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# Identification of MAPKAP kinase 2 as a major enzyme responsible for the phosphorylation of the small mammalian heat shock proteins

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MAP kinase-activated protein kinase-2 (MAPKAP kinase-2) phosphorylates the serine residues in murine heat shock protein 25 (hsp 25) and human heat shock protein 27 (hsp27) which are phosphorylated in vivo in response to growth factors and heat shock, namely $Ser^{15}$ and $Ser^{86}$ (hsp25) and $Ser^{15}$, $Ser^{78}$ and $Ser^{82}$ (hsp27). $Ser^{86}$ of hsp25 and the equivalent residue in hsp27 ($Ser^{82}$) are phosphorylated preferentially in vitro. The small heat shock protein is present in rabbit skeletal muscle and hsp25 kinase activity in skeletal muscle extracts co-purifies with MAPKAP kinase-2 activity throughout the purification of the latter enzyme. These results suggest that MAPKAP kinase-2 is the enzyme responsible for the phosphorylation of these small heat shock proteins in mammalian cells.

MAP kinase; Protein kinase; Heat shock; Protein phosphorylation; Growth factor

## 1. INTRODUCTION

We have recently identified a new protein kinase in rabbit skeletal muscle which is only active after it has been phosphorylated on a unique threonine residue by mitogen-activated protein kinase (MAP kinase) [1]. This enzyme, which has been termed MAP kinase activated protein kinase-2 (MAPKAP kinase-2) can be distinguished from S6 kinase-II (or MAPKAP kinase-1) [2], the only other protein kinase known to be activated by MAP kinase, by its response to inhibitors, failure to phosphorylate peptides related to the C-terminus of ribosomal protein S6 and by its amino acid sequence [1]. MAPKAP kinase-2 was originally identified by its ability to phosphorylate rabbit skeletal muscle glycogen synthase, which it labels preferentially on a serine located seven residues from the N-terminus. It also phosphorylates the first serine in the peptide KKPLNRTLSVASLPGLamide, which is related to the N-terminus of glycogen synthase, and this substrate is used to assay MAPKAP kinase-2 routinely [1].

Although glycogen synthase was the first substrate for MAPKAP kinase-2 to be identified, it is not clear whether it is phosphorylated by this protein kinase in vivo. Furthermore, since MAPKAP kinase-1 may phosphorylate more than one substrate in vivo (e.g. the glycogen-binding subunit of protein phosphatase-1 [3] and ribosomal protein S6 [4]), MAPKAP kinase-2 may also have a number of physiological substrates and thereby mediate several actions of extracellular signals which exert their effects through the activation of MAP kinase and its downstream targets. We were therefore interested in identifying potential physiological substrates for MAPKAP kinase-2.

Murine heat shock protein 25 (hsp25) and its human homologue heat shock protein 27 (hsp27) are small thermostable proteins present in almost all mammalian cells, which become phosphorylated in many cells in response to signals such as tumour necrosis factor (TNF) [5,6], interleukin-1 [6,7], platelet derived growth factor (PDGF) [7] and fibroblast growth factor (FGF) [7,8], as well as tumour-promoting phorbol esters [9,10] and heat shock [11–13]. Their physiological roles are unknown, although overexpression of hsp27 and hsp25 has been reported to increase the thermotolerance of some mammalian cells [14,15] and to inhibit cell proliferation [15]. Our interest in examining whether hsp25 and hsp27 were physiological substrates for MAPKAP kinase-2 was aroused by two observations. Firstly, several of the stimuli which trigger the phosphorylation of hsp25 and hsp27 in cells, such as PDGF, phorbol esters and heat shock, are known to activate MAP kinase [16,17]. Secondly, the amino acid sequences surrounding one of the in vivo phosphorylation sites in hopes (LNRQLSSG) [18] or hsp27 (LSRQLSSG) [8] are very similar to the sequence surrounding serine-7 of glycogen synthase in human (LNRTLSVS) [19] or rabbit (LSRTLSVS) [20] skeletal muscle. In this paper we demonstrate that MAPKAP kinase-2 (but not MAPKAP kinase-1) phosphorylates each of the serine residues in

