Phosphorylation of ribosomal protein S6 and a peptide analogue of S6 by a protease-activated kinase isolated from rat liver

Brian Gabrielli, Richard E.H. Wettenhall+, Bruce E. Kemp*, Michael Quinn and Lily Bizonova

Department of Biochemistry, La Trobe University, Bundoora, Victoria 3083, and **Department of Medicine, University of Melbourne, Repatriation General Hospital, Heidelberg, Victoria 3081, Australia*

Received 17 July 1984

A trypsin-activated protein kinase has been isolated from rat liver using a peptide analogue of ribosomal protein S6 as a substrate in kinase assays. The structure of the peptide, Arg-Arg-Leu-Ser-Ser-Leu-Arg-Ala, was based on a region of S6 containing both an insulin- and cyclic AMP-regulated phosphorylation site. The trypsin-activated protein kinase phosphorylated a corresponding site in the peptide analogue and ribosomal protein S6 that was distinct from the preferred site for cyclic AMP-dependent protein kinase. Ribosomal S6 contained at least one other major site for the trypsin-activated protein kinase.

Ribosomal protein S6 *Synthetic peptide analogue* *Protease-activated protein kinase* *Rat liver*

1. INTRODUCTION

A variety of polypeptide hormones and growth factors promote the phosphorylation of 40 S ribosomal protein S6 at multiple sites in mammalian cells [1–10]. There is appreciable overlap in the sites phosphorylated on S6 in response to the different growth regulators [5,6,8,9,11,12] indicating that similar S6 kinases may be involved in the different responses. A number of protein kinases have been shown to phosphorylate S6 including those regulated by cyclic AMP [13,14], cyclic GMP [15], Ca^{2+} [16] and phospholipid together with Ca^{2+} [17], as well as casein kinase I [18] and a group of protease-activated kinases (in addition to the Ca^{2+}-phospholipid dependent enzyme [11,19,20]). Evidence has been provided that a major protease-activated protein kinase is regulated by insulin in mouse 3T3 fibroblasts [11]. We have shown that some of the insulin-regulated phosphorylation sites in rat S6 are in close proximity with the major site phosphorylated by the cyclic AMP-dependent protein kinase sequence Arg-Arg-Leu-Ser-Ser-Leu-Arg-Ala-Ser-Thr-Ser-Lys-Ala-Ser-Glu-Glu-Ser-Gln-Lys [21]. The first serine residue is the preferred site for the cyclic AMP-dependent protein kinase [14]. Insulin appears to control the phosphorylation of one of the adjacent serines together with additional sites within rat hepatocytes [8]. To study the nature and specificity of the protease-activated protein kinase in these liver cells we have synthesised the peptide Arg-Arg-Leu-Ser-Ser-Leu-Arg-Ala termed S6-1 whose structure is based on the region of S6 containing one of the insulin-regulated phosphorylation sites. The peptide has proved to be a useful substrate for a protease-dependent protein kinase activity in rat liver. The preferred site for this enzyme in both peptide S6-1 and ribosomal protein S6 is distinct from the site preferred by cyclic AMP-dependent kinase.

+ To whom correspondence should be addressed

Abbreviations: Bt_2cAMP, N^6,O^2-dibutyryladenosine 3′:5′-monophosphate; Mops, morpholinopropanesulphonic acid; CM, carboxymethyl

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00145793/84/$3.00

2. MATERIALS AND METHODS

2.1. *Materials*

[γ-^{32}P]ATP was obtained from Amersham International (England); acrylamide and *N,N'*-methylenebisacrylamide from Eastman Kodak (USA), trypsin treated with tosylphenyl chloromethyl ketone from Worthington (USA); DE-52 cellulose and P-81 paper from Whatman (England); CM-Sepharose CL-6B and Pharmalytes for isoelectric focussing from Pharmacia (Sweden); Bt_2cAMP, histone type IIA, and soyabean trypsin inhibitor from Sigma (USA); casein from Matheson, Coleman and Bell (USA). Bovine serum albumin (essentially fatty acid free) was obtained from Sigma and treated with phenylmethylsulfonyl fluoride [22].

