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Comparison of Phosphorylation of Ribosomal Proteins from HeLa and Krebs II Ascites-Tumour Cells by Cyclic AMP-Dependent and Cyclic GMP-Dependent Protein Kinases

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Phosphorylation of eukaryotic ribosomal proteins *in vitro* by essentially homogeneous preparations of cyclic AMP-dependent protein kinase catalytic subunit and cyclic GMP-dependent protein kinase was compared. Each protein kinase was added at a concentration of 30nM. Ribosomal proteins were identified by two-dimensional gel electrophoresis. Almost identical results were obtained when ribosomal subunits from HeLa or ascites-tumour cells were used. About 50–60% of the total radioactive phosphate incorporated into small-subunit ribosomal proteins by either kinase was associated with protein S6. In 90min between 0.7 and 1.0mol of phosphate/mol of protein S6 was incorporated by the catalytic subunit of cyclic AMP-dependent protein kinase. Of the other proteins, S3 and S7 from the small subunit and proteins L6, L18, L19 and L35 from the large subunit were predominantly phosphorylated by the cyclic AMP-dependent enzyme. Between 0.1 and 0.2mol of phosphate was incorporated/mol of these phosphorylated proteins. With the exception of protein S7, the same proteins were also major substrates for the cyclic GMP-dependent protein kinase. Time courses of the phosphorylation of individual proteins from the small and large ribosomal subunits in the presence of either protein kinase suggested four types of phosphorylation reactions: (1) proteins S2, S10 and L5 were preferably phosphorylated by the cyclic GMP-dependent protein kinase; (2) proteins S3 and L6 were phosphorylated at very similar rates by either kinase; (3) proteins S7 and L29 were almost exclusively phosphorylated by the cyclic AMP-dependent protein kinase; (4) protein S6 and most of the other proteins were phosphorylated about two or three times faster by the cyclic AMP-dependent than by the cyclic GMP-dependent enzyme.

Initial observations on the phosphorylation of eukaryotic ribosomal proteins by Loeb & Blat (1970) and Kabat (1970) stimulated investigations by numerous laboratories on the phosphorylation *in vivo* and *in vitro* of ribosomal proteins from a variety of mammalian tissues (Rubin & Rosen, 1975; Goldberg & Haddox, 1977, and references therein). *In vitro*, several proteins from the small (40S) and large (60S) subunit are modified by the cyclic AMP-dependent protein kinase. However, *in vivo* only the phosphorylation of a small-subunit protein, S6, has been observed (Gressner & Wool, 1974; Anderson *et al.*, 1975; Traugh & Porter, 1976). In rat liver, phosphate incorporation into protein S6 was stimulated by glucagon (Blat & Loeb, 1971; Gressner & Wool, 1976*a*), dibutyryl cyclic AMP (Gressner & Wool, 1976*a*), cycloheximide, puromycin (Gressner & Wool, 1976*b*), ethionine (Treloar *et al.*, 1977) and under conditions of liver regeneration (Gressner & Wool, 1974). Stimulation has also been observed in rabbit reticulocytes (Cawthon *et al.*, 1974), pituitary slices (Barden & Labrie, 1973) and rat cerebral cortex (Roberts & Ashby, 1978) with cyclic AMP, and in baby-hamster kidney fibroblasts (Leader *et al.*, 1976) and HeLa cells (Lastick *et al.*, 1977) by the addition of serum. Since most of these conditions are associated with increased intracellular concentrations of cyclic AMP, involvement of cyclic AMP-dependent protein kinase in the phosphorylation of protein S6 has been assumed. More recently, the phosphorylation *in vivo* of other mammalian ribosomal proteins, S2 (Kaerlein & Horak, 1976; Rankine *et al.*, 1977; Roberts & Ashby, 1978) and S3 (Leader & Coia, 1978*b*) from the small subunit and L6, L14 (Leader & Coia, 1978*b*) and 'acidic' (Lastick *et al.*, 1977; Horak & Schiffmann, 1977; Leader & Coia, 1978*a*) proteins from the large subunit, has been reported. The enzyme that catalyses the phosphorylation of

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'acidic' ribosomal proteins *in vitro* has been identified as a cyclic nucleotide-independent protein kinase (Issinger, 1977; Kudlicki *et al.*, 1978). The protein kinases that are involved in the modification of the other ribosomal proteins *in vivo* are unknown.

During the last years a cyclic GMP-dependent protein kinase has been identified in several mammalian tissues (Kuo, 1974; Takai *et al.*, 1975; Gill *et al.*, 1976; Lincoln *et al.*, 1977; Flokerzi *et al.*, 1978). This enzyme has been purified to homogeneity from bovine lung (Gill *et al.*, 1976; Lincoln *et al.*, 1977) and bovine cardiac muscle (Flokerzi *et al.*, 1978) and shown to be distinct from the cyclic AMP-dependent protein kinases. So far, the nature of the substrates of the cyclic GMP-dependent protein kinase *in vivo* is unknown. It was possible that cyclic GMP-dependent protein kinase is involved *in vivo* in the modification of the above-mentioned ribosomal proteins. As a first attempt in this direction, the phosphorylation of ribosomal proteins *in vitro* by homogeneous preparations of cyclic GMP-dependent protein kinase was studied. The results obtained with this enzyme were compared with those obtained in the presence of cyclic AMP-dependent protein kinase catalytic subunit. This comparative approach was necessary in order to evaluate the specificity of possible occurring phosphorylation reactions. Previously it had been shown that *in vitro* both enzymes will phosphorylate identical proteins (Hashimoto *et al.*, 1976; Lincoln & Corbin, 1977; Khoo *et al.*, 1977; Chihara-Nikashima *et al.*, 1977; Blumenthal *et al.*, 1978; Lincoln & Corbin, 1978). It was hoped that by this approach a substrate specificity for either enzyme might be revealed. This possibility was supported by the notion that the organization of a large number of proteins within the ribosomal subunit might result in a differential accessibility of the phosphate-accepting site(s) of individual proteins for either kinase.