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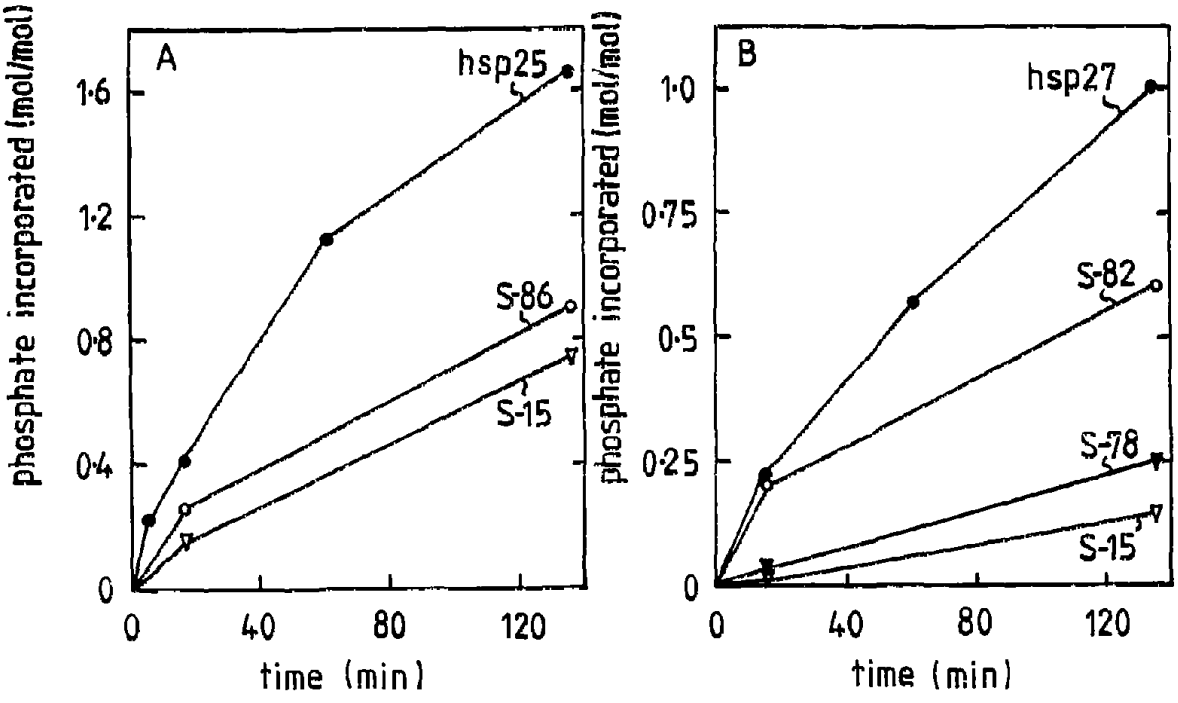


Fig. 1. Phosphorylation of hsp25 (A) and hsp27 (B) by MAPKAP kinase-2. Phosphorylation of hsp25 and hsp27 (50 $\mu$M) was carried out with 2 U/ml MAPKAP kinase-2 as described in Section 2. Calculation of phosphorylation stoichiometries was based on molecular masses of 25 kDa (hsp25) and 27 kDa (hsp27) and protein concentrations measured according to Bradford [30]. Values obtained by the Bradford procedure agreed with those obtained by amino acid analysis to ± 10%. Phosphate incorporated into hsp25 or hsp27 is denoted by the closed circles. Phosphorylation of $Ser^{15}$ (S-15, ▽) and $Ser^{86}$ (S-86, ○) of hsp25 and $Ser^{15}$, $Ser^{78}$ (S-78, ▼▼) and $Ser^{82}$ (S-82, ○) of hsp27 was determined by measurement of the $^{32}$P-radioactivity associated with the tryptic peptides containing each of these residues (Figs. 2 and 4).

hsp25 and hsp27 that are phosphorylated in vivo in response to mitogens or heat shock, that hsp25 kinase activity co-purifies with MAPKAP kinase-2 throughout the purification of the latter enzyme and that MAPKAP kinase-2 and the substrate small heat shock protein are co-expressed in rabbit skeletal muscle. These results strongly suggests that MAPKAP kinase-2 is an enzyme responsible for phosphorylating hsp25 and hsp27 in vivo.

## 2. MATERIALS AND METHODS

MAPKAP kinase-2 [1], recombinant murine hsp25 [21] and recombinant hsp27 [22] were purified as described. MAPKAP kinase-1 (Mr. C. Sutherland) [23] and the catalytic subunit of protein phosphatase 2A (Dr. D. Schelling) [24] were purified from rabbit skeletal muscle at Dundee by the investigators in parentheses. Calmodulin-dependent protein kinase-II from rat brain was provided by Dr. Angus Nairn (Rockefeller University, New York, USA). Phosphorylation of the standard peptide substrate KKPLNRTLSVASLPGLamide by MAPKAP kinase-2 and other protein kinases was carried out in the presence of EGTA, the specific peptide inhibitor of cyclic AMP-dependent protein kinase and the protein kinase inhibitor H7 (which does not inhibit MAPKAP kinase-2) as described [1] and one unit of activity (U) was that amount which catalysed the phosphorylation of one nmol of peptide in one min. When hsp25 and hsp27 were used as substrates, reactions were terminated by addition of 1.0 ml of 5% (w/v) trichloroacetic acid. After standing for 5 min, the suspensions were centrifuged for 2 min at 13,000 × $g$ and the supernatants discarded. The pellets were washed three times with 25% (w/v) trichloroacetic acid and analysed by Cerenkov counting. Phosphorylation of the small heat shock protein by purified MAPKAP kinase-2 was carried out in rabbit muscle extracts heated at 42°C for 10 min to inactivate protein kinases. The incubations were carried out at 30°C with 5 mM $MgCl_2$/0.1 mM [$\gamma$-$^{32}$P]ATP (0.1 Ci/mmol), with and without 0.5 U/ml MAPKAP kinase-2, and after 30 min the small heat shock protein was immunoprecipitated with 10 $\mu$l of an antiserum against an hsp25/hsp27 hybrid protein which cross-reacts between different mammalian species [25]. After secondary precipitation using Protein A-Sepharose (Pharmacia), proteins bound specifically were eluted by heating at 100°C in the presence of SDS, subjected to SDS/polyacrylamide gel electrophoresis and autoradiographed using a Bio Imaging Analyser BAS 2000 (Fuji). Amino acid analysis was carried out using a Waters PICOTAG System. Other materials and methods are detailed in [1].

