ISOLATION AND CHARACTERISATION OF CYCLIC AMP-DEPENDENT PHOSPHORYLATION SITES FROM RAT LIVER RIBOSOMAL PROTEIN S6

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1. Introduction

Protein S6 of the small (40 S) ribosomal subunit is the major phosphoprotein of mammalian ribosomes [1–3], and can be phosphorylated at multiple sites both in vitro and in vivo [1–3]. The primary consequences of S6 phosphorylation have not yet been established, although a relationship between the extent of phosphorylation and protein synthetic activity is suggested by several observations [3–7; cf. 1,8].

S6 can be phosphorylated by cyclic AMP-dependent protein kinase (3), but the existence of other S6 kinases is suggested by the increased phosphorylation observed in response to anabolic factors that do not alter cyclic AMP levels [4,6,9]. While the increased phosphorylation in these cases could result from suppression of phosphatase activity [10,11], the presence of both cyclic AMP-dependent and independent sites has been inferred from tryptic peptide maps of S6, obtained after exposure of HeLa cells to cyclic AMP-elevating agents or anabolic factors such as insulin [9].

A major limitation in studying S6 phosphorylation sites has been the problem of isolating sufficient quantities of ^{32}P-labelled S6 for detailed structural analyses. Here, brief tryptic digestion of ribosomes phosphorylated by the catalytic subunit of cyclic AMP-dependent protein kinase selectively released the S6 phosphorylation sites while leaving most of the other ribosomal proteins intact. This has allowed the isolation of these sites and determination of their amino acid sequences. Surprisingly, the two major serine residues phosphorylated by cyclic AMP-dependent protein kinase were adjacent in the primary structure of S6.

Abbreviations: MOPS, morpholinopropane sulphonic acid; HPLC, high-performance liquid chromatography; SDS, sodium dodecyl sulphate; TPCK, tosylphenylchloromethylketone; Pth, phenylthiohydantoin amino acid

2. Materials and methods

2.1. *Materials*

[γ-^{32}P]ATP was obtained from Amersham International (Buckinghamshire), Sephadex G-25 (superfine grade) and Sephadex G-15 from Pharmacia (GB) Ltd (Hounslow, Middlesex), acrylamide specially purified for electrophoresis from Fison's Scientific Apparatus (Loughborough, Leicestershire), puromycin hydrochloride from Sigma Chemical Co. (Poole, Dorset), Polygram ®CEL400 thin-layer cellulose plates (Macherey-Nagel) from Camlab (Cambridge) and trypsin treated with TPCK (Worthington) from Cambrian Chemicals (Croydon, Surrey). A highly purified preparation of the catalytic subunit of cyclic AMP-dependent protein kinase from rabbit skeletal muscle [12] was a gift from Dr B. A. Hemmings.

2.2. *Preparation of ribosomes*

Liver ribosomes were prepared as in [13] from 10–12 week old male Wistar rats which had been starved overnight. The ribosomes were further purified by incubation for 15 min at 33°C in 20 mM Tris–HCl buffer (pH 7.6) containing 0.2 mM puromycin, 500 mM KCl, 5 mM $MgCl_2$ and 1 mM dithiothreitol, followed by centrifugation through 1.45 M buffered sucrose [13] at 125 000 × g for 20 h. The recovery of ribosomes was usually ~16 mg RNA or 7 nmol/liver, assuming that 1 A_{260} unit is equivalent

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to 45 μg RNA and that the combined M_r of rRNA is 2.2×10^6 [14].

2.3. *Phosphorylation of ribosomes*

Ribosomes (3 mg RNA/ml) were incubated at 33°C in a reaction mixture containing buffer R (15 mM Tris–HCl (pH 7.0), 15 mM MOPS (pH 7.0), 25 mM KCl, 4 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mM EDTA, 0.1 mM EGTA, 3 mM NaF), 0.2 mM [γ-^{32}P]-ATP (50 000–100 000 cpm/nmol) and 2–10 U/ml of the catalytic subunit of cAMP-dependent protein kinase (see [12] for definition of units). Standard incubations were for 50 min (cf. fig.1), after which the reaction mixture was layered over 1.0 M sucrose in 20 mM Tris–HCl (pH 7.6), 100 mM KCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mM EGTA, and centrifuged at 125 000 × *g* for 12 h. The ribosomes were resuspended in buffer R at ~20 mg RNA/ml and stored at −70°C.

