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A stimulated S6 kinase from rat liver: identity with the mitogen activated S6 kinase of 3T3 cells

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A number of approaches were tested for their ability to induce S6 phosphorylation and S6 kinase activation in rat liver, including i.p. injection of insulin, sodium orthovanadate or cycloheximide, as well as refeeding starved animals. All treatments led to increased S6 phosphorylation and activation of the apparent same enzyme. The most potent activator of the S6 kinase in liver extracts was cycloheximide. Maximum activation was achieved in 20 min at 1 mg cycloheximide/100 g body weight, with half-maximal activation in 10 min. Based on these findings a large-scale kinase purification procedure was established involving seven steps of chromatography. Following the final step a major protein band of M_r 70 000 was revealed. The protein was purified 20 000-fold, had a sp. act. of 640 nmol/min/mg of protein towards S6, autophosphorylated and was inactivated by phosphatase 2A. Peptide maps of autophosphorylated material were identical to those derived from the mitogen-activated kinase of 3T3 cells.

Key words: cycloheximide/phosphatase 2A/rat liver/S6 kinase

Introduction

Activation of protein synthesis is an early obligatory response in biological processes requiring increased cell growth or restructuring, e.g. wound healing, development and tumorigenesis. In many systems it appears that this event is either triggered or facilitated by the multiple phosphorylation of 40S ribosomal protein S6 (Traugh and Pendergast, 1986; Kozma *et al.*, 1989). Such conclusions were initially derived from the studies of Gressner and Wool (1974a) who showed that partial hepatectomy, which was known to lead to increased protein synthesis, led to a rapid induction of S6 phosphorylation. Similar results were also obtained following unilateral denervation of rat diaphragm, which resulted in the phosphorylation of S6 in the denervated but not the innervated portion of the organ (Nielsen *et al.*, 1982). The conclusion that S6 phosphorylation plays an important role in these responses was complicated by the fact that injection of cycloheximide, a potent inhibitor of protein synthesis, also led to increased S6 phosphorylation in a large number of tissues and organs (Gressner and Wool, 1974b; Nielsen *et al.*, 1982). In view of later results showing that cycloheximide induces many early mitogenic responses, including the activation of c-*myc* (Elder *et al.*, 1984; Makino *et al.*, 1984; Greenberg *et al.*, 1986), its effect on S6 phosphorylation may not be so surprising. To understand the role of S6 phosphorylation in processes which require cell growth it will be necessary to identify the enzymes involved in regulating this event.

Recently it was shown that liver extracts from partially hepatectomized rats contain 2- to 8-fold more S6 kinase activity than extracts from sham-operated animals (Mizuta *et al.*, 1987; Nemenoff *et al.*, 1988). The conditions used to detect elevated kinase levels were first employed in this laboratory to extract a mitogen-activated S6 kinase from Swiss 3T3 cells (Novak-Hofer and Thomas, 1984). Dose–response studies with epidermal growth factor (EGF) indicated that S6 kinase activity in cell extracts paralleled increased S6 phosphorylation in the intact cell, indicating that this kinase activity was responsible for regulating the level of S6 phosphorylation *in vivo* (Novak-Hofer and Thomas, 1985; Novak-Hofer *et al.*, 1986). This enzyme was purified to homogeneity and shown to be a rare polypeptide of M_r 70 000 (Jenö *et al.*, 1988; Jenö *et al.*, 1989), which is activated *in vivo* by serine or threonine phosphorylation (Ballou *et al.*, 1988a). In addition to this kinase, a number of other kinases have been implicated in S6 phosphorylation, including the cyclic AMP dependent protein kinase (Del Grande and Traugh, 1982), two protease-activated kinases (Lubben and Traugh, 1983; Donahue and Masaracchia, 1984), protein kinase C (LePeuch *et al.*, 1983; Parker *et al.*, 1985), a kinase from *Xenopus* oocytes termed S6 kinase II (Erikson and Maller, 1985), and an enzyme of M_r 67 000 from bovine liver (Tabarini *et al.*, 1987). With the exception of the cyclic AMP dependent protein kinase, all of these enzymes, if added in sufficient amounts, phosphorylate S6 to high levels. To establish that the mitogen-activated kinase is the essential enzyme in regulating S6 phosphorylation it is important to identify the stimulated S6 kinase in other systems, especially those capable of providing sufficient material for further studies.

Here we have explored the ability of refeeding, cycloheximide, insulin and vanadate to induce S6 phosphorylation and stimulate S6 kinase activity in rat liver. Starving and refeeding led to a suppression and reactivation of protein synthesis in liver, respectively (Morgan and Peters, 1971; Pain *et al.*, 1978), and might mimic the effects of partial hepatectomy without the $\approx 70\%$ loss of tissue involved in this operation. Cycloheximide and insulin injection (Nielsen *et al.*, 1982) are known to lead to increased S6 phosphorylation, and it is thought that vanadate induces many of the same effects as insulin (Shechter and Ron, 1986). After finding that all treatments lead to stimulation of the same kinase, optimal conditions were established for the most potent agent and the enzyme was purified through seven steps of chromatography. Finally, the enzyme's sensitivity to phosphatase 2A and peptide maps of its autophosphorylated