## 3. RESULTS

### 3.1. *Phosphorylation of hsp25 and hsp27 by MAPKAP kinase-2*

hsp25 and hsp 27 were both phosphorylated by MAPKAP kinase-2 at similar rates to the standard peptide substrate KKPLNRTLSVASLPGLamide. The initial rate of phosphorylation of hsp25 (20 $\mu$M) was 55% of the rate at which the peptide substrate (30 $\mu$M) was phosphorylated. The $K_m$ for hsp25 phosphorylation (19 $\mu$M) was also similar to that of the peptide substrate (12

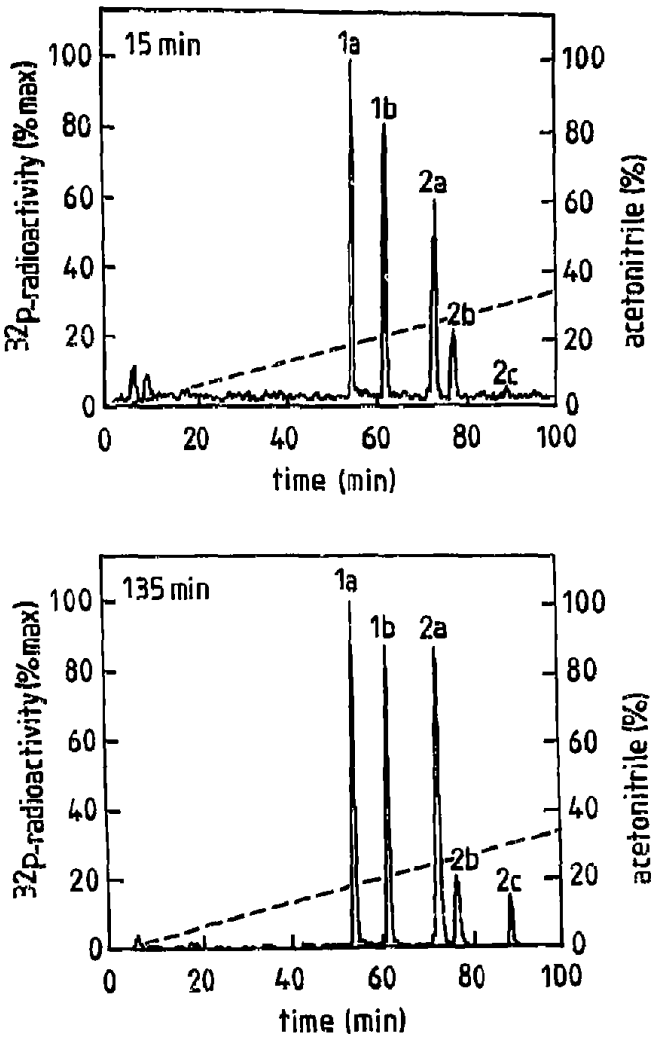


Fig. 2. Separation of $^{32}$P-labelled tryptic peptides from hsp25 by chromatography on a $C_{18}$ column. The trichloroacetic acid precipitated hsp25 from the 15 min (upper trace) and 135 min (lower trace) time points in Fig. 1A (2.5 nmol) were digested with trypsin (2 $\mu$g, 0.08 nmol) and chromatographed on a Vydac $C_{18}$ reverse phase HPLC column (Separations Group, Hesperia, California, USA) equilibrated in 0.1% (v/v) trifluoroacetic acid, as described previously [1]. $^{32}$P-radioactivity (full line) was recorded continuously with an on-line monitor. The acetonitrile gradient is shown by the broken line. The analysis of peptides 1a and 1b (containing Ser$^{86}$), and 2a, 2b and 2c (containing Ser$^{15}$) is presented in Section 3.2.

$\mu$M). hsp27 was phosphorylated at about half the rate of hsp25 (Fig. 1). The phosphorylation of hsp25 reached 1.7 mol/mol and the phosphorylation of hsp27 1.0 mol/mol after incubation for 2 h with 2 U/ml MAPKAP kinase-2. In contrast, MAPKAP kinase-1 or calmodulin-dependent protein kinase-II (data not shown), which both phosphorylate the peptide KKPLNRTLSVASLPGLamide efficiently, were unable to phosphorylate hsp25 or hsp27 at all when added at the same concentration (2 U/ml).