2.4. *Other analytical procedures*

Analysis of ribosomal proteins by SDS–polyacrylamide gel electrophoresis and subsequent autoradiography [6], and isoelectric focussing of phosphopeptides in polyacrylamide gels [15], were performed by standard procedures. Two-dimensional mapping of phosphopeptides was carried out on thin-layer cellulose using electrophoresis at either pH 3.5 [16] or pH 4.4 [17] as the first dimension, and chromatography in butanol/acetic acid/water/pyridine (15:3:12:10, by vol.) as the second dimension [16]. Peptides were further purified on a Beckman/Altex HPLC system using a Waters Associates μBondapak C_{18} reverse-phase column, and linear gradients of water/acetonitrile (0–40%) containing 0.1% trifluoroacetic acid. Amino acid analyses were performed on an LKB Biochrom 4400 amino acid analyser and automated sequence analyses on a Beckman 890C sequencer as in [18].

3. Results

3.1. *Selective cleavage of phosphopeptides from whole ribosomes*

Ribosomes were phosphorylated with the catalytic subunit of cyclic AMP-dependent protein kinase, the extent of phosphorylation depending on the catalytic subunit concentration (fig.1). Most of the radioactivity was present in S6, M_r app. = 34 000 [6] (fig.2).

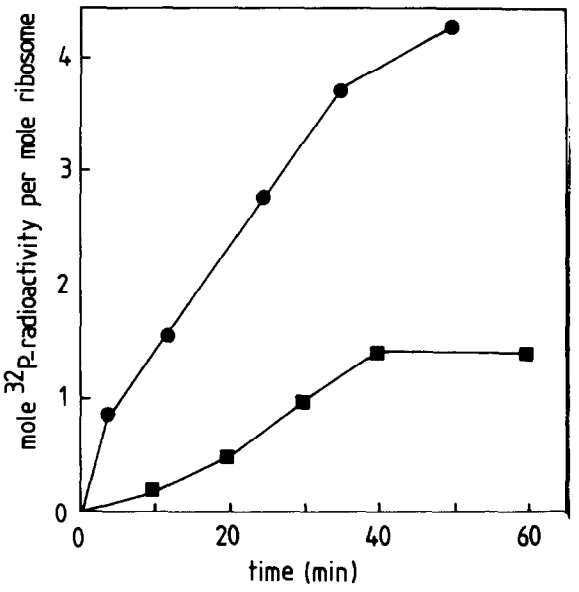

Fig.1. Phosphorylation of ribosomes by the catalytic subunit of cyclic AMP-dependent protein kinase. The standard reaction mixture (see section 2.3) contained either 2 U/ml (▪) or 10 U/ml (•) of protein kinase. Aliquots (20 μl) were removed at various time intervals, added to 0.5 ml 20 mM ATP in 10% (w/v) trichloroacetic acid, and the acid-insoluble radioactivity determined.

Incubation of the phosphorylated ribosomes with trypsin led to almost complete release of ^{32}P-radioactivity from S6 within 20 s (fig.2), and its appearance in trichloroacetic acid-soluble phosphopeptides (fig.3). The disappearance of ^{32}P-radioactivity from S6 was accompanied by the loss of a protein staining band at the same position, whereas most of the ribosomal proteins were unaffected by tryptic digestion for up to 60 s (fig.2).

The trichloroacetic acid-soluble phosphopeptides were analysed by gel filtration on Sephadex G-25 equilibrated in 1.0 M acetic acid. Two broad peaks of ^{32}P-radioactivity were resolved termed T1 (V_e/V_o = 1.3) and T2 (V_e/V_o = 1.6), corresponding to peptides of ~10–20 and ~5–8 residues, respectively (fig.3). The amounts of T1 and T2 decreased and increased respectively during tryptic digestion, suggesting that T1 and T2 contained phosphorylation sites derived from the same region of S6. This was confirmed by a separate prolonged digestion of T1 which was largely converted to T2 (not shown). However, a minor fraction of T1 was resistant to prolonged digestion (fig.3) suggesting the presence of an additional phosphorylation site(s).