Materials and Methods

Materials

ATP, cyclic AMP and cyclic GMP were purchased from Boehringer, Mannheim, Germany. [γ-^{32}P]ATP was prepared essentially by the method of Glynn & Chappell (1964). Other chemicals used were obtained at high purity from local companies.

Methods

Preparation of ribosomal particles. Ribosomes were isolated from Krebs II ascites-tumour cells propagated in Balb/c-strain mice and from HeLa cells (strain S3) grown in suspension cultures at a cell density of 3×10^5 cells/ml (Issinger & Beier, 1978). Ribosomal subunits were prepared as described by Blobel & Sabatini (1971). The subunits were separated on a hyperbolic sucrose density gradient in a buffer containing 500mM-KCl, 3mM-$MgCl_2$, 20mM-Tris/HCl, pH7.6, and 14mM-2-mercaptoethanol (Eikenberry *et al.*, 1970). The separated subunits were pooled, concentrated by centrifugation and stored at −70°C. The concentration of ribosomes and ribosomal subunits was calculated from an absorption coefficient of $A^{1\%}_{260}=130$.

Enzyme preparations. Catalytic subunit of bovine cardiac muscle cyclic AMP-dependent protein kinase (peak II) was purified by a slight modification (Hofmann *et al.*, 1977) of method B as described by Beavo *et al.* (1974). Cyclic GMP-dependent protein kinase was purified from bovine cardiac muscle as described previously (Flokerzi *et al.*, 1978). Each enzyme preparation showed one stained band after polyacrylamide-gel electrophoresis (7.5% acrylamide gels) in the presence of sodium dodecyl sulphate.

Phosphorylation of ribosomal proteins and preparation of samples for gel electrophoresis. The reaction mixture for the phosphorylation of ribosomal proteins contained: 30mM-Tris/HCl, pH7.4; 0.2mM-EGTA; 10mM-$MgCl_2$; 0.1mM- or 1.0mM-[γ-^{32}P]-ATP; 0.3μM-cyclic GMP, when cyclic GMP-dependent protein kinase was added; and either 40S subunit (2mg) or 60S subunit (3mg). The reactions were started for preincubated samples by the addition of cyclic AMP-dependent protein kinase (catalytic subunit) or cyclic GMP-dependent protein kinase at equal concentrations to separate but otherwise identical tubes. The samples were incubated at 37°C for the indicated time. The reactions were terminated by the addition of 0.2vol. of 1M-$MgCl_2$ (final concn. 166mM) and 2vol. of acetic acid (Hardy *et al.*, 1969). After incubation for 60min on ice, the precipitated RNA was removed by centrifugation (18000*g*, 20min). The supernatant containing the ribosomal proteins was dialysed against 7.5% (v/v) propionic acid/14mM-2-mercaptoethanol and was then freeze-dried (Issinger *et al.*, 1975). During the present study, ribosomal subunits from different preparations were used. In each experiment, in which phosphorylation of ribosomal proteins by cyclic AMP-dependent and cyclic GMP-dependent protein kinases was compared, ribosomal subunits from the same preparations were used. To minimize experimental errors in the results for either kinase, phosphorylation reactions, two-dimensional gel electrophoresis and radioautography were performed on the same day with identical buffers.

Two-dimensional gel electrophoresis of ribosomal proteins. Ribosomal proteins were separated by two-dimensional polyacrylamide-gel electrophoresis (Kaltschmidt & Wittmann, 1970; Howard & Traut, 1974) as described previously (Issinger & Beier, 1978). The freeze-dried ribosomal proteins were first suspended in 60μl of 8M-urea/10mM-dithiothreitol, and then 40μl of the running buffer for the first dimension (60mM-Tris/HCl, pH8.6; 6mM-EDTA;

80mM-boric acid; 6M-urea) was added. This mixture (100μl) was incubated for 15min at 37°C and was then divided into two equal portions. Samples migrating towards the cathode ('basic' proteins) were run for 12h at 2.5mA/tube and samples migrating towards the anode ('acidic' proteins) were run for 4h. Electrophoresis in the second dimension was carried out at pH4.5 and 100V for 12h. To minimize eventually occurring loss of phosphorylated proteins, gels of the first dimension were not dialysed against the running buffer of the second dimension. Gels were stained with Coomassie Brilliant Blue R250 and destained in methanol/water/acetic acid (9:9:2, by vol.). Individual proteins were identified by the nomenclature of Sherton & Wool (1974), which has been adapted to the ribosomal proteins from HeLa and Krebs II ascites-tumour cells (Issinger & Beier, 1978).

Radioautography of phosphorylated ribosomal proteins and determination of radioactivity. Destained gels were wrapped in clear plastic sheets and exposed directly against Agfa Osray T4 X-ray film. After identification of radioactive proteins, Coomassie Blue-stained spots were excised with a razor blade from the polyacrylamide gels. Gel pieces were dissolved at 100°C in 1ml of 30% (v/v) H_2O_2 and counted for radioactivity in a Triton X-100/toluene (1:2, v/v)-based scintillation fluid.