### 3.2. *Identification of the residues in hsp25 and hsp27 phosphorylated by MAPKAP kinase-2*

hsp25 phosphorylated by MAPKAP kinase-2 was digested with trypsin and three major $^{32}$P-labelled peptides were resolved by chromatography on a $C_{18}$ column (Fig. 2). Peptides 1a and 1b had identical amino acid compositions and the sequence of peptide 1a was QLSSGVSEIR. The first serine residue in this peptide, which corresponds to Ser$^{86}$ in hsp25, was identified as the site of phosphorylation (Fig. 3A). Peptide 1b did not yield any peptide sequence, indicating that its amino-terminus was blocked, presumably by cyclisation of the amino-terminal glutaminyl residue to a pyroglutaminyl residue. Its elution at a slightly higher concentration of acetonitrile than peptide 1a (Fig. 2) is consistent with the loss of a positively charged amino group at the N-terminus, while peptide 1a could be partially converted to peptide 1b by incubation at ambient temperature in 1 M acetic acid (data not shown). Peptide 2a had the sequence SPSWEPFR and the second serine residue (Fig. 3), which corresponds to Ser$^{15}$ in hsp25, was identified as the site of phosphorylation. Peptide 2b had the same amino acid sequence as peptide 2a. The reason for its elution at a slightly higher concentration of acetonitrile is unclear, but it could either result from some modification of the tryptophan residue or isomerisation of one of the proline residues. Peptide 2c had the sequence SPSWEPFRDWYPAHSR (data not shown) and resulted from incomplete cleavage of an Arg–Asp peptide bond. The relative rates of phosphorylation of Ser$^{15}$ and Ser$^{86}$ were calculated from the $^{32}$P-radioactivity associated with each tryptic phosphopeptide, and showed that Ser$^{86}$ was labelled preferentially (Fig. 1A). When $^{32}$P-labelled hsp 27 containing 1.0 mol phosphate/mol protein was digested with trypsin, four major $^{32}$P-peptides were resolved on the $C_{18}$ column (Fig. 4, lower trace). The peptides termed 1a and 1b eluted in the same positions as peptides 1a and 1b from the hsp 25 digest and their amino acid compositions (data not shown) and sequence (Fig. 3) demonstrated that they also corresponded to the unblocked and blocked forms of the peptide QLSSGVSEIR, these peptide sequences being conserved between hsp25 and hsp27. The first serine (Ser$^{82}$ in hsp27) was identified as the site of phosphorylation (Fig. 3). Peptide 2a in the hsp27 digest had the sequence GPSWDPFR and the serine residue, which corresponds to Ser$^{15}$ of hsp27, was the site of phosphorylation (Fig. 3). The sequence of peptide 2a in the hsp27 digest shows two conservative replacements from the corresponding peptide in the hsp25 digest. Peptide 3 was a tetrapeptide with the sequence ALSR, the serine residue (corresponding to Ser$^{78}$ of hsp27) being the site of phosphorylation (Fig. 3). This serine residue is replaced by asparagine in hsp25, explaining why no $^{32}$P-peptide corresponding to peptide 3 is present in the tryptic digest of hsp25.