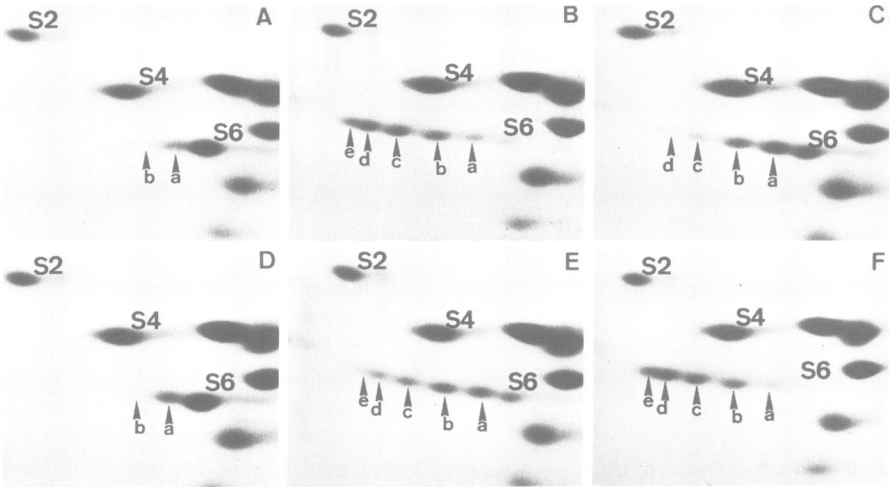

Fig. 1. Two-dimensional polyacrylamide gel analysis of 80S ribosomal proteins. Total 80S ribosomal proteins were analysed from rats starved for 24 h (**A**), starved for 24 h and refed for 1 h (**B**), starved for 24 h and injected with 2.5 U insulin/100 g body weight for 1 h (**C**), injected with PBS (**D**), injected with 3 mg sodium orthovanadate/100 g body weight for 1 h (**E**) or injected with 15 mg cycloheximide/100 g body weight for 1 h (**F**). The volume injected was 4 ml in all cases.

sites were compared with those derived from the mitogen-activated mouse enzyme.

Results

S6 phosphorylation

Each of the approaches outlined above was first tested for its ability to induce S6 phosphorylation. The results showed that in either starved animals (Figure 1A) or normally fed animals injected with phosphate-buffered saline (PBS) (Figure 1D), most of S6 resided in the unphosphorylated form with a small amount in derivatives S6a and S6b, containing 1 and 2 mol of phosphate respectively. Refeeding (Figure 1B) and cycloheximide injection (Figure 1F) were the most potent activators of S6 phosphorylation, causing the appearance of the tetra- and penta-phosphorylated derivatives S6d and S6e respectively. These were followed in potency by vanadate (Figure 1E) and insulin injection (Figure 1C). The results with insulin were identical for starved and normally fed animals (unpublished data). Thus, refeeding starved animals and sodium orthovanadate injection, two methods not previously reported to induce S6 phosphorylation, proved to be as efficient as insulin and cycloheximide in triggering this response.

S6 kinase activation

All of the approaches tested led to increased S6 kinase activity in liver extracts. In addition, regardless of the mode of activation, the kinase eluted from cation- and anion-exchange columns in the same position as a single peak of activity (our unpublished data). However, the ability to stimulate S6 kinase activity did not parallel the induction of S6 phosphorylation (cf. Table I and Figure 1). For example, cycloheximide injection was almost 3-fold more efficient than refeeding in stimulating S6 kinase activity even though both treatments were equally potent in inducing S6 phosphorylation. Therefore, the level of kinase activity was not a measure of the extent of S6 phosphorylation, consistent with recent results from this laboratory (Olivier *et al.*, 1988).

Table I. Activation of S6 kinase in whole cell extracts

	Sp. act. (pmol/min/mg)	Fold increase
(A) Animals starved for 24 h		
Control	3.81	1
Re-fed	6.65	1.75
Insulin	4.38	1.15
(B) Non-starved animals		
Control	4.02	1
Vanadate	7.70	1.92
Cycloheximide	18.77	4.67

Extracts were prepared from rat liver and assayed for S6 kinase activity as described in Materials and methods.

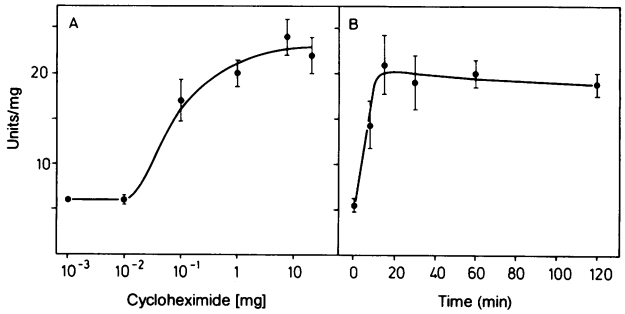

Fig. 2. Effect of dose and time of cycloheximide treatment on S6 kinase activity. Animals were injected with the indicated amount of cycloheximide/100 g body weight for 1 h (**A**) or with 1 mg cycloheximide/100 g body weight for the indicated times (**B**). Points represent the average value of two independent experiments indicated by bars. Liver extracts were prepared and tested for S6 kinase activity as described in Materials and methods.