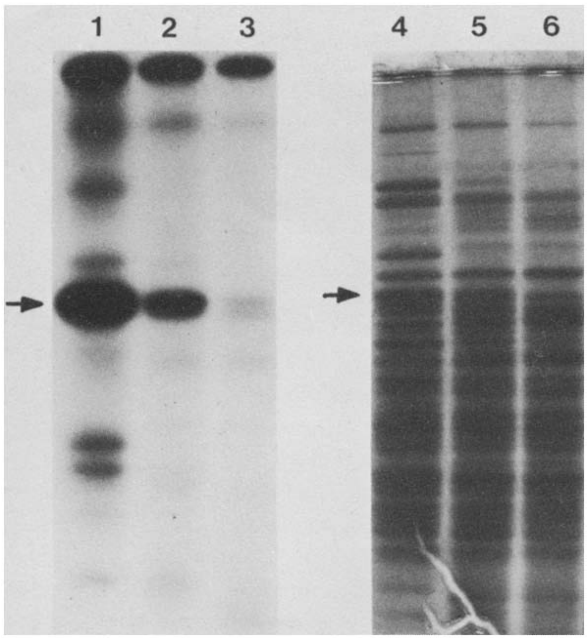

Fig.2. Tryptic digestion of phosphorylated ribosomes. ^{32}P-Labelled ribosomes (1.4 nmol/ml) in buffer R (section 2.3) were incubated at 33°C with trypsin (60 μg/ml). Aliquots (25 μl) were removed at various time intervals and the trichloroacetic acid-insoluble material analysed by 15% SDS–polyacrylamide gel electrophoresis and subsequent autoradiography: (1–3) autoradiogram; (4–6) Coomassie blue-stained electrophoretogram; (1,4) undigested ribosomes; (2,5) after 10 s trypsin digestion; (3,6) after 20 s digestion. Tryptic digestion for up to 75 s gave electrophoretograms that were indistinguishable from 20 s digestion time point (not shown). The arrows denote the position of ribosomal protein S6, M_r = 34 000.

3.2. *Purification of tryptic phosphopeptides*

Phosphopeptides were isolated on a large scale from 100–120 nmol batches of ribosomes (1.4 nmol/ml) and digested with trypsin (60 μg/ml) for 45 s at 33°C. After treatment with trichloroacetic acid, the soluble fraction was acidified with 0.1 vol. glacial acetic acid, and passed over Sephadex G-15 equilibrated with 1.0 M acetic acid, to remove [γ-^{32}P]-ATP and trichloroacetic acid. The phosphopeptides eluting near the void volume were lyophilised, redigested with trypsin, gel filtered on Sephadex G-25 and the T1 and T2 peaks pooled separately (fig.3).

Individual phosphopeptides were isolated from the T1 region by HPLC, the two major peaks of ^{32}P-radioactivity eluting at 22.5% (peptide T1a) and 33.5% (peptide T1b) acetonitrile. The peptides were further purified by peptide mapping using electrophoresis at pH 4.4 in the first dimension [17].

Peptides from the T2 region were initially fractionated by peptide mapping (fig.4). The [^{32}P]peptides were eluted with 0.1 M acetic acid and further purified by HPLC; the major phosphopeptides eluting at 22% (T2a), 23% (T2b) and 22% (T2c) acetonitrile. The most abundant species, T2b, was pure at this stage (table 1), whereas T2a and T2c required further purification by peptide mapping (pH 4.4 electrophoresis).