Results

Phosphorylation of ribosomal subunits by cyclic AMP- and cyclic GMP-dependent protein kinases

In a first attempt to demonstrate phosphorylation of ribosomal proteins by cyclic GMP-dependent protein kinase, the total trichloroacetic acid-precipitable radioactive phosphate incorporated into ribosomal subunits from HeLa and ascites-tumour cells was determined and compared with the amount incorporated by cyclic AMP-dependent protein kinase (Table 1). Almost identical results were obtained when ribosomal subunits from HeLa and ascites-tumour cells were used; e.g. cyclic AMP-dependent protein kinase incorporated respectively 455 and 400pmol of phosphate/10min into 60S-subunit proteins. Under the same conditions, about half the amount of phosphate that was found in the 60S subunit was incorporated into the 40S subunit. In the presence of the cyclic GMP-dependent protein kinase, roughly equal amounts of phosphate were incorporated into the 60S and 40S subunits, regardless of the source from which the subunits were obtained. As shown in Table 1, the phosphorylation reaction was stimulated by cyclic GMP, indicating that it was caused by the cyclic GMP-dependent protein kinase and not by a different contaminating protein kinase. The ratio of phosphorylation of the 60S and 40S subunits by the cyclic AMP-dependent to that by the cyclic GMP-dependent protein kinase was 4.5 and 2.3 respectively.

Identification of major phosphorylated ribosomal proteins. The results shown in Table 1 suggested that cyclic GMP-dependent protein kinase phosphorylated ribosomal proteins. But these results did not reveal which proteins were phosphorylated by each kinase, since only total trichloroacetic acid-precipitable protein was determined. Therefore experiments were carried out in which ribosomal proteins were first phosphorylated by either kinase and subsequently separated by two-dimensional gel electrophoresis.

Phosphorylation of 40S-subunit proteins. Proteins S2, S3, S4, S6, S7, S9, S11 and S16 from the 40S subunit contained most of the radioactive phosphate, regardless of whether cyclic AMP-dependent or

Table 1. *Phosphorylation of ribosomal subunits by cyclic AMP-dependent and cyclic GMP-dependent protein kinases*
Ribosomal subunits were phosphorylated at 30°C for 10min. The concentration of ATP was 0.1mM and that of cyclic AMP-dependent protein kinase catalytic subunit and cyclic GMP-dependent protein kinase was 10nM. Total incubation volume was 0.11ml. Reactions were terminated and trichloroacetic acid-precipitable radioactivity was determined as described by Reimann *et al.* (1971). The ratio was obtained by dividing the amount of phosphate incorporated by cyclic AMP-dependent protein kinase by that transferred by cyclic GMP-dependent protein kinase.

Ribosomal subunit		Phosphate incorporated (pmol/10min)			
		Cyclic AMP-dependent protein kinase	Cyclic GMP-dependent protein kinase		
Type	(μg)	(*A*)	−cyclic GMP (*B*)	+cyclic GMP (*C*)	Ratio (*A*/*C*)
HeLa cells					
60S	52	455	23	101	4.5
40S	49	210	15	87	2.4
Ascites-tumour cells					
60S	32	400	12	86	4.6
40S	35	205	31	90	2.3

Table 2. *Identification of major phosphorylated proteins of the 40 S subunit*

Proteins of the 40S subunit from ascites-tumour cells (A) and HeLa cells (H) were incubated for 15min at 37°C in the presence of 0.1mM-ATP (specific radioactivity 635c.p.m./pmol). Enzymes were added at a concentration of 30nM each. Total incubation volume was 0.45 and 0.76ml for subunits from HeLa cells and ascites-tumour cells respectively. Individual samples for each kinase were subjected to two-dimensional gel electrophoresis, and radioactive protein spots were localized by radioautograms. Thereafter radioactive spots were excised and counted for radioactivity. For further details see also the Materials and Methods section and the legend to Table 1.

Protein	Source of ribosomes	Radioactivity (c.p.m.) Cyclic AMP-dependent protein kinase	Cyclic GMP-dependent protein kinase	Ratio
S2	A	2800	2370	1.2
	H	1098	1851	0.6
S3	A	11460	5827	1.9
	H	9250	6282	1.5
S4	A	13930	2399	5.8
	H	14235	4342	3.3
S6	A	72457	27942	2.6
	H	120672	57474	2.1
S7	A	21977	503	43.7
	H	16572	507	29.0
S9	A	2213	2325	1.0
	H	2830	2090	1.4
S11	A	4925	653	7.5
	H	941	399	2.3
S16	A	2561	275	9.3
	H	1380	119	11.5

Table 3. *Identification of major phosphorylated proteins of the 60 S subunit*

Proteins of the 60S subunit from ascites-tumour cells (A) and HeLa cells (H) were incubated and processed under identical conditions as described in the legend to Table 2. Total incubation volume was 0.45ml for each subunit.

Protein	Source of ribosomes	Radioactivity (c.p.m.) Cyclic AMP-dependent protein kinase	Cyclic GMP-dependent protein kinase	Ratio
L3	A	8821	—	—
	H	18421	1982	9.3
L6	A	25135	10637	2.4
	H	6953	—	—
L15	A	11239	2670	4.2
	H	11950	1672	7.2
L18	A	24826	13668	1.8
	H	10509	6204	1.7
L19	A	15502	7889	2.0
	H	13948	4820	2.9
L21	A	11253	1218	9.2
	H	7841	1069	7.3
L28	A	8386	5353	1.6
	H	—	—	—
L29	A	8943	2348	3.8
	H	8553	2093	4.0
L34	A	19108	8448	2.2
	H	12930	5513	2.3
L35	A	27124	9621	2.8
	H	10472	1970	5.3
L36	A	22588	2968	7.6
	H	6244	795	7.8

