THE PHOSPHORYLATION OF RIBOSOMAL PROTEIN S6 IN BABY HAMSTER KIDNEY FIBROBLASTS

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SUMMARY

Ribosomal protein S6 was extensively phosphorylated in pre-confluent but not in post-confluent baby hamster kidney fibroblasts. This appears to be the first example of increased phosphorylation of S6 under physiological conditions where the cellular concentration of cyclic AMP is not elevated. The extent of the phosphorylation of S6 was also independent of alterations in the protein synthetic activity of the cells, suggesting that the biological role of this phosphorylation may be unrelated to the functional ability of the ribosomes.

INTRODUCTION

The extent of phosphorylation of ribosomal protein S6 (nomenclature of Sherton and Wool (1)) can be altered by a number of stimuli. Cyclic AMP administered directly to both rabbit reticulocytes (2) or rat liver (3) causes an increased phosphorylation of S6, a situation also found in the livers of glucagon-treated (4), partial hepatectomised (5), or diabetic (6) rats, where increased concentrations of cyclic AMP have been observed. Although inhibitors of protein synthesis can also stimulate the phosphorylation of S6 (7, 3), their effects may be indirect and it has been suggested (5) that the phosphorylation of S6 might, in fact, increase the synthesis of certain proteins. Despite this, careful studies with phosphorylated and dephosphorylated ribosomes failed to reveal any differences in protein synthetic activity in vitro (8,9), and the

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biological role of the phosphorylation of S6 remained unresolved. We thought it might be useful, therefore, to examine this phosphorylation using animal cells in culture, where the conditions of growth can affect both the rates of protein synthesis (10) and the cellular concentrations of cyclic AMP (11). Our results with baby hamster kidney fibroblasts, presented here, demonstrate that increased phosphorylation of ribosomal protein S6 need not be associated with either elevated concentrations of cyclic AMP or greater protein synthesis, and suggest the need for a reappraisal of the role of the phosphorylation.

MATERIALS AND METHODS

BHK21/C13 cells, an established line of hamster fibroblasts (12), were grown in rotating 80 oz. roller bottles in Eagle's medium (Glasgow modification (13)) containing 10% (v/v) calf serum and 10% (v/v) tryptose phosphate broth at 37°C in an atmosphere of 95% air/5% CO_2 (14). Cells were 'seeded' at a density of 2×10^7 cells per bottle in 180 ml medium, which was normally replaced after three days and every two days thereafter. The cells reached confluence after about four days but continued to grow for three or four days thereafter, albeit at a slower rate. Those cells designated pre-confluent were harvested after two or three days' growth, whereas those designated post-confluent were harvested after five to eight days' growth. In some experiments BHK21/C13/PyY cells, a variant which had been transformed by polyoma virus (15), were used. These grew more rapidly than the parent strain, reaching confluence after about three days.

In experiments in which the ribosomal proteins were labelled with (^{32}P) orthophosphate the medium was replaced by medium (50 ml) from which orthophosphate and tryptose phosphate broth had been excluded. (^{32}P) orthophosphate (5mCi) was then added to two of the twenty bottles used and incubation continued for a further 3 hr before harvesting the cells. In experiments where the cells were not labelled, the medium was not replaced before harvesting the cells, unless specifically stated in the text.

Cells were removed from the bottles with a rubber scraper and ribosomes isolated, essentially as described by Ascione and Arlinghaus (16). Ribosomal subunits were prepared from these (17) and the ribosomal protein extracted with acetic acid (1). Two-dimensional gel electrophoresis, in which the first dimension contained 4% acrylamide (3.3% bisacrylamide) and 6M urea (pH 8.7) and the second dimension contained 18% acrylamide (1.4% bisacrylamide) and 6M urea (pH 4.5), was performed as previously described (18).