Cycloheximide induction

Since cycloheximide injection was the most potent treatment for raising kinase activity and was easily administered to multiple animals, the most efficient conditions of kinase activation were established using this drug. In the concentration range of 1–30 mg of cycloheximide/100 g body

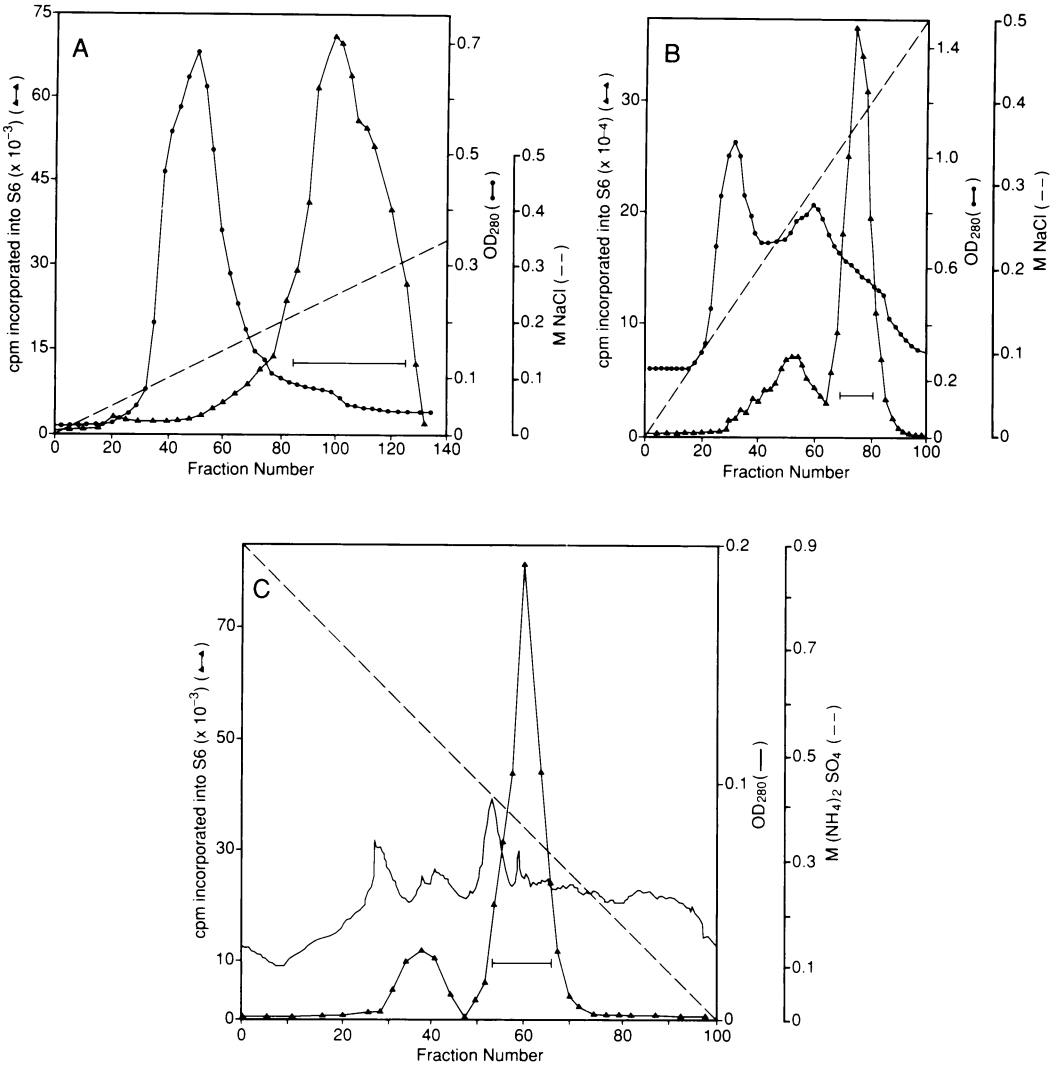

Fig. 3. Elution profile of S6 kinase on (**A**) FFS, (**B**) FFQ and (**C**) phenyl TSK (see Materials and methods). Bar indicates pooled fractions.

Table II. Purification of S6 kinase from rat liver

Steps of purification	Volume (ml)	Total protein (mg)	Sp. act. (nmol/min/mg)	Total activity (nmol/min)	Recovery (%)	Relative purification
Liver extract	4000	100 000.00	0.03	3380	100.0	1.0
FFS pool	2050	4 400.00	0.45	1960	58.0	13.2
FFQ pool	110	198.00	2.98	590	17.5	101.0
Phenyl pool	104	4.50	26.70	120	3.6	789.0
Mono S pool	5	1.08	110.00	110	3.3	3000.0
S300 pool	53	0.15	320.00	79	2.3	9300.0
ATP pool[a]	7	–	–	33	1.0	–
Mono Q pool	0.5	0.05	640.00	32	0.9	19 000.0

Protein content and S6 kinase activity were determined as described in Materials and methods.
[a]The protein concentrations in the ATP fraction could not be determined by the methods previously outlined (Jenö *et al.*, 1989) due to the limiting amount of protein and the presence of ATP.

weight, maximal activation was obtained between 1 and 7.5 mg (Figure 2A). At 1 mg/100 g body weight half-maximal activation was observed within 10 min, and the full effect was achieved between 20 and 40 min and remained at this level for at least 2 h (Figure 2B). For practical reasons, in all subsequent experiments animals were treated with 1 mg/100 g body weight for 1 h. Under these conditions the extent of S6 phosphorylation was identical to that obtained at the higher concentration of cycloheximide employed in Figure 1 (our unpublished data).