cyclic GMP-dependent protein kinase was added (Table 2). Small amounts of radioactivity were associated with proteins S10, S25 and S26. The amount of phosphate incorporated per protein spot was quite similar when ribosomal subunits from HeLa cells or ascites-tumour cells were used. In agreement with results obtained previously *in vivo* (Gressner & Wool, 1974; Leader *et al.*, 1976), protein S6 was the most heavily phosphorylated protein. In the presence of cyclic AMP-dependent protein kinase, between 50 and 60% of the total amount of radioactivity incorporated into the 40S-subunit proteins was incorporated into protein S6. About 35% of the total amount of radioactivity was associated with proteins S3, S4 and S7, and 5–10% with the other proteins. Since protein S4 is located directly 'north-west' of protein S6 (see also Fig. 1*a*) and protein S6 is the most heavily phosphorylated protein, unambiguous identification of S4 as a phosphoprotein is difficult. In the presence of cyclic GMP-dependent protein kinase, more than 60% of the total amount of radioactivity was present in protein S6. Roughly 14% was associated with protein S3 and about 17% with proteins S2, S4 and S9.

Surprisingly, protein S7 was only very slightly phosphorylated by cyclic GMP-dependent protein kinase. The ratios of the amounts of phosphate incorporated into individual proteins by cyclic AMP-dependent protein kinase to that by cyclic GMP-dependent protein kinase varied from 0.6 to 1.4 for proteins S2 and S9 to about 35 for protein S7, and were between 2 and 3 for protein S6.

Phosphorylation of 60S-subunit proteins. Phosphorylation of the 60S-subunit proteins by cyclic AMP-dependent protein kinase and cyclic GMP-dependent protein kinase showed an almost even distribution of radioactivity between several proteins. No equivalent to protein S6 was found. The major phosphorylated proteins found here were L6, L18, L34 and L36 (Table 3). About one-third of the label of protein S6 was incorporated by cyclic AMP-dependent protein kinase into each of these proteins, when ribosomes from ascites-tumour cells were used. With the exception of protein L36, cyclic GMP-dependent protein kinase incorporated about half as much phosphate into these proteins as did cyclic AMP-dependent protein kinase. These proteins were also the major phosphorylated proteins from the HeLa-cell 60S subunit. For unknown reasons less phosphate was associated with the proteins derived from HeLa cells compared with those from ascites-tumour cells. About 11 proteins are potential substrates for each kinase (L3, L6, L15, L18, L21, L28, L29, L34, L35 and L36). Another five proteins of the large subunit were phosphorylated by the cyclic AMP-dependent protein kinase (L4, L9, L24, L27 and L37), none of which was a substrate for the cyclic GMP-dependent protein kinase (results not shown). These latter proteins were phosphorylated only when ATP at high specific radioactivity was used, and usually less than 100c.p.m. was incorporated. Of the major phosphorylated proteins (L6, L18, L19, L34, L36), protein L36 has a phosphorylation ratio (cyclic AMP-dependent protein kinase relative to cyclic GMP-dependent protein kinase) of about 7, whereas all the other strongly phosphorylated proteins had comparable ratios of about 2. In no instance was phosphorylation of 'acidic' proteins by either kinase detected.

Time course of the phosphorylation of individual proteins

The results of the experiments shown in Tables 2 and 3 suggested that with a few exceptions most of the identified phosphoproteins were phosphorylated by either kinase. However, certain proteins appeared to be a preferred substrate for one of the two protein kinases. Since only one time point during the phosphorylation reaction was taken, it could not be ascertained that these differences were due to the different protein kinases used. Differential denaturation of the ribosomal subunits might also have resulted in an apparent substrate specificity. To minimize this possibility, time courses of the phosphorylation reaction were studied. A total incubation time of 90min was chosen, since it was assumed that at this time possible denaturation of the ribosomal subunits should be equal in each set of experiments. In addition, phosphate incorporation was determined also after 5 and 30min incubation. The 5min time point was included in order to gain some information in the initial rates of phosphorylation of individual proteins. In contrast with the previous experiments, the ATP concentration was raised to 1mM, and, to ensure that the reaction could proceed for well over 30min, additional [γ-^{32}P]ATP and enzymes were added after this time. Only ribosomal subunits from ascites-tumour cells were used for this experiment, since large quantities of each subunit were required. The results of these experiments are presented in two ways, by using radioautography of the gels and by determination of the radioactivity of excised proteins by liquid-scintillation spectrometry. It is well known that both methods have certain disadvantages, which can result in an apparent substrate specificity for each enzyme.

Phosphorylation of 40S-subunit proteins. The radioautograms of 40S-subunit proteins (Fig. 1) suggested that after 5min protein S6 was already phosphorylated, regardless of which kinase was present (Figs. 1*b* and 1*f*). After 30min (Figs. 1*c* and 1*g*) a strong increase in the phosphorylation of protein S6 by either enzyme was observed. In addition, proteins S3 and S7 were phosphorylated by cyclic AMP-dependent protein kinase (Fig. 1*c*). In the presence of