3.3. *Amino acid compositions of the phosphopeptides*

The amino acid compositions of the peptides sug-

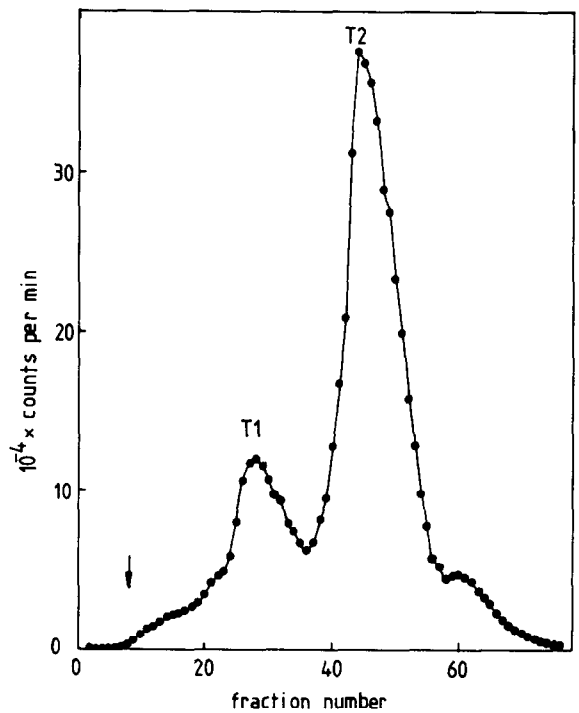

Fig.3. Gel filtration of ^{32}P-labelled tryptic peptides on Sephadex G-25 (superfine). The column (146 cm × 1.5 cm) was equilibrated with 1.0 M acetic acid. The freeze dried material from Sephadex G-15 (section 3.2) prepared from 100 nmol [^{32}P]ribosomes, was dissolved in 1.5 ml 0.1 M $NaHCO_3$ and redigested with trypsin (50 μg/ml) for 9 h at 33°C. The digest was acidified with 0.1 vol. glacial acetic acid and trichloroacetic acid (final conc. 10% (w/v)) was added to precipitate trypsin. After centrifugation, the acidified digest was applied to the column at a flow rate of 18 ml/h. Fractions of 1.2 ml were collected after 60 ml had passed through the column. The arrow denotes the position of the void volume (V_o).

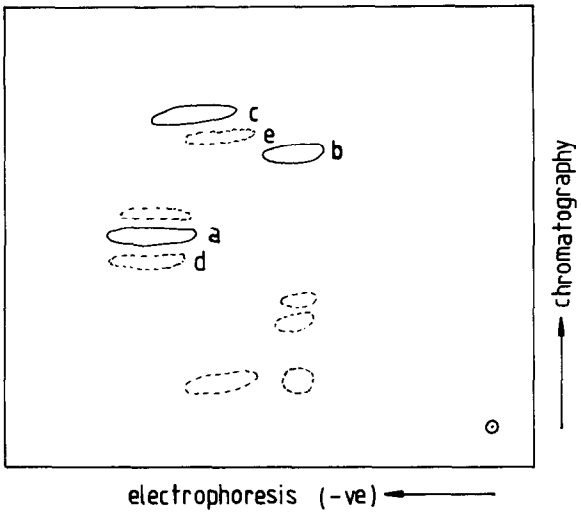

Fig.4. Separation of ^{32}P-labelled tryptic peptides (T2 fraction) by two-dimensional mapping. The pooled T2 fraction (fig.3) was lyophilized, redissolved in 200 μl 0.1 M acetic acid, and 50 μl aliquots applied to thin-layer cellulose plates. Electrophoresis was done at pH 3.5. The [^{32}P]peptides a,b,c,d and e represent the peptides T2a, T2b, T2c, T2d and T2e, respectively (table 1, fig.5). These 5 peptides together accounted for >80% of the total ^{32}P-radioactivity. Peptide T2b (b) alone accounted for 60%.

gested that T1a, T2a, T2b and T2c were all related (table 1). The compositions of T2b and T2c were identical (Ser_2, Leu_2, Arg_2) while T2a contained one additional arginine residue. The larger peptide T1a was also rich in serine, leucine and arginine but contained additional threonine, alanine and lysine residues. This peptide is of special interest because it contains three phosphate groups (see section 4). Peptide

Table 1
Compositions of the major tryptic phosphopeptides isolated from ribosomes

Description	Peptides[a]				
	T1a	T1b	T2a	T2b	T2c
1. Amino acid					
Asp/Asn	–	1.4	–	–	–
Thr	1.1	1.1	–	–	–
Ser	3.1	1.2	1.8(2)	2.0(2)	2.0(2)
Glu	–	0.9	0.5(0)	–	–
Gly	–	1.2	–	–	–
Ala	1.1	3.6	–	–	–
Val	–	0.7	–	–	–
Ile	–	1.6	–	–	–
Leu	1.9	1.1	2.1(2)	1.9(2)	1.9(2)
Lys	1.0	1.0	–	–	–
Arg	1.8	–	3.2(3)	2.1(2)	2.2(2)
Total	10	14	7	6	6
2. Spec. act. (cpm/nmol) × 10^{-3}	27.0	10.9	19.6	18.2	10.2
3. Recoveries[b] (nmol/100 nmol ribosomes)	5	6	6	48	10