Purification scheme

The first steps employed in the purification of S6 kinase from rat liver were cation-exchange chromatography on Fast

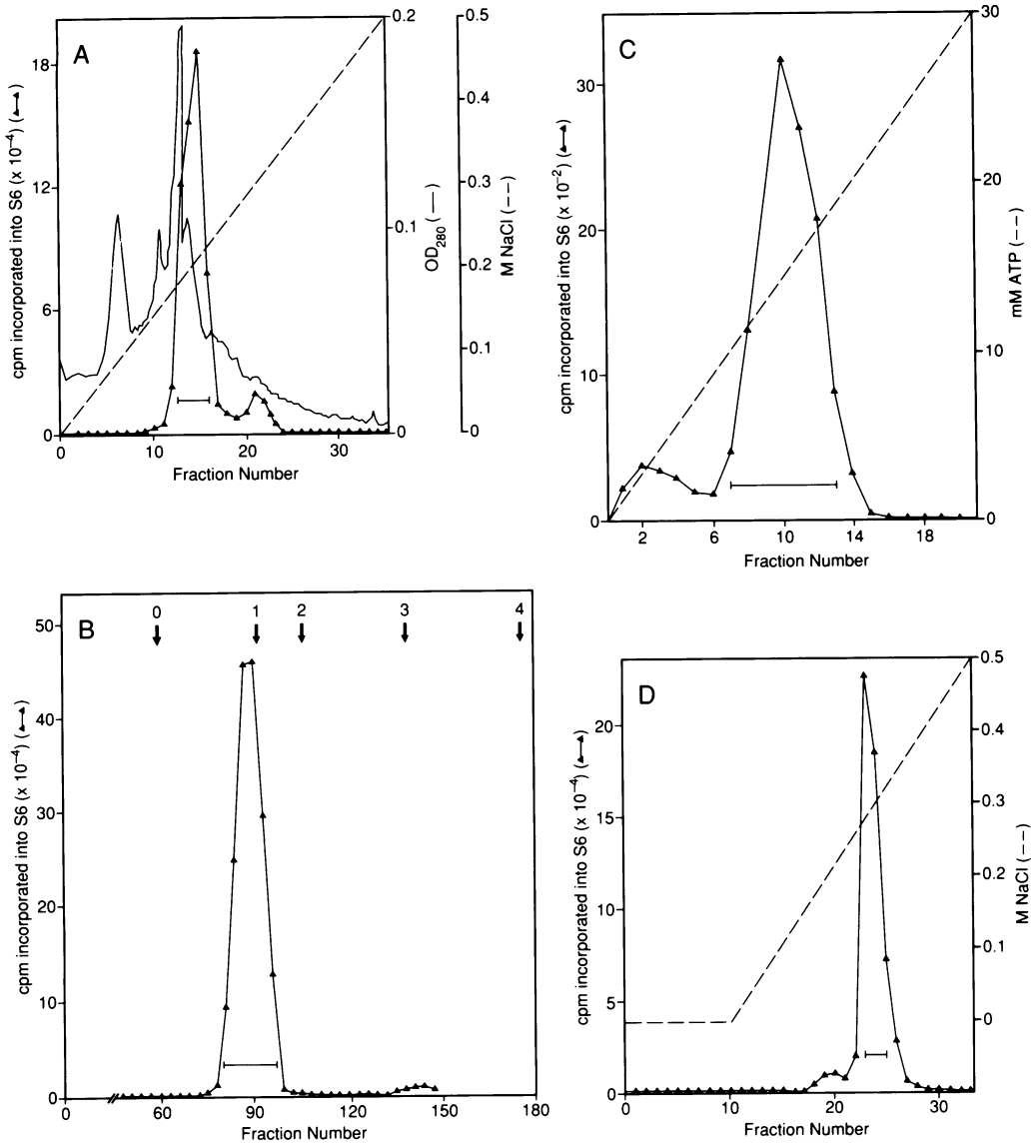

Fig. 4. Elution profile of S6 kinase on (**A**) Mono S, (**B**) Sephacryl S300, (**C**) ATP–agarose (type 4) and (**D**) Mono Q (see Materials and methods). Bar indicates pooled fractions.

Flow S (Figure 3A), to remove endogenous phosphatases (Ballou *et al.*, 1988b; Jenö *et al.*, 1989), followed by anion-exchange chromatography on Fast Flow Q (Figure 3B). The kinase pool was then applied to a Phenyl TSK column where it was resolved into two peaks (Figure 3C). The minor peak varied between 10 and 15% of the total activity applied to the column. At present this kinase peak has not been analysed further. The Phenyl TSK step resulted in an 8- to 10-fold purification of the kinase and reduced total protein >40-fold (Table II). The kinase pool was next resolved on Mono S (Figure 4A), which also served to concentrate the enzyme for purification on Sephacryl S300 (Figure 4B), where the kinase emerged with a $M_r \approx 80\,000$. The sizing column was run in the presence of a low salt concentration so that the enzyme could next be applied to ATP–agarose (type 4) equilibrated in the same buffer. After elution from ATP–agarose (Figure 4C), final purification was achieved on a 250 μl Mono Q column (Figure 4D). This column served to concentrate the kinase as well as to resolve it from free ATP used to elute the protein in the previous step of purification. Finally, the proteins present in the active pools were subjected to SDS–PAGE and analysed by silver staining (Figure 5). After the last step of chromatography three minor proteins of M_r 52 000, 54 000 and 120 000 were revealed, and a single major protein band of M_r 70 000 (Figure 5, lane I). This major protein band migrated at the identical mol. wt previously described for the mitogen-activated mouse kinase (Jenö *et al.*, 1988).