2.2. *Isolation of protease-activated protein kinase*

Livers isolated from 2–3-month-old normal-fed male Wistar rats were cooled to 4°C, homogenized in 10 mM Tris–HCl buffer (pH 7.6) containing 2 mM $MgCl_2$, 0.1 mM EDTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 0.3 M sucrose [22]. The methods of [22] were used to prepare the post-ribosomal supernatant fraction, to adjust the pH to 8.5 by the addition of 1/10 vol. of 100 mM Tris–HCl buffer (containing 20 mM EDTA, 100 mM 2-mercaptoethanol, 0.2% (w/v) NaN_3), to batch adsorb the supernatant fractions to DEAE–cellulose (DE-52, Whatman) and to elute proteins with a 0–0.5 M NaCl gradient. Fractions containing the trypsin-activated protein kinase were pooled, dialysed against 15 mM potassium phosphate buffer (pH 6.4) containing 10 mM EDTA, 10 mM 2-mercaptoethanol and 0.02% NaN_3, batch-adsorbed to CM-Sepharose and the proteins eluted with a 0–0.3 M KCl gradient [22]. Column fractions (5 ml) containing trypsin-activated protein kinase activity were pooled, concentrated by precipitation with 70% (w/v) $(NH_4)_2SO_4$ and the enzyme dissolved in 10 mM Tris–HCl buffer (pH 8.5) containing 2 mM EDTA, 10 mM 2-mercaptoethanol and 0.02% NaN_3 at concentrations in the range 3–10 mg protein $\cdot$ ml^{-1} and stored at −70°C.

2.3. *Kinase assay*

Aliquots (15 μl) of the column fractions containing 5–60 μg protein were incubated with 0.2 μg trypsin in a reaction volume of 18 μl at 30°C for 2–3 min depending on the overall protein concentration of the column fractions [22]. Proteolysis was terminated by the addition of soyabean trypsin inhibitor (6 μg in 3 μl). Trypsinised column fractions (10-μl aliquots) were assayed for histone and casein kinase activities as in [22]. The protein kinase activity with the synthetic peptide S6-1 was assayed in a reaction volume of 55 μl containing 15 μl trypsin-treated or control column fractions, 7 mM $MgCl_2$, 26 mM KCl, 0.7 mM EGTA, 0.7 mM EDTA, 0.7 mM dithiothreitol, 0.2 mM [γ-^{32}P]ATP (100–400 cpm $\cdot$ pmol^{-1}) and 30 μM peptide S6-1. The reaction was initiated by adding the enzyme fraction and usually terminated after 5 min. Peptide phosphorylation was determined by binding to P-81 papers [23]. Cyclic AMP-dependent protein kinase was assayed by incubating 15-μl aliquots of column fractions with peptide S6-1 in same reaction mixture as above in the presence or absence of Bt_2cAMP (10^{-6} M).

2.4. *Phosphorylation of 40 S ribosomal subunits*

Rat liver ribosomes or 40 S ribosomal subunits [14,24] were incubated at 33°C in a reaction volume of 50 μl containing 15 μl trypsin-treated or untreated kinase fractions, and buffer (pH 7.4) containing 15 mM Tris–HCl, 15 mM Mops, 25 mM KCl, 4 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mM EGTA, 25 mM NaF and 0.2–0.8 mM [γ-^{32}P]ATP (200–400 cpm $\cdot$ pmol^{-1}). The method of processing the phosphorylated proteins by SDS-polyacrylamide gel electrophoresis was described previously [7,21].

2.5. *Other procedures*

Peptide S6-1 having the structure Arg-Arg-Leu-Ser-Ser-Leu-Arg-Ala was synthesised with a free COOH-terminal form by the Merrifield solid phase synthesis procedure using a Beckman 990 Synthesiser [25]. The peptide was purified and characterized as in [26]. Protein determination [27], counting of ^{32}P radioactivity by Cerenkov counting in a liquid scintillation spectrometer [14], and isoelectric focussing of phosphopeptides in polyacrylamide gels [14,28] were performed as described.