Quantitative analysis of the $^{32}$P-labelled tryptic pep-

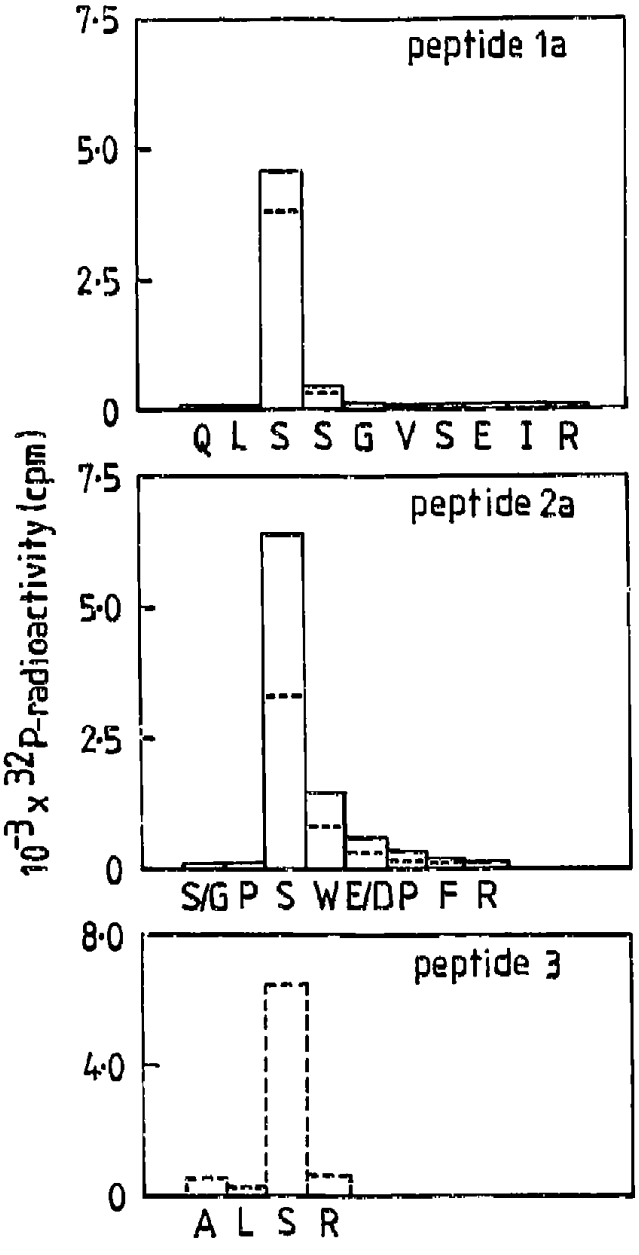


Fig. 3. Identification of the serine residues in hsp25 and hsp27 phosphorylated by MAPKAP kinase-2. The $^{32}$P-labelled peptides in Figs. 2 and 4 were coupled covalently to an arylamine membrane and subjected to solid phase sequencing on an Applied Biosystems 470A/120A sequencer [1]. Conventional gas phase sequencing was also used to confirm the assignment of amino acid residues in the hsp25 peptides. The figure shows $^{32}$P-radioactivity released and the amino acid residue identified (single letter code) after each cycle of Edman degradation. The full lines show the results obtained with peptides from hsp25 and the broken line results from the corresponding peptides of hsp27. In peptide 2a, the N-terminal residue is serine in hsp25 and glycine in hsp27, and the fifth residue is glutamic acid in hsp25 and aspartic acid in hsp27. $^{32}$P-radioactivity (cpm) applied to sequencer was: 8,000 (peptide 1a, hsp25); 8,500 (peptide 1a, hsp27); 13,000 (peptide 2a, hsp25); 8,000 (peptide 2a, hsp27); 17,000 (peptide 3, hsp27).

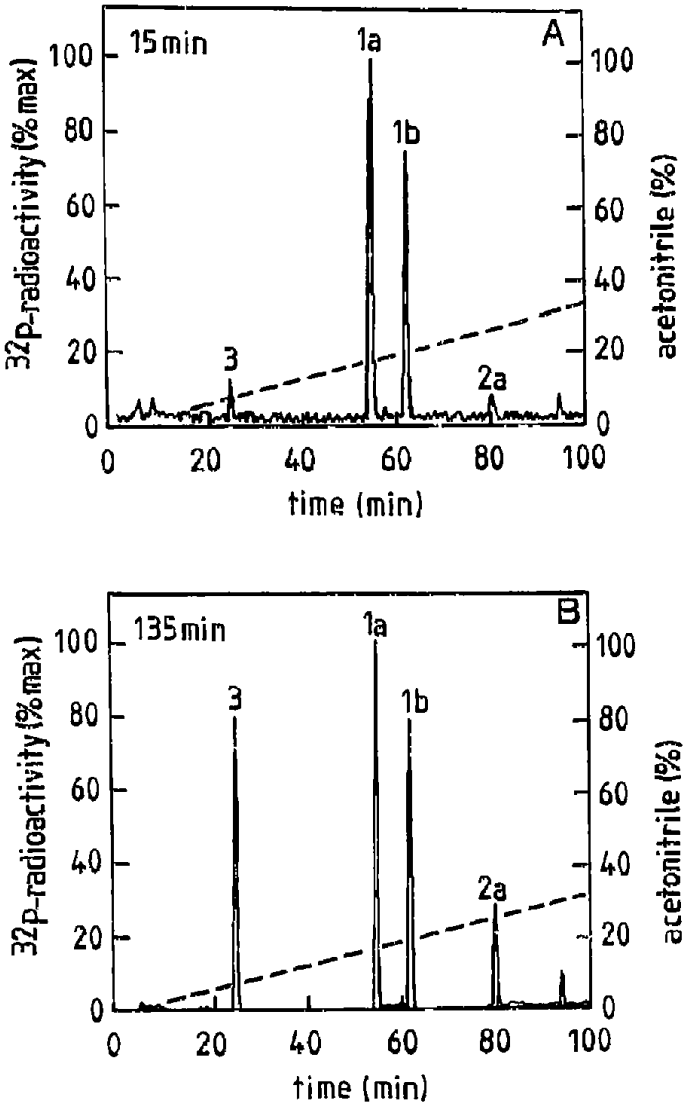


Fig. 4. Separation of $^{32}$P-labelled tryptic peptides from hsp27 by chromatography on a $C_{18}$ column. The experiment was carried out in an identical manner to that described in Fig. 2 using the 15 min (A) and 135 min (B) time points from the hsp27 phosphorylation reaction (Fig. 1B).

tides at various times of phosphorylation revealed that Ser$^{82}$ in hsp27 is phosphorylated much faster than either Ser$^{15}$ or Ser$^{78}$ (Fig. 1B). For example, after 15 min when phosphorylation was only 0.2 mol/mol, about 90% of the phosphate was present in Ser$^{82}$, 5% in Ser$^{15}$ and 5% in Ser$^{78}$ (Figs. 1B and 4A).