Autophosphorylation

A portion of the material from the Mono Q column was incubated with [γ-^{32}P]ATP to test whether this kinase would autophosphorylate. The results show that under these conditions only the protein of M_r 70 000 became phosphorylated (Figure 5, lane K). In addition, the ability to autophosphorylate directly paralleled S6 kinase activity on the Mono Q column (our unpublished data). Based on the purification scheme outlined in Table II, the sp. act. of the enzyme was 640 nmol/min/mg protein, consistent with values reported for other purified kinases (see Jenö *et al.*,

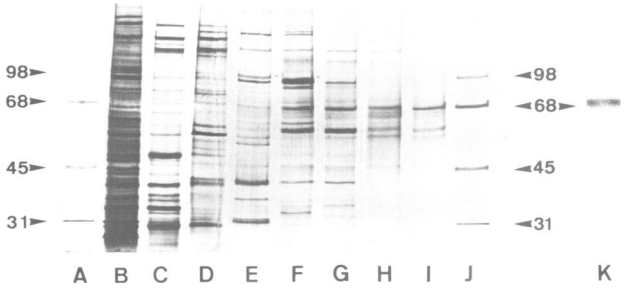

Fig. 5. SDS–PAGE of S6 kinase purification at each step of chromatography. Samples containing the indicated amount of protein were electrophoresed and stained with silver as described under Materials and methods. **Lanes A** and **J**, mol. wt markers (50 ng/protein); **lane B**, liver extract (5 μg); **lane C**, FFS pool (2.1 μg); **lane D**, FFQ pool (1.8 μg); **lane E**, phenyl TSK pool (1.4 μg); **lane F**, Mono S pool (1.1 μg); **lane G**, Sephacryl S300 pool (0.7 μg); **lane H**, ATP–agarose pool (0.4 μg); **lane I**, Mono Q pool (0.2 μg). **Lane K**, autoradiogram of the Mono Q pool incubated alone with [γ-^{32}P]ATP (see Materials and methods).

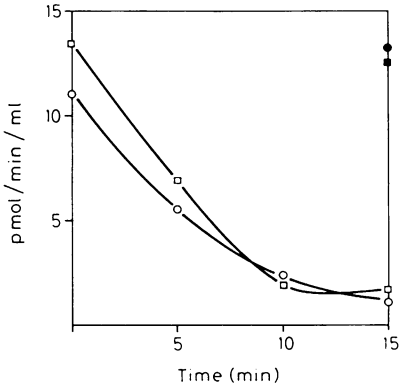

Fig. 6. Phosphatase 2A inactivation of Swiss mouse 3T3 cell or rat liver kinase. The peak fractions from the Mono S column (Figure 3A and C) of either Swiss mouse 3T3 cells (□) or rat liver (○) were treated for the indicated times in the absence (closed symbols) or presence (open symbols) of rabbit skeletal muscle phosphatase 2A as previously described (Jenö *et al.*, 1988).

1989). Thus, the ability to autophosphorylate, and the final sp. act. of the kinase indicated that the protein of M_r 70 000 is the enzyme stimulated by cycloheximide.

Comparison with the Swiss 3T3 cell enzyme

As stated earlier, the Swiss mouse 3T3 cell enzyme is activated by phosphorylation, is preferentially dephosphorylated by a type 2A phosphatase, and, like the rat enzyme, autophosphorylates. To determine whether these two molecules are equivalent, they were tested for sensitivity to phosphatase 2A and their peptide maps were compared following autophosphorylation. The results show that both enzymes were stable at 37°C for 15 min in the absence of phosphatase 2A (Figure 6). However, the addition of phosphatase 2A led to the rapid inactivation of both enzymes at approximately equivalent rates. Next, both molecules were incubated with cyanogen bromide or chymotrypsin following autophosphorylation. In both cases almost identical patterns were observed (Figure 7). Thus, based on phosphatase sensitivity and peptide map analysis, the two enzymes are judged to be equivalent.

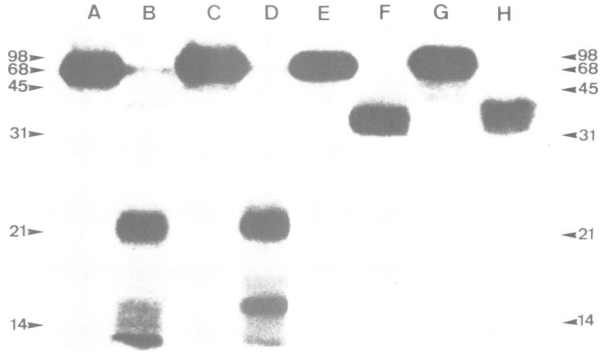

Fig. 7. Peptide maps of autophosphorylated S6 kinase from cycloheximide treated rat liver (**lanes A, B, E** and **F**) or from serum stimulated Swiss 3T3 cells (**lanes C, D, G** and **H**). The kinase preparations were autophosphorylated followed by electrophoresis (**lanes A, C, E** and **G**) or treated with either chymotrypsin (**lanes B** and **D**) or cyanogen bromide (**lanes F** and **H**) prior to electrophoresis (see Materials and methods).