Sucrose density gradient analysis of polysomes was performed by layering 1.8ml post-nuclear cell extract (16) onto a 10 - 50% linear gradient (37ml) of sucrose in a solution containing 50mM Tris-HCl (pH 7.6), 200mM KCl, 5mM $MgCl_2$ and centrifuging for 2 hr at 82,500g in a SW27 rotor of a Beckman ultracentrifuge at 2°C. The gradients were then pumped through the flow-cell of a Gilford Model 240 recording spectrophotometer and analysed at 260nm.

The concentration of cyclic AMP in BHK cells was determined by a modification (19) of the protein-binding competition assay of Gilman (20), using a kit supplied by the Radiochemical Centre, Amersham, Bucks, U.K. A trichloroacetic acid extract of the cells was prepared for assay by ether extraction, followed by elution with 2N formic acid from a Dowex-1 (formate) column (21). The values obtained were corrected for the recovery of (^3H) cyclic AMP added to the original extract and expressed in terms of the total cellular protein, determined by a modification of the Lowry method (22).

RESULTS AND DISCUSSION

The phosphorylation of ribosomal protein S6 involves the formation of derivatives of the protein containing up to five phosphoryl groups, and the more phosphorylated of these can be visualised in the stained gels obtained after subjecting the protein to two-dimensional electrophoresis in systems involving separation on the basis of charge in the first dimension (3, 5, 23, 24). This is illustrated for pre-confluent BHK cells in Fig. 1 where an anodic 'tail' to the left of, and slightly upward from, the parent S6 corresponds to the radioactivity associated with the protein. This 'tail' was far more extensive than we had previously observed in Krebs II ascites cells (23) and indicated the possibility of detecting gross changes in the phosphorylation of S6, merely by examining stained two-dimensional gels of unlabelled ribosomal proteins. We decided to adopt this approach to studying the phosphorylation as it circumvented two problems associated with the use of (^{32}P) orthophosphate: the effect of the state of cellular growth on the uptake of phosphate (25), and the fact that the phosphate-deficient medium

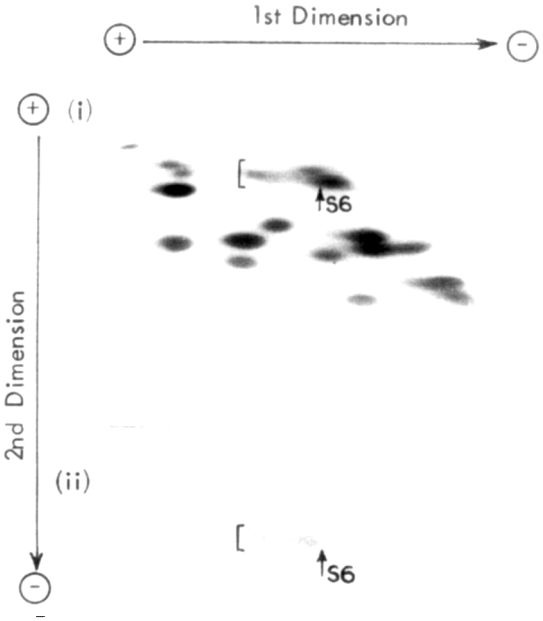

Figure 1. Phosphorylation of ribosomal protein S6 in pre-confluent BHK cells incubated with (^{32}P) orthophosphate. The upper frame (i) shows a stained gel and the lower frame (ii) the relevant area of the corresponding autoradiograph following two-dimensional gel electrophoresis of ribosomal protein (200µg) from the 40S subunit. The arrow and parenthesis marks, indicating the beginning and end of the tail of S6, are in identical positions in both frames.

we used to label the cells altered the condition of protein synthesis being considered (see Fig. 3, below).