3. RESULTS

3.1. *Detection of protease-activated protein kinase using synthetic peptide S6*

Chromatography of the post-ribosomal fraction of rat liver on DE-52 cellulose resulted in a broad peak of trypsin-dependent protein kinase activity which was readily detected with the synthetic peptide substrate S6-1 (fig.1a). The S6-1 protein kinase activity was increased by more than 10-fold by trypsin-treatment in the region chromatographing midway between the peaks of casein kinase activities and just after the major peaks of histone kinase and type I cyclic AMP-dependent kinase activities (fig.1a). A particular advantage of using S6-1 rather than histone (fig.1a) as a substrate was the relatively low background of peptide phosphorylation in the absence of protease treatment. The peptide also served as a substrate for cyclic AMP-dependent kinases when Bt_2cAMP was included in the reaction mixture (fig.1a).

Chromatography of the trypsin-activated protein kinase on CM-Sepharose resulted in a discrete peak of activity eluting at about 0.06 M KCl (peak I, fig.1b). The specific activity of the protein kinase was increased from 113 to 774 pmol ^{32}P transferred to S6-1 · min^{-1} · mg $protein^{-1}$ by this step. The concentrated enzyme catalysed the transfer of up to 0.8 mol phosphate per mol peptide (fig.2). A second broader peak of trypsin-insensitive protein kinase activity eluted between 0.1 and 0.2 M KCl (peak II). The latter activity was inhibited up to 70% by the Walsh heat-stable inhibitor of cyclic AMP-dependent kinase [29] whereas the trypsin-sensitive activity (peak I) was insensitive to the inhibitor (not shown). The proportion of the peak II activity could be reduced by pretreating the post-DE52 fraction with Bt_2cAMP (10^{-5} M) and rechromatographing on DE52 cellulose prior to CM-Sepharose chromatography [22] (not shown).

3.2. *Phosphorylation of ribosomal proteins by the trypsin-activated kinase*

The capacities of the trypsin-activated protein kinase to phosphorylate liver ribosomes were investigated. Trypsin activation of the kinase stimulated the phosphorylation of S6 as well as that of a 17-kDa and a 23-kDa protein in both 80 S and 40 S preparations (fig.3). The phosphorylations of S6 and the 17-kDa species were more extensive and were increased 10- and 20-fold, respectively, by trypsin activation (table 1). The stimulation of activity was not as great with concentrated enzyme