[a] Hydrolyses were done in 5.7 N HCl, 5 mM phenol for 24 h at 110°C. Serine and threonine were corrected for 10% and 5% destruction, respectively. Impurities <0.2 mol are omitted. The numbers in parentheses represent residues determined by sequence analysis

[b] Recoveries refer to ribosomes phosphorylated with 10 μg protein kinase/ml (fig.1). At 2 μg protein kinase/ml, recoveries of T2b and T2c were only 3 and 10 nmol/100 nmol ribosomes, respectively

Table 2
Automated sequencer analyses on peptides T2b and T2c

Step	Sequence residue		^{32}P-radioactivity (cpm)	
	T2b	T2c	T2b	T2c
1	Arg[a,b]	Arg[a,b]	76	82
2	Leu[a,b]	Leu[a,b]	72	173
3	Ser[b]	Ser[b]	592	1570
4	Ser[b]	Ser[b]	696	576
5	Leu[a,b]	Leu[a]	607	399
6	Arg[b]	–	426	251

[a] Pth-amino acids detected by HPLC
[b] Identified by amino acid analysis after back hydrolysis of Pth-amino acids with HI [18]. Pth-serine is converted to Ala on back hydrolysis

T1b appeared quite unrelated, being devoid of arginine.

3.4. *Primary structures of peptide T2b and T2c*

Automated sequencer analyses (table 2) confirmed that these peptides had the same sequence: Arg–Leu–Ser–Ser–Leu–Arg. A burst of ^{32}P-radioactivity occurred on the third cycle with peptide T2c and declined thereafter, suggesting that the serine residue at this position was phosphorylated. A burst of ^{32}P-radioactivity also occurred at the third cycle with peptide T2b, but the radioactivity increased further at the fourth cycle before declining in subsequent cycles (table 2). This suggested that both serine residues were phosphorylated in peptide T2b.

The notion that peptides T2c and T2b represented mono- and di-phosphorylated derivatives of the same peptide was substantiated by the following observations. The migration characteristics of the peptides during peptide mapping indicated that T2b was more acidic than T2c. The isoelectric points of T2c and T2b (pH 8.0 and 4.5, respectively) estimated by isoelectric focussing (fig.4) were consistent with the presence of an extra phosphate moiety on T2b. Furthermore, the ^{32}P-radioactivity/nmol peptide was 2-fold higher in peptide T2b than T2c (table 2). Thus, it can be concluded that the structures of peptides T2b and T2c are:

T2b – Arg–Leu–Ser(P)–Ser(P)–Leu–Arg

T2c – Arg–Leu–Ser(P)–Ser–Leu–Arg

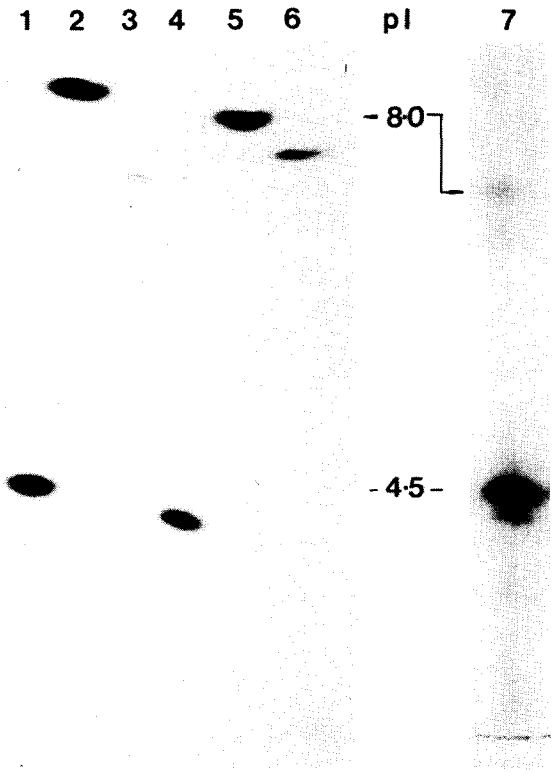

Fig.5. Analysis of ^{32}P-labelled tryptic peptides derived from S6, by isoelectric focussing in polyacrylamide gels. Tryptic peptides were released from whole ribosomes (1–6) or purified S6 (7): (1) peptide T2b; (2) T2d; (3) T2a; (4) T1a; (5) T2c; (6) T2e; (7) digest of purified S6 (from a separate gel). After completion of isoelectric focussing, pH gradients were estimated using a surface electrode.

