended in 3 μ l of acetic acid, and diluted to 60 μ l with water.

Mass spectra were recorded on a TSQ-70 triple quadrupole instrument (Finnigan-MAT, San Jose, CA, U.S.A.). Aliquots from the total digest, dissolved in 5% acetic acid, were injected into the electrospray ionization source from a fused-silica micro-

capillary h.p.l.c. column by gradient elution [16]. The initial analysis provides a map of peptide abundance at a given m/z in the total digest versus elution time. Thereafter, the total digest was fractionated by conventional narrow-bore reverse-phase h.p.l.c. Peaks of 214 nm absorbance were analysed on the TSQ70 triple quadrupole instrument, and selected peptides were sequenced by collision-activated dissociation [16,17] and/or on gas phase sequencer 473A (Applied Biosystems, Foster City, CA, U.S.A.).

RESULTS AND DISCUSSION

Purification of MAP kinase activator from rabbit skeletal muscle

Skeletal muscle extracts from fed rabbits contained factor(s) which potentially stimulated the activity of recombinant wild-type p42^{mapk} (Table 1). Activator was purified approx. 2000-fold using the purification steps outlined in Table 1 (see the Materials and methods section). The first three steps were performed batchwise and were designed to rapidly decrease the total protein to amounts compatible with preparative-scale f.p.l.c. One major peak and two minor peaks of MAP kinase activator were reproducibly observed following Q-Sepharose column chromatography, as previously observed by Nakielnny *et al.* [6]. The principal peak from Q-Sepharose was purified for analysis; the minor peaks were not studied further.

Correlation of activator activity with 45–50 kDa protein bands by Superose 12 chromatography

Results from a run of the final gel filtration step are shown in Fig. 1. Activity eluted in this and other preparations (results not shown) between BSA (68 kDa) and carbonic anhydrase (30 kDa). The peak appeared too broad to be accounted for by a single species, determined by comparisons with peak widths for homogeneous proteins applied under identical conditions (results not shown).

Comparison of the activity data (Fig. 1a) with protein staining (Fig. 1b) was insufficient to unambiguously assign activity to individual bands. A group of 45–50 kDa bands and a doublet of 59–60 kDa bands correlated best with the activity. To resolve these ambiguities, we devised a protocol for renaturing the activator after SDS/PAGE.

Renaturation of MAP kinase activator

Fractions 25–38 from Superose 12 chromatography (Fig. 1) were pooled and concentrated. An aliquot of the pooled activator was analysed after SDS/PAGE (10% gel). The Coomassie Blue-stained gel is shown in Fig. 2(a); the principal bands are numbered for reference. Bands 3, 4, 5, 6 and 7 are the bands which correlated with activity in Fig. 1.

Each numbered band was excised carefully, and processed for renaturation (see the Materials and methods section). The results of assays for K52R phosphotransferase activity (left) and activation of wild-type p42^{mapk} (right) are shown in Fig. 2(b).

MAP kinase activator activity was found in bands 6 and 7, having apparent molecular masses of 48 and 46 kDa respectively. Renatured protein from each band both phosphorylated K52R and activated wild-type p42^{mapk} (Fig. 2). Neither renatured band phosphorylated MBP (results not shown). In additional experiments, an entire lane of gel was divided and analysed; activity was again found only in slices corresponding to 45–48 kDa proteins (results not shown). K52R phosphorylated by either band contained both phosphotyrosine and phosphothreonine (results not shown), as expected from the dual phosphorylation requirement for activation of MAP kinase [1]. This demonstrated the ability of excised and renatured bands to phosphorylate

Table 1. Purification of rabbit skeletal muscle MAP kinase activator

Values are the average of two preparations. Each preparation used two rabbits. One unit of MAP kinase activator activity was defined as the amount of enzyme that would activate the B-form of recombinant MAP kinase, resulting in the phosphorylation of MBP at a rate of 1 nmol/min under standard assay conditions.