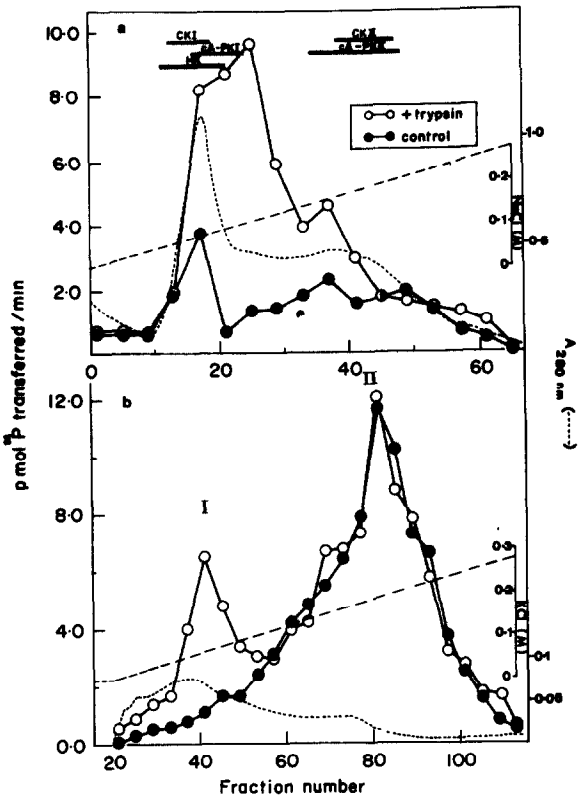

Fig.1. Fractionation of protein kinase by ion exchange chromatography. (a) The post-ribosomal fractions from 5 livers were batched-absorbed to 100 ml DE-52 cellulose [22]. The washed DE-52 was transferred to a column (3.2 × 12 cm) and eluted with an 800 ml linear gradient (0–0.5 M) of NaCl. Column fractions (8.7 ml) were assayed for trypsin-activated protein kinase activities using synthetic peptide S6-1 as substrate with (○—○) and without (●—●) trypsin treatment. Regions of cyclic AMP-dependent protein kinase (types I and II are denoted as cA-PKI and cA-PKII, respectively), casein kinase (CKI and CKII) and histone kinase (HK) activities are also marked. A broad region of histone kinase activity encompassing fractions 30–40 is not illustrated. (b) The trypsin-sensitive protein kinase activity in De-52 fractions 20–30 was rechromatographed on CM-Sepharose (3.2 × 12 cm column). Proteins were eluted with a 500 ml linear gradient (0–0.3 M) of KCl. Column fractions (5 ml) were assayed for trypsin-dependent protein kinase activities as in a.

preparations due to an increased basal activity, apparently resulting from activation by endogenous proteases [20,22]. The rates of phosphorylation of S6 and the 17-kDa protein by the trypsin-activated kinase were similar (table 1). The stoichiometries reached values of 1.7 and 2.2 mol phosphate per mol S6 and the 17-kDa protein, respectively, during incubation for 80 min with concentrated preparations of the trypsin-activated kinase (table 1). Maximum stoichiometries of phosphorylation of S6 required ATP concentrations of 0.8 mM ATP (table 1).

3.3. *Phosphorylation sites in S6 and synthetic peptide S6-1*

The sites of phosphorylation for the trypsin-activated kinase within ribosomal protein S6 and the synthetic peptide S6-1 were investigated by tryptic peptide mapping in isoelectric focussing gels. Preparations of both phosphorylated S6 excised from the electrophoretograms (fig.3) and phosphorylated synthetic peptide S6-1 were digested with trypsin under similar conditions. Tryptic digests of peptide S6-1 phosphorylated with the trypsin-activated protein kinase contained only one major species (band 3, fig.4, track 2) which comigrated with a monophosphorylated form of the Leu-Ser-Ser-Leu-Arg peptide (submitted). This species remained the most prominent species when the stoichiometry of phosphorylation increased to maximum values (~0.8 mol phosphate per mol peptide) (fig.2). The tryptic digests also contained some of the mono and diphospho forms of the Arg-Leu-Ser-Ser-Leu-Arg sequence [14] (bands 2 and 4, respectively; fig.4) but these species were always minor components when compared with the band 3 species.

A phosphopeptide comigrating with the monophosphorylated form of the Leu-Ser-Ser-Leu-Arg peptide was also a major species in tryptic digests of ribosomal S6 phosphorylated with preparations of the trypsin-activated protein kinase (band 3, fig.4, tracks 5,6,8). The monophosphorylated Arg-Leu-Ser-Ser-Leu-Arg tryptic peptide (band 2) and

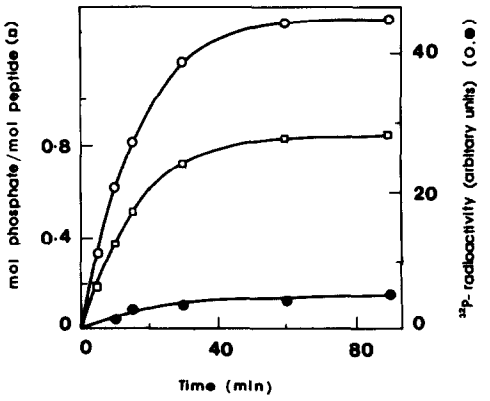

Fig.2. Phosphorylation of peptide S6-1 by trypsin-activated protein kinase. The peak I post-CM-Sepharose protein kinase fraction (30 μg protein/30 μl assay) was used: (□—□) stoichiometry of peptide S6-1 phosphorylation determined with P-81 paper binding assay [23]. (●—●), (○—○) ^{32}P radioactivity associated with tryptic derivatives of ^{32}P-S6-1 referred to as bands 2 and 3 in the isofocussing analysis (fig.4) and quantitated by densitometric analysis of the autoradiograms [7] of the dried isofocussing gels: (●—●) band 2, (○—○) band 3.