### 3.3. *Co-purification of hsp25 kinase activity with MAPKAP kinase-2*

We have developed a method for purifying MAPKAP kinase-2 to homogeneity from skeletal muscle by which the enzyme is purified 85,000-fold within 3 days [1]. The procedure involves batchwise chromatography on CM-Sephadex, precipitation at 45% ammonium sulphate, chromatography on DEAE-cellulose, successive gradient elutions from S-Sepharose, Mono S and Mono Q and gel-filtration on Superose 12. The purified enzyme contains two components whose apparent molecular masses estimated by SDS/poly-

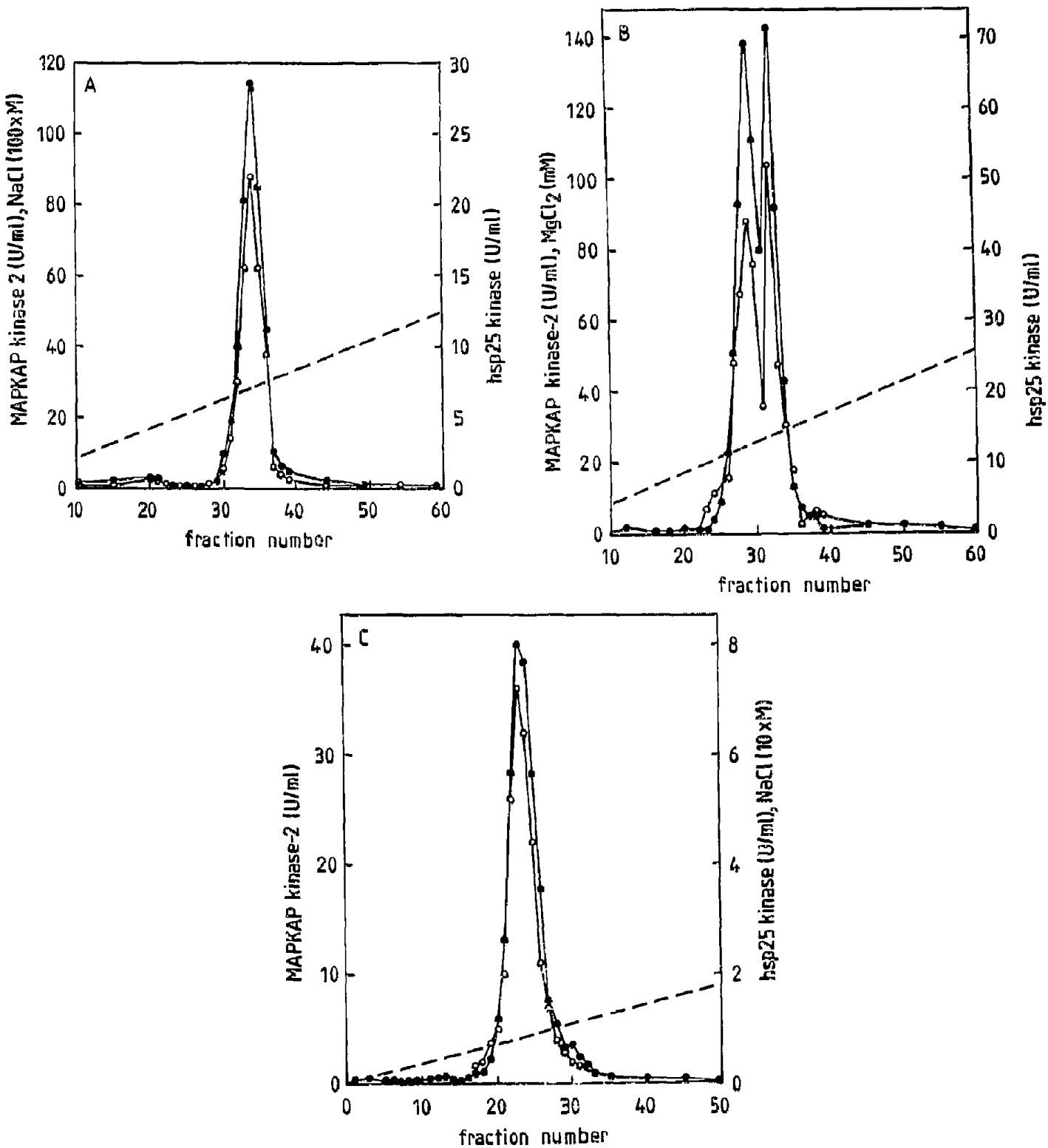


Fig. 5. Co-purification of hsp25 kinase activity with MAPKAP kinase-2 on S-Sepharose, Mono S and Mono Q. MAPKAP kinase-2 was assayed using the peptide KKPLNRTLSVASLPGLamide at 30 $\mu$M (closed circles) and hsp25 kinase using hsp25 at 6 $\mu$M (open circles). The broken lines denote the salt gradients. (A) The flowthrough fractions from DEAE Cellulose were applied to a column of S-Sepharose (10 × 1.6 cm) equilibrated at ambient temperature in 20 mM MOPS, pH 7.0, 1 mM EDTA, 5% (v/v) glycerol, 0.01% (w/v) Brij 35, 1 mM benzamidine and 0.1% (v/v) 2-mercaptoethanol (Buffer A). After washing with equilibration buffer until the absorbance at 280 nm was < 0.02, the column was developed with a 200 ml linear salt gradient to 0.5 M NaCl in the same buffer. The flow rate was 3 ml/min and fractions of 2.5 ml were collected. (B) The enzyme from S-Sepharose was dialysed for 90 min into Buffer A containing 50% (v/v) glycerol, then diluted 4-fold in Buffer A and applied to a Mono S column (5 × 0.5 cm) equilibrated at ambient temperature in Buffer A. After washing with buffer until the absorbance at 280 nm was < 0.02, the column was developed with a 40 ml linear salt gradient to 0.1 M $MgCl_2$ in Buffer A. The flow rate was 1.0 ml/min and fractions of 0.5 ml were collected. (C) The enzyme from Mono S was dialysed against Buffer A and applied to a column of Mono Q (5 × 0.5 cm) equilibrated in Buffer A. After washing with 10 ml of buffer A, the column was developed with a 40 ml linear salt gradient to 0.3 M NaCl. The flow rate was 1.0 ml/min and fractions of 0.5 ml were collected. Further details of the preparation are given in [1].

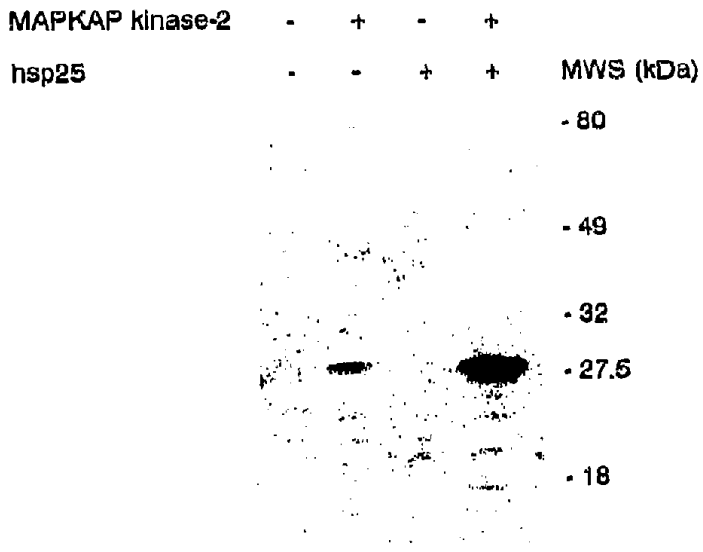