3.5. *Proof that peptides T2b and T2c are derived from S6*

To verify that these tryptic peptides were really derived from S6, ^{32}P-labelled S6 was purified from ^{32}P-labelled ribosomes by acetic acid extraction [6] and polyacrylamide gel electrophoresis at pH 4.1 in the presence of urea [19]. Following tryptic digestion of the purified S6, isoelectric focussing revealed a major [^{32}P]peptide that comigrated with T2b (fig.5). In addition, a phosphopeptide comigrating with T2c, and a species in the region of T1a and T1b (isoelectric point ~4.3) were also apparent (fig.5).

4. Discussion

The isolation of S6 phosphopeptides in sufficient quantities for structural determinations was greatly

facilitated by the finding that these sites could be released selectively from whole ribosomes by limited tryptic digestion. This method may have an even wider application for the isolation of other sites, such as those phosphorylated in response to anabolic factors, or any regions on ribosomal proteins like S6 [20], that occupy exposed positions on the surface of ribosomes.

In the case of S6, the yields of phosphopeptides were remarkably high, considering the number of purification steps. The combined yield of the related group of peptides T1a, T2a, T2b and T2c was >60%. In view of this, site duplication within the primary structure of S6 or duplicate copies of S6 on the ribosome must be considered a possibility. In fact, 4.5 mol phosphate were incorporated/mol ribosome at high protein kinase concentrations (fig.1), only a minor proportion of which appears to be due to phosphorylation of proteins other than S6 (fig.2B). Clarification of this point will require the determination of the complete primary structure of S6.

The discovery of adjacent phosphoserine residues in S6 provides a further example of the clustering of phosphorylation sites in proteins, which is apparently a common phenomenon [21]. In fact, the clustering of phosphorylation sites in S6 may be even more extensive. The decapeptide T1a, whose composition suggested that it included the T2b/T2c sequence, appeared to contain 3 phosphate moieties (table 1). This peptide was recovered in low yields, and no other peptide that might be derived from the third site was detected. Thus the third phosphorylation site may only be labelled very slowly, casting doubt on its physiological significance.

This work indicates that cyclic AMP-dependent protein kinase phosphorylates two major sites on S6 from rat liver (the adjacent phosphoserine residues in T2b/T2c) with the possibility of two minor phosphorylation sites (contained within peptides T1a and T1b). These results are in agreement with studies on the phosphorylation of rabbit reticulocyte S6 by cyclic AMP-dependent protein kinase in vitro, which showed that ⩽2 mol phosphate could be incorporated/mol ribosome [22]. Tryptic peptide maps showed two major phosphopeptides that appear to resemble peptides T2b and T2c. As observed in table 1, the proportion of the more acidic species increased when incubations were performed at high protein kinase concentrations [22]. These similarities suggest that the two phosphopeptides from rabbit reticulocyte S6 may be analogous to peptides T2b and T2c, and therefore represent mono- and di-phosphorylated derivatives of the same peptide.

The phosphorylation of adjacent serine residues on S6 can be rationalised in terms of the known site specificity of cyclic AMP-dependent protein kinase. Many of the phosphorylation sites in physiological substrates have the structures Arg–Arg–X–Ser or Lys–Arg–X–Y–Ser [23]. Peptide T2b therefore resembles both types of site, since being a tryptic peptide, it must have the structure:

$$\begin{matrix}\text{Arg}\\ \text{Lys}\end{matrix}\text{–Arg–Leu–Ser–Ser–}$$

Most of the phosphate in the mono-phosphorylated derivative T2c was in the first serine (table 2) suggesting that this residue is preferentially phosphorylated. This is as expected from studies with synthetic peptides [23].

The isolation and structural analysis of peptides T2b and T2c provide the molecular basis for elucidating the phosphorylation of S6 in vivo. Both the mono- and di-phosphorylated derivatives are formed in isolated hepatocytes, especially after incubation with the hormone glucagon [24].

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