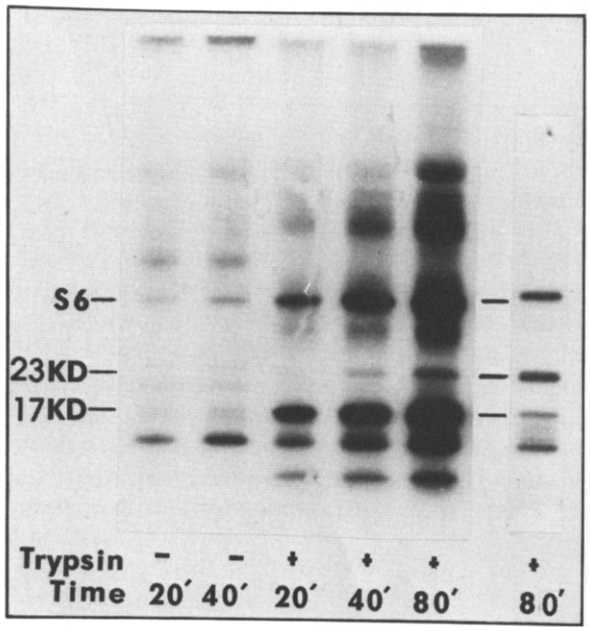

Fig.3. SDS-polyacrylamide gel analysis of phosphorylated ribosomal proteins. Ribosomes (tracks 1–5) and 40 S ribosomal subunits (track 6) were phosphorylated by the post-CM-Sepharose peak I protein kinase (5 μg protein per 50 μl assay). Trypsin activations and reaction times are indicated in the figure. M_r values were estimated as in [7]. Tracks are numbered from left to right.

the species having a p*I* value ~4.6 (band 4), and to a lesser extent those with p*I* values ~3.8 (band 7) and 3.6 (band 8), were also increased in response to trypsin activation, particularly in digests of S6 phosphorylated with concentrated preparations of the protein kinase (fig.4, track 8). The most obvious species detected in digests of S6 phosphorylated with the protein kinase preparation not subjected to trypsin treatment was the monophosphorylated Arg-Leu-Ser-Ser-Leu-Arg peptide (band 2, fig.4, track 4; cf track 7 where the band 3 species was also prominent due to partial activation of the concentrated kinase preparation by endogenous protease). However, the intensity of this band was appreciably less than in digests of S6 phosphorylated by the trypsin-activated kinase.

3.4. *Cyclic AMP-dependent kinase*

A striking difference in the tryptic phosphopeptides generated from ribosomal S6 and peptide S6-1 phosphorylated with the cyclic AMP-dependent (fig.4, tracks 3 and 9) and protease-activated kinases (fig.4, tracks 2,5,6 and 8) was that the monophosphorylated Leu-Ser-Ser-Leu-Arg species (band 3) was only prominent in digests of substrates phosphorylated with the protease-activated kinase. Other major differences were that the mono and diphosphorylated forms of the Arg-Leu-Ser-Ser-Leu-Arg (band 5) [14] and, in the case of S6, the Arg-Leu-Ser(PO_4)-Ser(PO_4)-Leu-Arg-Ala-Ser(PO_4)-Thr-Ser-Lys species (band 6) [21] were prominent only in digests of the substrates phosphorylated by cyclic AMP-dependent kinase (fig.4, tracks 3 and 9).

4. DISCUSSION

Knowledge of the structure of S6 in the region of two important phosphorylation sites has enabled the synthesis of a peptide analogue of this region of S6 for use as a substrate in S6 kinase assays. The peptide proved to be an effective substrate for the detection and isolation of a trypsin-activated protein kinase from rat liver.