Fig. 6. Expression of the small heat shock protein in rabbit skeletal muscle and its phosphorylation by MAPKAP kinase-2. Heat-inactivated extracts of rabbit skeletal muscle were incubated in the presence of Mg[$\gamma$-$^{32}$P]ATP with and without the addition of MAPKAP kinase-2 (Section 2). In two further reactions, purified murine hsp25 was added to serve as an internal marker protein. After phosphorylation, the small heat shock proteins were immunoprecipitated with a specific antibody and the immunoprecipitates analysed by SDS/polyacrylamide gel electrophoresis. The figure shows an autoradiograph of the gel and molecular weight standards (MWS) are marked.

acrylamide gel electrophoresis are 60 kDa and 53 kDa, respectively. These species have identical specific activities and the amino acid sequences of six peptides isolated from each component were found to be identical, indicating that that they are either closely related isozymes or derived from the same gene [1].

hsp25 kinase activity co-purified with MAPKAP kinase-2 activity through batchwise chromatography on CM-Sephadex and precipitation with ammonium sulphate, and like MAPKAP kinase-2 activity, all the hsp25 kinase activity did not bind to DEAE-cellulose equilibrated in 5 mM MOPS, pH 7.0 (data not shown). Like MAPKAP kinase-2 [1], the hsp25 kinase activity in the DEAE-cellulose eluate (and at all subsequent steps) was completely inactivated by preincubation for 30 min with 5 mU/ml protein phosphatase 2A (see [24] for definition of units). After chromatography on DEAE-cellulose, hsp25 kinase activity exactly co-purified with MAPKAP kinase-2 through successive chromatographies on S-Sepharose, Mono S and Mono Q (Fig. 5). The partial resolution of MAPKAP kinase-2 and hsp25 kinase activity into two components on Mono S (Fig. 5) is only observed occasionally and may reflect some separation of the 60 kDa and 53 kDa forms of the enzyme. When assayed with the standard peptide substrate at 30 $\mu$M and hsp25 at 20 $\mu$M, the activity ratio MAPKAP kinase-2/hsp25 kinase was 1.6 (after S-Sepharose), 1.6 (after Mono S) and 1.7 (after Mono Q), which compares to 1.8 in the muscle extract, 2.2 in the CM-Sephadex eluate and 1.7 for homogeneous preparations of MAPKAP kinase-2 purified through the final gel-filtration step.