Purification step	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Purification (fold)	Yield (%)
Extract	1142	37243	0.031	1	100
DEAE-cellulose	1286	25462	0.051	2	110
Q-Sepharose (batch)	1599	1390	1.15	37	140
Phenyl-Sepharose	642	445	1.44	46	56.2
Q-Sepharose (column)	90	96	0.94	30	7.9
S-Sepharose	22	0.46	47.83	1593	1.9
Phenyl-Superose	21	0.42	50.00	1613	1.8
Superose 12	4.2	0.063	66.67	2151	0.37

Fig. 1. Superose 12 chromatography of MAP kinase activator
The concentrated pool of activator (80 μ l) was chromatographed in equal aliquots in three runs on a 3.2/30 Superose 12 column equilibrated with buffer A (pH 7.4)/0.1 M-NaCl/0.02 % Brij 35. The flow rate was 40 μ l/min, and 20 μ l fractions were collected; runs were pooled by collection into the same tubes. (a) MAP kinase activator activity assayed by activation of p42^{mapk}. (b) Aliquots (3 μ l) of the indicated fractions were analysed by SDS/10 %-PAGE and silver staining.

MAP kinase on the regulatory tyrosine and threonine sites, and is the strongest evidence so far obtained for activation of MAP kinase by a dual-specificity kinase.

Because the active bands are closely spaced, the conclusion that two distinct bands are active depends on our ability to excise them without cross-contamination. All that we conclude at this juncture is that, in multiple experiments wherein bands 6 and 7 were sufficiently separated, activity was present in both bands. Thus we feel that cross-contamination cannot explain our findings, but we do not exclude this as a possibility.

Fig. 2. Renaturation of MAP kinase activator after SDS/PAGE
Fractions 25–38 (Fig. 1) were pooled and concentrated to 120 μ l. An aliquot (16 μ l; 11 μ g of total protein) was subjected to SDS/PAGE on a 10 % gel, and processed for renaturation of individual bands (see the Materials and methods section). (a) Gel after Coomassie Blue staining, major bands appearing are numbered for discussion. (b) Activity after renaturation of indicated bands (from a) by phosphorylation of K52R (left) and by activation of p42^{mapk} (right). Lanes 0 are controls without renatured sample. Denatured BSA is also phosphorylated by the activated MAP kinase, even though native BSA is not a good substrate.

No activity was detected in any other band. In particular, no activity was detected in bands 3 and 4. These two bands undergo endogenous phosphorylation and are labelled when partially purified activator is incubated with [γ -³²P]ATP/Mg (results not

Fig. 3. Sequence similarity of peptides from band 6 to smg p25 GDI and from band 7 to protein kinases

Peptide sequences were searched against GenBank (release 71), PIR (release 31) and SWISS-Prot (release 21) databases using the FASTA algorithm [18]. Shaded areas indicate identity; numbers in parentheses indicate residue position from the polypeptide *N*-terminus. (a) Sequences of three tryptic peptides from band 6 (Pep6-1, -2 and -3) aligned with smg p25 GDI. PEP6-2 did not terminate in K/R and may be the *C*-terminus; X indicates this ambiguity. (b) Sequences of two peptides from band 7 (PEP7-1 and -2) aligned with sub-domains II and VI of the two protein kinases with the highest scores for PEP7-1 (PBS2 and SPK1⁺) and the two protein kinases with the highest scores for PEP7-2 (STE7 and BYR1).

shown). The absence of activity in other bands indicates that the renaturation assay is specific. However, this approach cannot exclude the possibility that additional bands contain activity, but are not renaturable by the procedures used.

Mass spectrometry and partial sequence analysis

The remainder of the pooled activator from Superose 12 was subjected to preparative electrophoresis for structural analysis. The gel was stained and the bands corresponding to 3, 4, 5, 6 and 7 were excised (results not shown). A small piece of each band was also analysed for activity after renaturation, with result: identical to those obtained analytically (results not shown). Each protein band was digested with trypsin, and aliquots of the total digests were analysed for peptides by mass spectrometry, using on-line micro-capillary reverse-phase h.p.l.c. and electrospray ionization. Comparison of the major peptides present showed that the proteins in bands 6 and 7 were distinct, and not derived from bands 3, 4 or 5 (results not shown). Sequences of three major peptides (PEP6-1, -2 and -3) from band 6 and of two major peptides from band 7 (PEP7-1 and -2) were determined (Fig. 3). These sequences were compared with current protein sequence databases using the FASTA algorithm [18].