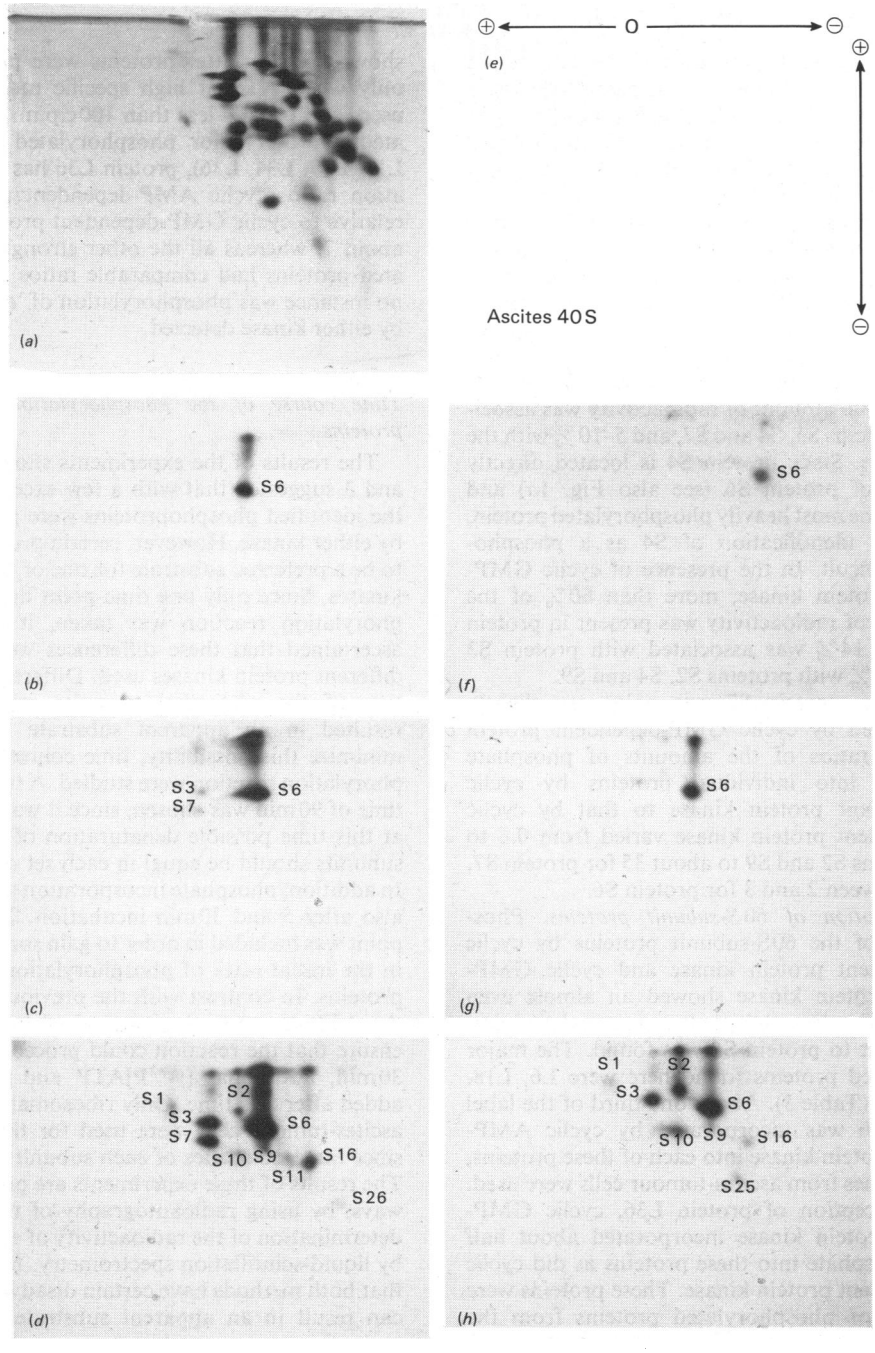

Fig. 1. *Radioautograms of the time course of phosphorylation of* 40*S ribosomal proteins*

Small ribosomal subunits from ascites-tumour cells (6.5mg) were incubated at 37°C in the presence of 1mM-ATP. Total incubation volume was 1.5ml and reactions were started by addition of cyclic AMP-dependent protein kinase catalytic subunit and cyclic GMP-dependent protein kinase (30nM) to separate tubes. After 5, 30 and 90min, 0.48ml portions were removed from the tubes and treated as described in the Materials and Methods section. After 30min, one-third of the initially added amounts of either enzyme and [γ-^{32}P]ATP were added again. All gels were exposed for 12h at the same day. (*a*) Protein stain pattern; (*e*) schematic drawing of protein stain; (*b*)–(*d*) and (*f*)–(*h*), radioautograms of phosphorylated proteins in the presence of cyclic AMP-dependent and cyclic GMP-dependent protein kinase respectively. Incubation time was for 5min (*b*, *f*), 30min (*c*, *g*) and 90min (*d*, *h*). To avoid obstruction of radioactive spots, only some of the phosphorylated proteins have been given a number.

cyclic GMP-dependent protein kinase no phosphorylation of protein S7 occurred, but proteins S2 and S3 were slightly phosphorylated. After 90min (Fig. 1*d*) proteins S1, S2, S3, S4, S6, S7, S9, S10, S11, S16 and S26 were phosphorylated by cyclic AMP-dependent protein kinase. Proteins S3, S7 and S6 were the most predominant phosphorylated ones. When cyclic GMP-dependent protein kinase was used, similar but not identical results were obtained (Fig. 1*h*). Proteins S1, S2, S3, S6, S9, S10, S16 and S25 were phosphorylated, but not proteins S7 and S11. Although the radioautograms indicated that during the first 5min only protein S6 was phosphorylated by either kinase, determination of the total amount of phosphate in each protein spot showed that those proteins that were phosphorylated during the 90min time period already contained radioactive phosphate at the 5min time point. However, different amounts of phosphate were found in each protein (results not shown, but see also Fig. 3). Obviously, the rate of phosphorylation was distinct for each protein. The highest rate was obtained for the phosphorylation of protein S6, which result was already expected from the values shown in Tables 2 and 3. Inspection of Fig. 1 suggests that cyclic AMP-dependent protein kinase has a broader affinity towards different ribosomal proteins than the cyclic GMP-dependent protein kinase.