The trypsin-activated kinase resembled major protease-activated protein kinases previously

Table 1

Phosphorylation of ribosomal proteins by the protease-activated kinase

Reaction conditions	Incubation time (min)	Stoichiometry (mol ^{32}P/mol protein)			
		S6[a]		17-kDa protein[a]	
		Control	Trypsin	Control	Trypsin
Peak I[b]					
(0.2 mM ATP)	20	0.01	0.10	0.005	0.11
	30	0.02	0.15	0.01	0.15
	40	0.02	0.23	0.14	0.25
	80	–	0.48	–	
Peak I, concentrated[c]					
ATP, 0.2 mM	40	0.33	0.8	–	1.5
0.4 mM	40	–	1.4	–	2.6
0.8 mM	40	–	1.7	–	2.2

[a] Stoichiometry determined from ^{32}P radioactivity associated with bands of S6 and the 17-kDa protein excised from SDS-polyacrylamide electrophoretograms of 80 S ribosomal proteins as in fig.2

[b] Peak I protein kinase fraction eluted from CM-Sepharose column, 5 μg/50 μl assay (fig.1b)

[c] Peak I kinase fraction concentrated by ammonium sulphate precipitation, 40 μg/5 μl assay

isolated from rabbit reticulocytes (PAK II [11,15,19]) and mouse lymphosarcomas (histone H4 kinase [20]) in their chromatographic behaviour and abilities to phosphorylate S6. Further, in the case of the histone H4 kinase, the amino acid sequence in the region of the H4 phosphorylation site [20] exhibits some similarities with the preferred site for the liver protease-activated kinase in ribosomal protein S6 (see below). Other protein kinases sensitive to mild trypsin treatment could have been present in the liver trypsin-activated kinase preparations. The presence of an enzyme resembling rabbit reticulocyte protease-activated kinase I [15,22] could explain the appreciable phosphorylation of the 23-kDa (S10) ribosomal protein [19]. Another possible enzyme is the Ca^{2+}-phospholipid-dependent protein kinase that can be activated proteolytically [30], can phosphorylate S6 [17] and appears to be present in liver cells [31]. However, this enzyme may have been eliminated by the CM-Sepharose step because of its low affinity for cation-exchange resins [32].

Tryptic mapping indicated that the liver protease-activated protein kinase preferentially phosphorylated a site that was distinct from the preferred site for cyclic AMP-dependent protein kinase. The predominance of the monophosphorylated Arg-Leu-Ser-Ser-Leu-Arg species in the tryptic digests of S6-1 phosphorylated with the