The major protein in band 6 was found to have significant sequence similarity to a bovine protein, smg p25 GDI [9] (Fig. 3a). GDI, a protein of 447 amino acids, was identified as a factor in bovine brain which inhibited the dissociation of GDP from smg p25, a member of the ras superfamily of small GTP-binding proteins. An 11-amino-acid peptide (GRDWNVDLIPK) was an exact match, and two other peptides had significant identities as indicated. Furthermore, these sequence similarities occurred at both the *N*-terminus and the *C*-terminus of GDI. The comparison strongly suggests that the major protein in band 6 is a GDI-related protein. Since smg p25 GDI is not thought to be a protein kinase, it is possible that the activator activity detected in band 6 is due to a minor fraction of the sequenced kinase in band 7 co-migrating with the GDI-related protein (see below).

It must be stated that authentic smg p25 GDI does not interact with p21^{ras} [19], and inhibition of GDP dissociation should result in inhibition of ras activation, based on current knowledge. However, given that p21^{ras} is likely to function for activation at some point upstream of MAP kinase [20], the presence of a co-

purifying factor with a sequence related to proteins interacting with small GTP-binding proteins may be significant.

A search of the protein sequence databases with peptide sequences from band 7 revealed significant similarity to protein kinase catalytic domains [21,22], but did not match exactly any known protein sequences (Fig. 3). PEP7-1 and PEP7-2 are predicted to derive from conserved protein kinase sub-domains II and VI respectively. PEP7-1 is most similar to sub-domain II of SPK1⁺ (58% identity) [10] and PBS2 (50% identity) [23]. PEP7-2 is most similar to sub-domain VI of STE7 [11] and BYR1 [12], with 67% and 75% identity respectively. PBS2 and SPK1⁺ were found as genes conferring resistance to polymyxin B and staurosporine respectively [23,10]. Polymyxin B and staurosporine are inhibitors of protein kinase C. SPK1⁺, BYR1 and STE7 have all been directly implicated in cell cycle control. SPK1⁺ is a 45 kDa protein kinase which has sequence similarity to KSS1 and FUS3, and is required for conjugation in yeast [10]. BYR1 was found as a gene which can restore some downstream functions of RAS in yeast [13]. STE7 appears to function upstream from FUS3 in the conjugation pathway and is a candidate for phosphorylating and activating FUS3 [4,12]. Since both p42^{mapk} and FUS3 require dual phosphorylation at Thr-Glu-Tyr for activity [1,4], it is reasonable to expect the MAP kinase activator to be related to STE7.

Sub-domain II contains a conserved alanine and the lysine required for catalysis, two residues *C*-terminal from the alanine [21]. The conserved alanine is present in PEP7-1; the lysine (assumed) is missing because an arginine terminates the tryptic peptide. Sub-domain VI contains residues between the conserved Asp and Asn which have been correlated with substrate specificity of the serine/threonine and tyrosine kinases [21,22]. DLKPEN and DLAARN have been found most frequently in serine/threonine and tyrosine kinases respectively [22]. Almost all putative dual-specificity kinases have a small uncharged residue replacing E or R [22], for example DLKPAN in weel and DVKPSN in mik1, a functional homologue of weel in *Saccharomyces pombe*. Significantly, the peptide sequence obtained for PEP7-2 (DVKPSN) matches sequences from putative dual-specificity kinases [22].

The results of the present study provide the best evidence so far obtained that the MAP kinase activator is a dual-specificity protein kinase. Closely spaced 48 and 46 kDa bands present in an activator preparation from skeletal muscle phosphorylate a kinase-defective MAP kinase mutant on tyrosine and threonine, and also activate wild-type MAP kinase. The results argue against contributions from any proteins differing substantially in SDS/PAGE mobility. Sequencing of the major proteins in the 48 and 46 kDa bands identified a GDI-related protein and a putative protein kinase respectively. A partial sequence of the protein kinase is consistent with it being capable of phosphorylating threonine and tyrosine residues. It is important to note that, although PEP7-1 and PEP7-2 have some sequence similarity to SPK1⁺ [and to a lesser extent, to KSS1 and FUS3 as well (results not shown)], the sequenced kinase is not a MAP kinase. Purified activator has no MBP kinase activity, and its chromatographic behaviour is distinct from that of MAP kinase (results not shown). We cannot exclude the possibility that MAP kinase activator activity present with the GDI-related protein is also due to the sequenced protein kinase, either as cross-contamination or as a result of retardation of a fraction of the kinase by additional phosphorylation(s).

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