Phosphorylation of 60S-subunit proteins. In contrast with the results obtained with the 40S proteins, nearly all of the proteins that were phosphorylated during the time course incorporated radioactive phosphate during the first 5min of incubation when cyclic AMP-dependent protein kinase was added (Fig. 2*b*). In the presence of cyclic GMP-dependent protein kinase the overall phosphorylation was less pronounced. Only protein L6 was significantly phosphorylated during the first 5min of incubation (Fig. 2*a*). After 30min an increase in the overall intensity of phosphorylation and phosphorylation of additional proteins was observed (Figs. 2*c* and 2*g*). After 90min of incubation phosphorylation of additional proteins (Figs. 2*d* and 2*h*) by either enzyme was not detectable. Similar to the results obtained with the 40S-subunit proteins, most proteins that were phosphorylated by the cyclic AMP-dependent protein kinase were also phosphorylated by the cyclic GMP-dependent protein kinase, although in general to a lesser extent. But a few exceptions are evident. Protein L29 was only weakly phosphorylated by the cyclic GMP-dependent protein kinase (Figs. 2*g* and 2*h*). Proteins L3 and L9 appear to be almost exclusively phosphorylated by the cyclic AMP-dependent protein kinase, although only small amounts of phosphate were incorporated into these proteins. The contrary appears to be true for protein L15, which after 90min was phosphorylated more by the cyclic GMP-dependent protein kinase than by the cyclic AMP-dependent protein kinase.

Apparent phosphorylation rates. From the radioautograms it is evident that different amounts of phosphate were incorporated into individual proteins by each protein kinase. This impression was confirmed by direct measurement of the radioactivity associated with each protein spot. These measurements suggested four types of phosphate incorporation. Representative results are shown in Fig. 3. (1) Phosphorylation of a protein by cyclic AMP-dependent protein kinase which is only marginally phosphorylated by cyclic GMP-dependent protein kinase. Proteins S7 and L29 are representatives of this type of phosphorylation. (2) Equal or very similar phosphate incorporation by either kinase (protein S3). (3) A protein is more readily phosphorylated by the cyclic GMP-dependent than by the cyclic AMP-dependent protein kinase. Proteins S2, S10 and L5 belong to this category. (4) A protein is a better substrate for the cyclic AMP-dependent protein kinase, but may also be phosphorylated by cyclic GMP-dependent protein kinase. Most proteins belong to this category. As typical representatives proteins S6 and L19 are shown. In general the differences observed between cyclic AMP-dependent and cyclic GMP-dependent phosphate incorporation into individual proteins at the 90min time point were also found at the earlier time points. As is evident from Fig. 3, linear rates were not always obtained during the 90min incubation. In addition, biphasic phosphorylation reactions were observed (proteins S3, S7 and S10). These latter phosphorylation reactions are most likely the result of structural alterations of the ribosomal subunits during the long incubation period. These alterations might make these proteins available as substrates for the protein kinases. However, as is evident from Fig. 3, marked differences in the affinity of these proteins for cyclic AMP-dependent and cyclic GMP-dependent protein kinase exist. This finding might indicate that the presumed structural alterations are not identical with a denaturation of the ribosomal proteins during the incubation period.

Stoichiometry of phosphorylation. The stoichiometry of phosphorylation for different proteins was estimated by using the following assumptions. (1) The protein spot on the final gel contained 50% of that amount that was present in the incubation reaction. This assumption appears to be reasonable, since preparation of the sample for gel electrophoresis involves a precipitation, dialysis and freeze-drying step. In addition, not all of the protein present in the first gel enters the second gel. Considering all these steps, 50% recovery may even be an overestimation of the actual amount of protein present in a single spot. (2) It was further assumed that 40S and 60S subunits contain 56% and 45% protein respectively (Wool & Stöffler, 1974). The number of individual