Discussion

Regenerating rat liver has long served as a model for studying growth control (Bucher, 1967). Recently two laboratories have shown that partial hepatectomy leads to a 2- to 8-fold activation of S6 kinase activity. However, when formulating a strategy for purifying this enzyme, we argued that this treatment would be impractical because of the great loss of tissue during the operation and the difficulty in carrying out partial hepatectomy on a large number of animals. These considerations were taken into account in screening techniques known to induce S6 phosphorylation in animals, e.g. insulin or cycloheximide injection, or approaches we reasoned might lead to increased S6 phosphorylation, e.g. fasting and feeding as well as vanadate injection. Cycloheximide was the most potent agent in stimulating S6 kinase activity and S6 phosphorylation. A number of hypotheses have been proposed to explain the mechanism by which cycloheximide induces many of the mitogenic responses (Elder *et al.*, 1984; Makino *et al.*, 1984; Greenberg *et al.*, 1986). The most likely explanation, however, appears to be its direct inhibitory effect on translation. This possibility is supported by the fact that puromycin also induces S6 phosphorylation (Gressner and Wool, 1974b) even though its mode of action in inhibiting protein synthesis is distinct from that of cycloheximide. Whether this effect is due to blocking the synthesis of a short-lived inhibitory protein or whether it represents a more complex stress response awaits future studies.

There appears to be a single kinase responsible for regulating S6 phosphorylation in rat liver, which is identical to the mitogen-activated kinase of 3T3 cells (Jenö *et al.*, 1988, 1989). At least two lines of evidence support this argument. First, when liver extracts from any of the four approaches used to induce S6 phosphorylation are analysed on cation- or anion-exchange columns the kinase activity always elutes as a single entity at an identical salt concentration (our unpublished data), indicating that all of them represent the same enzyme. Second, based on peptide maps of autophosphorylated material, this enzyme is equivalent to the mitogen-activated S6 kinase from 3T3 cells (Figure 7).

Final proof that these molecules are equivalent awaits further immunological and genetic studies. In addition, it must be established that this kinase phosphorylates all the S6 sites observed *in vivo* (Krieg *et al.*, 1988a). It should also be noted that we have selected for optimal conditions leading to S6 kinase activation in rat liver and recovery of activity through the purification. We have found, however, that in some early preparations the small peak of activity on the Mono Q column, in fractions 19–21 (Figure 4D), can be quite pronounced (our unpublished data). This material autophosphorylates at M_r 68 000, has a lower sp. act. towards S6, and probably represents a less highly phosphorylated form of the kinase which could have been generated either by incomplete activation or dephosphorylation during its purification. Again, this conclusion will be tested with antibodies and peptide maps.

From biochemical studies it is clear that the mitogen-activated S6 kinase is distinct from protein kinase C as well as the cyclic AMP-dependent protein kinase and the two protease-activated kinases PAKII and H4PK (Lubben and Traugh, 1983; Donahue and Masaracchia, 1984). It has recently been proposed that the enzyme activated in regenerating rat liver is equivalent to the partially proteolysed Ca^{2+}/phospholipid independent form of protein kinase C (Mizuta *et al.*, 1987). The first step of purification employed by these authors, however, is cation-exchange chromatography, which would concentrate the S6 kinase with phosphatase 2A leading to a severe reduction of total kinase activity (Ballou *et al.*, 1988b). This may explain the low level of kinase activation observed by Mizuta *et al.* (1987). The mitogen-activated S6 kinase appears to be also distinct from amphibian S6 kinase II (Erikson and Maller, 1985). Unlike this enzyme the mitogen-activated S6 kinase appears to be much less abundant, is significantly smaller in size, is strongly inhibited by Mn^{2+} (Jenö *et al.*, 1989), and autophosphorylates on threonine as well as serine residues (Ballou *et al.*, 1988a; Sturgill *et al.*, 1988). More importantly, preliminary sequence data shows no significant homology with this kinase or any other kinase described to date (our unpublished data). The case is less clear for the M_r 67 000 bovine liver enzyme (Tabarini *et al.*, 1987). The mol. wt and abundance of these two enzymes are similar and both are inhibited by μM concentrations of Mn^{2+} in the presence of mM concentrations of Mg^{2+}. However, their final sp. act. towards S6 are quite distinct: 22 nmol/min/mg protein for the bovine enzyme versus 640 nmol/min/mg protein for the mitogen-activated S6 kinase. The difference in activity could be explained by the fact that the bovine enzyme was isolated from animals under conditions where S6 phosphorylation was not activated.