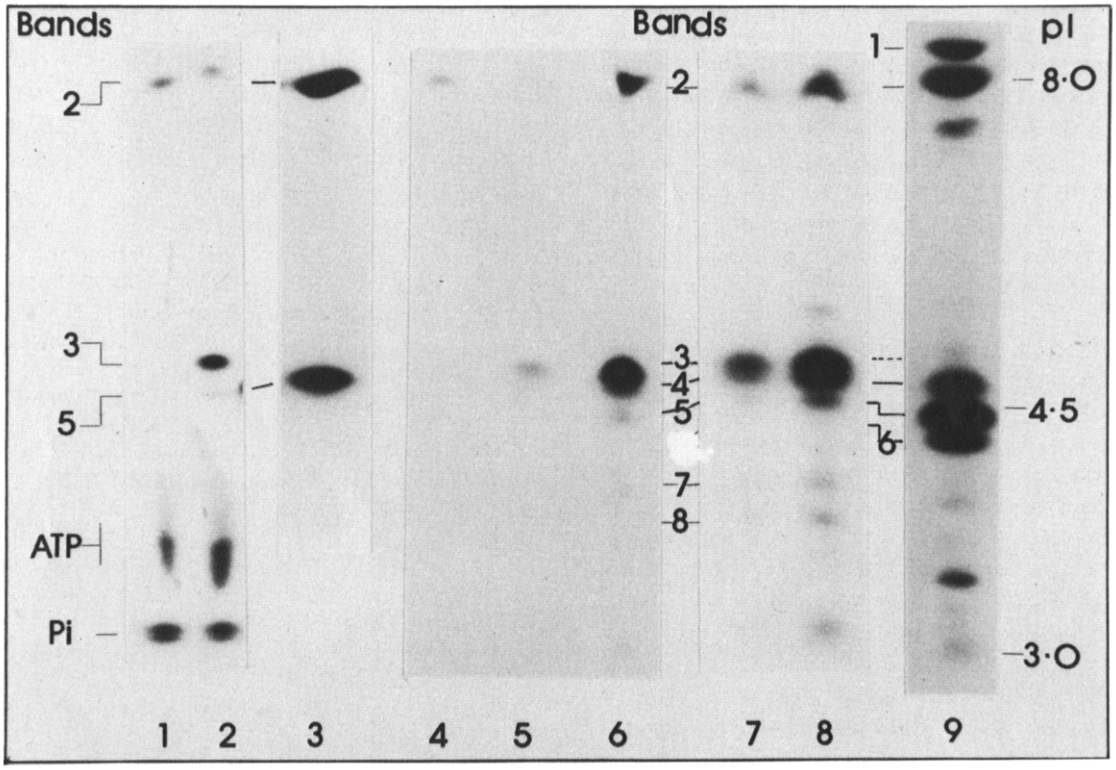

Fig.4. Analysis of ^{32}P-tryptic peptides by isoelectric focussing in polyacrylamide gels. Tryptic digests of peptide S6-1 phosphorylated with the catalytic subunit of cyclic AMP-dependent protein kinase (0.1 μg) (track 3) and the post-CM-Sepharose peak I protein kinase (20 μg protein/30 μl assay) pre-incubated with (track 2) and without (track 1) trypsin. Tryptic digests of ^{32}P-S6 recovered from SDS-polyacrylamide gels (fig.3): Ribosome phosphorylated with 0.5 μg cyclic AMP-dependent protein kinase (track 9) and with 5 μg (tracks 4–6) and 30 μg (tracks 7,8) of the peak I protein kinase preincubated with (tracks 5,6,8) and without (tracks 4,7) trypsin. Each isofocussing analysis was calibrated with a reference preparation of ^{32}P-phosphopeptides containing the mono (band 2) and diphospho (band 3) forms of the Arg-Leu-Ser-Ser-Leu-Arg peptides.

cyclic AMP-dependent kinase was attributable to the first of the adjacent serine residues being preferentially phosphorylated by this enzyme. A phosphate at this site inhibits tryptic cleavage of the NH_2-terminal arginine residue [14]. More effective tryptic cleavage of this arginine residue should have occurred when only the second of the serine residues was phosphorylated. This would have resulted in the selective formation of the Leu-Ser-Ser(PO_4)-Leu-Arg tryptic peptide which was apparent when S6-1 was phosphorylated with the protease-activated protein kinase. Thus it is concluded that the protease-activated protein kinase preferentially phosphorylated the second serine residue in peptide S6-1.

The similarities in the patterns of tryptic phosphopeptides for digests of S6 and peptide S6-1 indicate that the specificities of the two protein kinases are reflected in the phosphorylation of the different serine residues in the 8-residue peptide S6-1 and do not involve long-range conformational influences of the type that have been observed in other phospho-proteins (e.g. [33]). Some overlap in the specificities of the two protein kinases was indicated by the presence of the Arg-Leu-Ser(PO_4)-Ser(PO_4)-Leu-Arg species in tryptic digests of both S6 and peptide S6-1 phosphorylated with the kinases. The phosphorylation of adjacent serine residues by non-casein kinase is uncommon and has only been observed in vitro with unusually high concentrations of cyclic AMP-dependent kinases [14,33]. The phosphorylation of additional sites in protein S6 by the protease-activated kinase was indicated by the presence of other phosphopeptides in tryptic digests of S6 unrelated to the tryptic derivatives of the S6-1 sequence (fig.4) [34]. However, only one of these additional sites appeared to be phosphorylated strongly by the rat liver enzyme (band 4, fig.4). At least 5 phosphorylation sites have been inferred from the electrophoretic heterogeneity of S6 exhibited during two-dimensional polyacrylamide gels analyses [1–4]. Some of these sites may not have been phosphorylated efficiently by the protease-activated and cyclic AMP dependent kinases under the in vitro conditions employed. Alternatively, rat liver may contain additional S6 protein kinases that were not detected by our procedure.

ACKNOWLEDGEMENTS

This work was supported by grants from the National Health and Medical Research Council of Australia and the Victorian Anti-Cancer Society. We thank Sue Veinet for assistance in the purification of the synthetic peptide.

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