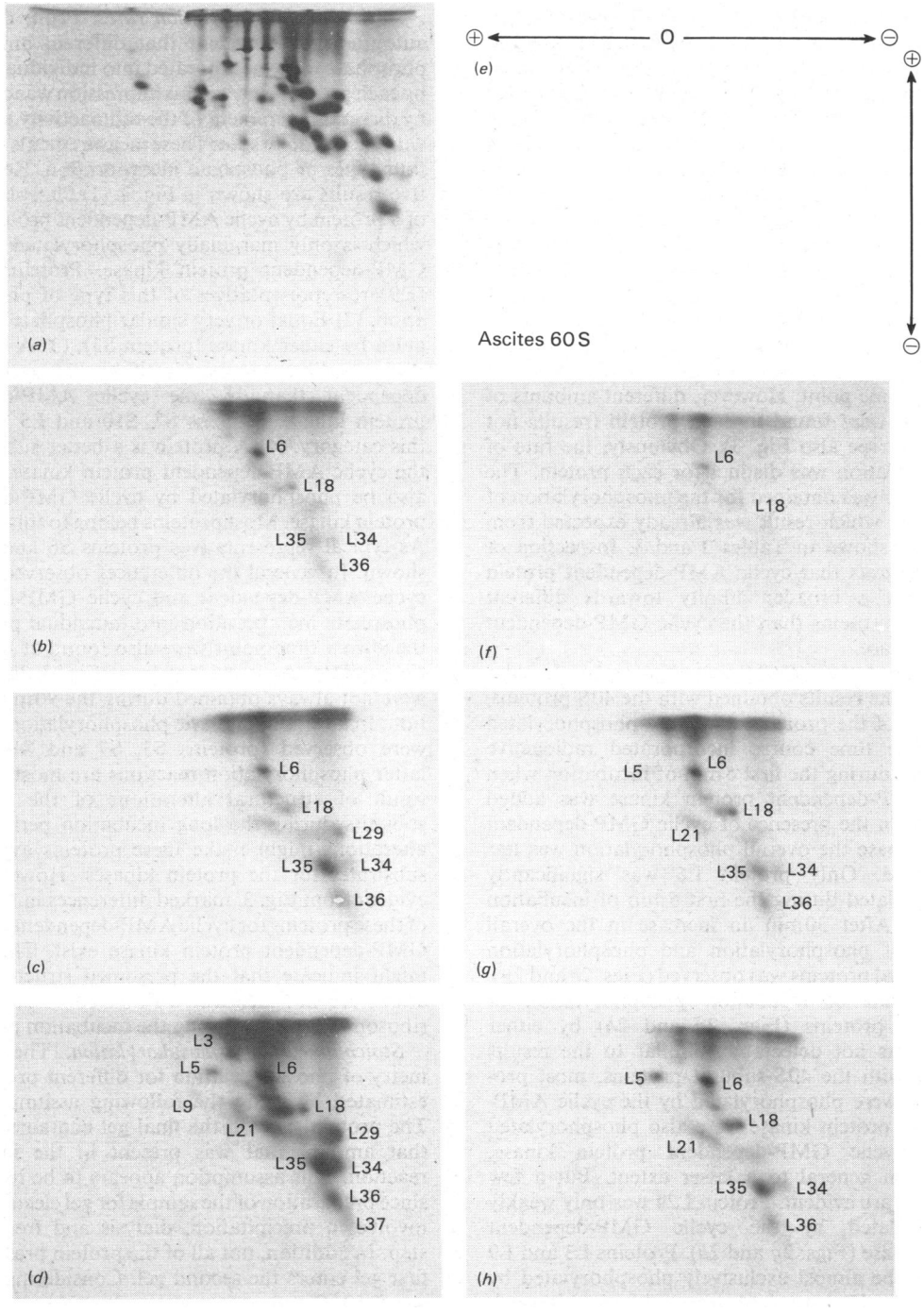

Fig. 2. *Radioautograms of the time course of phosphorylation of* 60 *S ribosomal proteins*
Large ribosomal subunits from ascites-tumour cells (10 mg) were incubated and processed as described in the legend to Fig. 1. The lettering system (*a–h*) is identical with that given in the legend to Fig. 1.

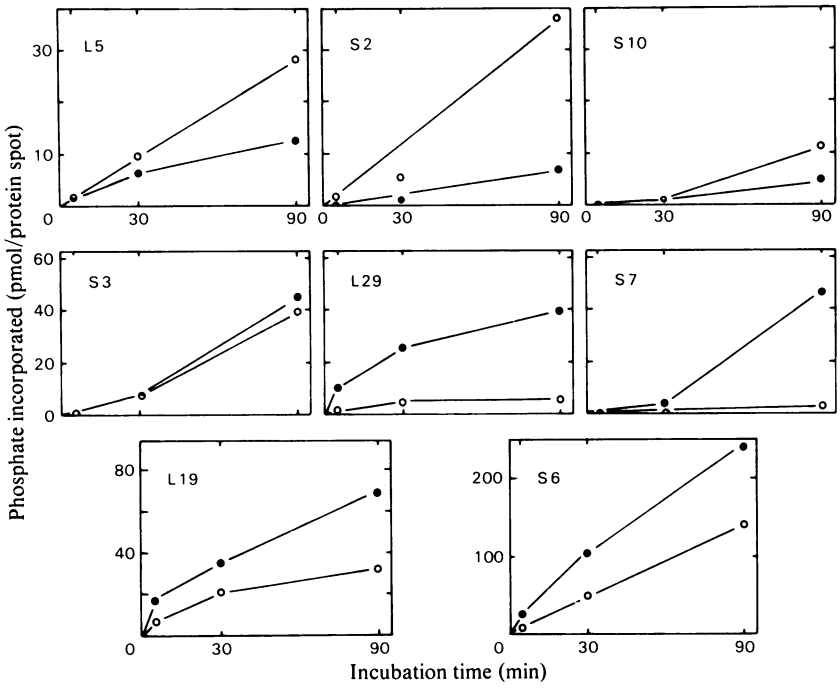

Fig. 3. *Representative phosphorylation rates obtained in the presence of cyclic AMP-dependent and cyclic GMP-dependent protein kinases*

Data given were obtained from the experiments shown in Figs. 1 and 2. Representative radioactive spots were excised from the corresponding gels and were counted for radioactivity after solubilization of the gel. For experimental details see the legends to Figs. 1 and 2 and the Materials and Methods section. (●) and (○), amounts of phosphate incorporated in the presence of cyclic AMP-dependent and cyclic GMP-dependent protein kinase respectively. Note the differences in scales.