Over the past year we demonstrated that the mitogen-activated S6 kinase was selectively inactivated by a type 2A phosphatase (Ballou *et al.*, 1988b) and activated *in vivo* by serine/threonine phosphorylation (Ballou *et al.*, 1988a). These results supported the model of a phosphorylation cascade, and indicated that there is at least one additional protein kinase which may couple growth factor receptor tyrosine kinases to the activated S6 kinase (Ballou *et al.*, 1988a). However, more recently we discovered that the S6 kinase is biphasically activated, with the second phase of activation under the control of protein kinase C (Susa *et al.*, 1989), implying that the mechanism of activation may be more complex than a simple cascade. This complexity is also supported by the fact that the kinase is multiply phosphorylated (Ballou *et al.*, 1988a) and that to date we have not been able to reactivate the kinase, following phosphatase 2A treatment, with any known protein kinases, including protein kinase C and the MAP 2 kinase (Ray and Sturgill, 1987; Sturgill *et al.*, 1988). Now that sufficient amounts of the mitogen-activated S6 kinase are available from rat liver it will be possible to produce antibodies, sequence and clone the enzyme. These tools will play an essential role in elucidating the mechanism of kinase activation.

Materials and methods

Materials

Fast flow S Sepharose (FFS), Fast Flow Q Sepharose (FFQ), Sephacryl S-300, ATP–agarose Type 4, and mol. wt markers for gel filtration, including ferritin (440 kd), bovine serum albumin (BSA, 68 kd), ovalbumin (45 kd) and ribonuclease (13 kd), were obtained from Pharmacia. Mol. wt markers for SDS–PAGE, including phosphorylase b (98 kd), BSA (68 kd), ovalbumin (45 kd), carbonic anhydrase (31 kd), trypsin inhibitor (21 kd) and lysozyme (14.4 kd) were obtained from Bio-Rad. Cycloheximide and insulin were obtained from Calbiochem. Sodium orthovanadate was purchased from BDH and [γ-^{32}P]ATP (3000 Ci/nmol) from Amersham. All other chemicals used were of analytical grade. Phosphatase 2A was kindly provided by A.R.Olivier and L.M.Ballou.

Solutions

Buffer A contained 5 mM ethylenedinitrilo-tetraacetic acid (EDTA), 1 mM dithiothreitol (DTT), 1 mM benzamidine and 15 mM pyrophosphate, pH 6.8 at 4°C. Buffer B contained 1 mM EDTA, 10 mM NaF, 1 mM DTT, 1 mM benzamidine and 20 mM triethanolamine, pH 7.4 at 4°C. Buffer C contained 10 mM $MgCl_2$, 1 mM EDTA, 1 mM benzamidine, 20 mM β-glycerol phosphate, 20 mM Tris–HCl, pH 7.4 at 4°C. Buffer D contained 10 mM EDTA, 1 mM ethylenebis(oxyethylenenitriol)tetraacetic acid (EGTA), 2 mM DTT, 0.1% Triton X-100 and 10 mM KPO_4, pH 6.8 at 4°C. Buffer E contained 10 mM NaF, 1 mM DTT, 1 mM benzamidine, 0.1 mM EDTA, 0.1% Triton X-100 and 10 mM morpholino-sulphonic acid, pH 7.4 at 4°C. Buffer F contained 1 mM EDTA, 1 mM DTT, 1 mM benzamidine, 0.1% Triton X-100 and 20 mM triethanolamine, pH 7.4 at 4°C.

Protein determination

Unless otherwise stated, protein concentrations were measured by either the Lowry assay or a fluorometric assay as previously described (Jenö *et al.*, 1989).

Protein kinase assays

S6 kinase activity was measured with rat liver 40S subunits as described earlier (Jenö *et al.*, 1989), except when assaying column fractions the ATP concentration was lowered from 30 to 5 μM. Autophosphorylation was carried out as previously described (Jenö *et al.*, 1988).

Gel electrophoresis

Phosphorylated 40S subunits or column fractions were electrophoresed as described (Jenö *et al.*, 1989) on 15% polacrylamide gels containing 0.07% bis-acrylamide according to Laemmli (1970). Silver staining was carried out according to Oakley *et al.* (1980). Two-dimensional gel electrophoresis of 80S ribosome proteins was carried out as previously described (Krieg *et al.*, 1988b).

Peptide maps

Aliquots of purified rat liver or Swiss mouse 3T3 enzyme were autophosphorylated at 37°C for 30 min as previously described (Jenö *et al.*, 1988), and separated by SDS–PAGE. The kinase was located by autoradiography, excised and eluted as previously described (Martin-Pérez and Thomas, 1983). Aliquots containing 10 000 c.p.m. of ^{32}P-labelled protein were either digested with chymotrypsin (Worthington) or cleaved chemically with cyanogen bromide (Fluka). Untreated protein or peptides were separated on 15% acrylamide gel containing 0.5% bis-acrylamide. Gels were stained, dried and autoradiographed as previously described (Novak-Hofer and Thomas, 1984).

Treatment of rats

Adult male Wistar rats weighing ~300 g were injected i.p. with either cycloheximide, insulin or sodium orthovanadate made up in PBS at the concen-

trations indicated in the text. For starved animals food was removed for 24 h prior to refeeding. After the indicated times of treatment, animals were killed by decapitation, the livers removed, washed in PBS, weighed and homogenized in 2.5 vol of ice-cold buffer A made 0.1 mM in phenylmethylsulphonylfluoride (PMSF) in a Sunbeam XPA blender operated twice at full speed for 30 s. All subsequent steps of purification were carried out at 4°C. The homogenate was poured through cheese cloth to remove lipid and then centrifuged at 27 500 *g* for 30 min (Sorval RCB-2 centrifuge, GSA rotor). Supernatants were either frozen and stored in liquid N_2 or immediately processed as described below.