About 30% of the protein kinase activity in muscle extracts measured with either hsp25 or the standard peptide substrate was not retained by CM-Sephadex. The peptide kinase activity not retained by this cationic exchange resin is distinct from MAPKAP kinase-2, because it is eluted from Mono Q at a far higher concentration of NaCl (0.4 M) and is not inactivated by protein phosphatase-2A. This protein kinase is devoid of hsp25 kinase activity and indeed no protein kinase with significant activity towards hsp25 was detected when the Mono Q fractions were assayed with this substrate. It is possible that the hsp25 kinase activity in the CM-Sephadex flowthrough fractions is the summation of many protein kinases each of which has trace hsp25 kinase activity.

Although small heat shock proteins have been identified in a wide variety of mammalian tissues, they had not been identified previously in rabbit skeletal muscle from which MAPKAP kinase-2 is purified. In order to demonstrate the biological relevance of the phosphorylation reaction, it was therefore important to show that this small shock protein was present in skeletal muscle and that it could be phosphorylated by MAPKAP kinase-2. Heat-treated muscle extracts were therefore incubated with MAPKAP kinase-2 and Mg[$\gamma$-$^{32}$P]ATP and subjected to immunoprecipitation using an antiserum against an hsp25/hsp27 hybrid protein [25] as described in Section 2. Analysis by SDS/polyacrylamide gel electrophoresis clearly revealed that a single phosphoprotein had been immunoprecipitated from the extracts which co-migrated with hsp25 (Fig. 6).

## 4. DISCUSSION

In this paper we have demonstrated that the small mammalian heat shock proteins are phosphorylated by MAPKAP kinase-2 at similar rates to the synthetic peptide KKPLNRTLSVASLPGLamide which is the standard substrate used to assay this enzyme. Since the $V_{max}$ towards this peptide (about 5 $\mu$mols/min/mg) is comparable to that of other protein serine/threonine kinases towards their best substrates, hsp25 and hsp27 are clearly good substrates for MAPKAP kinase-2. The high degree of specificity of MAPKAP kinase-2 for these small heat shock proteins is emphasized by the failure of two other protein kinases (MAPKAP kinase-1 [26] and calmodulin-dependent protein kinase-II [27], that phosphorylate Arg-Xaa-Xaa-Ser motifs, to phosphorylate hsp25 or hsp27 at all. The importance of MAPKAP kinase-2 in the phosphorylation of hsp25 and hsp27 in vivo is supported by three further findings. Firstly, under our assay conditions, hsp25 kinase activity and MAPKAP kinase-2 activity co-purify throughout the isolation procedure for the latter enzyme. Sec-

ondly MAPKAP kinase-2 and the substrate small heat shock protein are co-expressed in rabbit skeletal muscle. Thirdly, the residues on hsp25 and hsp27 phosphorylated by MAPKAP kinase-2 in vitro are the same as those reported to be phosphorylated in intact cells. In mouse Ehrlich ascites tumour cells $Ser^{86}$ was shown to be the major site of in vivo phosphorylation and $Ser^{15}$ the minor site [18]. In chinese hamster ovary cells, human HeLa cells and human mammary tumour cell line MCF-7, the major site of phosphorylation was $Ser^{82}$ while $Ser^{78}$ was phosphorylated to a lesser extent. More minor phosphorylation of $Ser^{15}$ also appeared to take place, although this was not established definitively [8]. The relative amounts of phosphate present in each site in vivo are consistent with their relative rates of phosphorylation by MAPKAP kinase-2 in vitro. It is well established that certain heat shock conditions increase the phosphorylation of the small heat shock proteins in vivo [11–13] and our results now suggest that this is explained by increased activity of MAPKAP kinase-2 which is itself activated by the heat shock-inducible MAP kinase activity [17].

Under our assay conditions cyclic AMP-dependent protein kinase is inhibited by the inclusion of its specific peptide inhibitor and calcium-dependent protein kinases (such as protein kinase C) by the inclusion of EGTA. Cyclic AMP-dependent protein kinase and protein kinase C are both capable of phosphorylating hsp25 in vitro at $Ser^{15}$ and $Ser^{86}$, albeit with low efficiency [18], but it is unlikely that either enzyme phosphorylates hsp25 in vivo for several reasons. Firstly, cyclic AMP-elevating agonists do not appear to stimulate the phosphorylation of hsp25 [7]. Secondly, depletion of protein kinase C by prolonged treatment of cells with phorbol myristate acetate has no effect on the phosphorylation of hsp25 induced by tumour necrosis factor or interleukin-1 [7].,

It has recently been shown that the small heat shock proteins have chaperone-like properties in vitro [22]. As also found for hsp90 [28], these chaperone-like properties are not influenced by the addition of ATP and the release of proteins bound to the heat shock proteins is not accompanied by ATP depletion. It will, however, clearly be of interest to examine whether the chaperone-like properties of these proteins are regulated by their degree of phosphorylation. Since phosphorylation of the small heat shock proteins is catalysed by MAPKAP kinase-2 and one of the enzymes which is capable of dephosphorylating these proteins is the calcium-dependent protein phosphatase 2B [29], this raises the intriguing possibility that intracellular chaperoning is regulated by at least two cellular signal transduction systems.

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