proteins per 40S and 60S subunit was estimated as 29 (average M_r 21000) and 35 (average M_r 23000) respectively (Issinger & Beier, 1978). By using these assumptions it was estimated that between 0.7 and 1.0mol of phosphate was incorporated/mol of protein S6 and between 0.1 and 0.2mol of phosphate/mol of the other major phosphorylated proteins at the 90min time point. Very similar values can be derived from the data given in Tables 2 and 3 for ribosomal proteins from ascites-tumour cells, if the 15min values are extrapolated to a 90min time point. The number obtained for protein S6 in this study is somewhat lower than that reported for the same protein derived from reticulocytes (DuVernay & Traugh, 1978); in that report between 1.0 and 1.7mol of phosphate was incorporated/mol of protein S6 by cyclic AMP-dependent protein kinase *in vitro*. *In vivo*, incorporation of up to 6mol of phosphate/mol of protein S6 has been observed (Gressner & Wool, 1974; Anderson *et al.*, 1975; Kaerlein & Horak, 1976). The reason for the differences between the results *in vivo* and *in vitro* are unknown and may be only apparent, since the phosphate content of protein S6 used for the present study is unknown. However, the differences observed could also indicate that *in vivo* cyclic AMP-dependent protein kinase phosphorylates only one or two sites, whereas additional sites are modified by other protein kinases. Multiple-site phosphorylation of one protein by several different protein kinases has been demonstrated with several proteins (Nimmo & Cohen, 1977).

Discussion

Studies *in vitro* are useful tools for the analysis of certain reactions that might occur *in vivo*, since studies *in vitro* can be carried out under well-defined conditions. For a number of reasons, however, it is quite obvious that results obtained *in vitro* may be not at all related to the situation occurring *in vivo*. On the other hand, studies *in vitro* might have application for the future analysis of the role of ribosomal-protein phosphorylation on the mechanism of protein synthesis. To our knowledge this is the first study in which homogeneous preparations of cyclic AMP-dependent protein kinase catalytic subunit and

of cyclic GMP-dependent protein kinase have been used. Up to 10 ribosomal proteins were phosphorylated significantly by each protein kinase. Similar results have been obtained previously *in vitro* (Stahl *et al.*, 1972; Eil & Wool, 1973; Ventimiglia & Wool, 1974). In contrast with these observations, Traugh & Porter (1976) reported that in rabbit reticulocyte ribosomes only protein S6 is phosphorylated by cyclic AMP-dependent protein kinase *in vitro*. The reasons for the discrepancy between this report and our results are not known, but may have been caused by differences in the source of ribosomes, in the incubation conditions and in the enzymes used. On the other hand the observed discrepancy may be more apparent than real, since in our experiments homogeneous preparations of cyclic AMP-dependent protein kinase catalytic subunit at rather high but physiological concentrations were used, whereas Traugh & Porter (1976) presumably used low concentrations of a partially purified cyclic AMP-dependent protein kinase. Nevertheless it is evident from the experiments shown that protein S6 is the major phosphorylated ribosomal protein. The physiological significance of the phosphorylation of the other ribosomal proteins is uncertain. Evidence indicates that under certain conditions not only protein S6 but also protein S2 (Kaerlein & Horak, 1976; Rankine *et al.*, 1977; Roberts & Ashby, 1978) and proteins S3, L6 and L14 are phosphorylated *in vivo* (Leader & Coia, 1978*b*). Our observation that several ribosomal proteins are phosphorylated *in vitro* is in line with these reports, since phosphorylation reactions occurring *in vivo* should also occur *in vitro*.

The protein kinases that are responsible for the modification of the above-mentioned proteins *in vivo* are unknown. It has been suggested that protein S6 is phosphorylated by cyclic AMP-dependent protein kinase *in vivo* (Gressner & Wool, 1976*a*). Later reports pointed out that moderate elevations of the cyclic AMP concentration within cells, by which concentrations cyclic AMP-dependent protein kinases were presumably activated, did not increase significantly the phosphorylation of protein S6 (Leader *et al.*, 1976). In the present report it is shown that, at equal concentrations of enzymes, which are within the physiological range for both enzymes, cyclic GMP-dependent protein kinase phosphorylates protein S6 at about half the rate that is obtained in the presence of the catalytic subunit of cyclic AMP-dependent protein kinase. The results shown would favour the idea that *in vivo* cyclic AMP-dependent protein kinase is responsible for the phosphorylation of protein S6. However, the results do not exclude the possibility that this protein can also be phosphorylated by a cyclic GMP-dependent protein kinase *in vivo*, since only total phosphate incorporated into protein S6 was measured. It is possible that both enzymes phosphorylate different sites in this protein, since up to six phosphate groups have been detected in protein S6.

Protein S2 was phosphorylated about 4 times faster by cyclic GMP-dependent than by cyclic AMP-dependent protein kinase. Although less than stoichiometric amounts of phosphate were incorporated, this result is of interest. To our knowledge this is the first known protein that is phosphorylated *in vitro* faster by cyclic GMP-dependent than by cyclic AMP-dependent protein kinase. On the other hand, phosphorylation *in vivo* of protein S2 has been observed, indicating that phosphorylation of this protein may be of physiological significance (Kaerlein & Horak, 1976; Rankine *et al.*, 1977; Roberts & Ashby, 1978). It is possible that cyclic GMP-dependent protein kinase catalyses the modification of this protein *in vivo*. Similar considerations may be pertinent with regard to protein S3, which was phosphorylated at equal rates by either kinase and is also phosphorylated *in vivo* (Leader & Coia, 1978*b*).

In general, the results of the present study may indicate that organization of several proteins in large particles may result in selective substrate specificity for cyclic nucleotide-dependent protein kinases *in vivo*, which is not observed when phosphorylation of a single purified protein is studied *in vitro*. This interpretation is supported by previous reports which showed apparent specific phosphorylation of membranes from several smooth-muscle tissues and intestinal brush-border membranes by cyclic GMP-dependent protein kinase (Casnelli & Greengard, 1974; DeJonge, 1976).

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