FFS chromatography

In the large-scale preparation of the cycloheximide-induced kinase described here, extracts were prepared from the livers of 50 rats, or 870 g of tissue. Following centrifugation the extract was made 0.1 mM in PMSF and diluted with 2 vol of a buffer containing 1 mM $KH_2PO_4-K_2HPO_4$, pH 6.8 (dilution buffer), giving a final conductivity of $\approx$2.3 mS. The extract was then loaded onto a 600 ml FFS column (11.3 × 10 cm) at 250 ml/min using a Watson–Marlow 501 U pump. It was necessary to handle the sample as rapidly as possible to avoid inactivation of the kinase by the phosphatases and proteases. After loading the sample the column was washed at the same flow rate with 1.8 l of a solution containing one part buffer A and two parts dilution buffer, then developed at 100 ml/min with a 6 l linear salt gradient from 0 to 0.5 M NaCl made up in the same mixture of buffer A and dilution buffer. No kinase activity was detected in the flow-through or wash fractions. The fractions were diluted 1 to 10 for assaying kinase activity as described above. The fractions indicated in Figure 3A were pooled, made 3 μM in 8-Br-cAMP and 0.1 mM in PMSF, then incubated on ice for 10 min.

FFQ chromatography

The pool from the FFS column was diluted with 5 vol of 1 mM triethanolamine, pH 7.4, made 0.1 mM in PMSF and applied at a flow rate of 225 ml/min onto a 100 ml FFQ column (5 × 5.1 cm) equilibrated in buffer B. The column was washed at the same flow rate with buffer B until the OD_{280} dropped to basal levels. The column was then developed with a 1 l linear NaCl gradient from 0 to 0.5 M salt in buffer B. No kinase activity was detected in the flow-through or wash. The fractions were diluted 1 to 20 for assaying activity. The fractions indicated in Figure 3B were pooled.

Phenyl TSK chromatography

The pool from the FFQ column was diluted with 1 vol of 1.8 M ammonium sulphate in buffer C, made 0.1 mM in PMSF, filtered through a 0.22 μm Nalgene filter and applied at a flow rate of 4 ml/min to a 54 ml Phenyl TSK column (21.5 mm × 15 cm) equilibrated in buffer C containing 0.9 M ammonium sulphate. The column was washed in the same buffer until the OD_{280} dropped to the basal level. The column was then developed with a 540 ml linear gradient of ammonium sulphate from 0.9 to 0 M salt in buffer C. The fractions were diluted 1 to 40 for assaying activity. No kinase activity was detected in the flow-through or wash. The fractions indicated in Figure 3C were pooled and dialysed against buffer D overnight.

Mono S chromatography

When the dialysed pool reached a conductivity of $\leq$1.8 mS it was made 0.1 mM in PMSF and applied at a flow rate of 1 ml/min onto an HR 5/5 Mono S column (0.5 × 5 cm) equilibrated in buffer D. The column was washed in buffer D until the OD_{280} dropped to basal levels. The column was then developed with a linear salt gradient of NaCl from 0 to 0.5 M salt. It was important that the conductivity was below 2 mS when the column was being loaded; otherwise, two peaks of activity were observed. Such artefacts were also observed if incompatible buffers were employed (see Pharmacia FPLC Handbook). The fractions were diluted 1 to 20 for assaying kinase activity as described above. No kinase activity was detected in the flow-through or wash. The fractions indicated in Figure 4A were pooled.

Sephacryl S300 chromatography

The pool from the Mono S column was made 0.1 mM in PMSF and applied at a flow rate of 26 ml/h to a 450 ml Sephacryl S300 column (2.6 × 85 cm) equilibrated in buffer E. The fractions were diluted 1 to 2 for assaying kinase activity as described above. The fractions indicated in Figure 4B were pooled.

ATP–agarose (type 4) chromatography

The pool from the Sephacryl S300 column was made 0.1 mM in PMSF and applied at a flow rate of 0.6 ml/min to a 5 ml ATP–agarose column (1 × 6.4 cm) equilibrated in buffer E. The column was washed at the same flow rate with buffer E until the OD_{280} dropped to basal levels. The column was then developed with a linear gradient of ATP from 0 to 30 mM in buffer E. The fractions were diluted 1 to 2000 for assaying kinase activity. No kinase activity was detected in the flow-through or wash. The fractions indicated in Figure 4C were pooled.

Mono Q chromatography

The ATP pool was diluted with 7 vol of buffer F, made 0.1 mM in PMSF and applied at a flow rate of 1 ml/min to a 250 μl Mono Q column (0.5 × 1.3 cm) equilibrated in buffer F. The column was washed with 2 ml of buffer F and developed at 0.1 ml/min with a linear gradient from 0 to 0.5 M NaCl in buffer F. All fractions were collected by hand directly from the end of the column to avoid mixing. The fractions were diluted 1 to 100 for assaying kinase activity. No kinase activity was detected in the flow-through or wash. The fractions indicated in Figure 4D were pooled and stored in liquid N_2.

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