When we examined the extent of the phosphorylated derivatives of S6 in unlabelled BHK cells (Fig. 2) it was clear that this was much lower in post-confluent than in pre-confluent cells. This was somewhat surprising in view of reports that the cyclic AMP concentrations of growing cells are generally lower than those of

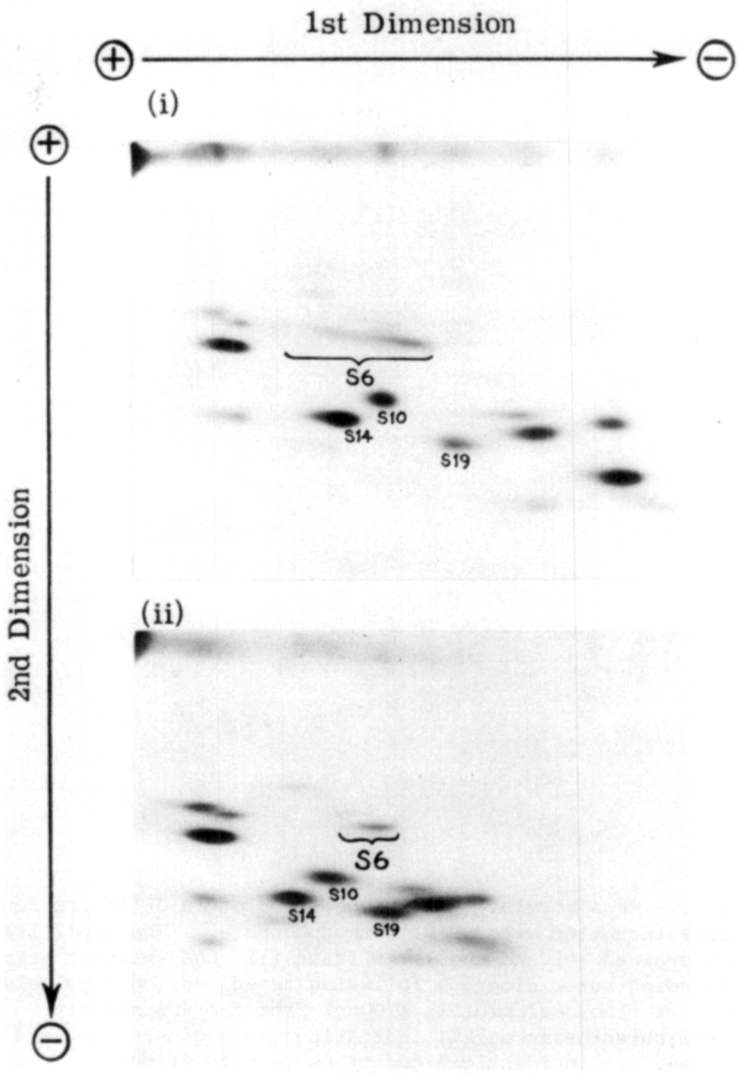

Figure 2. The phosphorylation of ribosomal protein S6 in pre-confluent and post-confluent BHK cells. The frames show stained two-dimensional gels of ribosomal protein from the 40S subunits of unlabelled (i) pre-confluent, and (ii) post-confluent BHK cells. The parentheses delineate the visible extent of protein S6 and its derivatives and the positions of proteins S14, S10 and S19 are indicated as reference points.

resting cells. We thus felt it necessary to measure the levels of this nucleotide in our own cells and found that there was only

a small difference in the concentration of cyclic AMP in the pre-confluent and post-confluent BHK cells (1.8 and 2.4 pmole/mg protein, respectively) which was not, in fact, statistically significant. The difference between these results and those of Rudland et al (11) (who found values of 7.6 and 13.2 pmoles cyclic AMP per mg of protein in growing and resting BHK cells, respectively) is probably due to their use of cells whose growth had been completely arrested, in contrast to the post-confluent cells in this study. What is important is that the concentration of cyclic AMP in pre-confluent BHK cells is not elevated and yet there is extensive phosphorylation of ribosomal protein S6. This is, to our knowledge, the first time such an increase has been observed under physiological conditions, independently of increased levels of cyclic AMP.

It seemed possible that the difference between the phosphorylation of S6 in pre-confluent and post-confluent cells was related to different rates of protein synthesis in these cells. Because the comparative incorporation of radioactive amino acids into cellular protein presents difficulties of interpretation

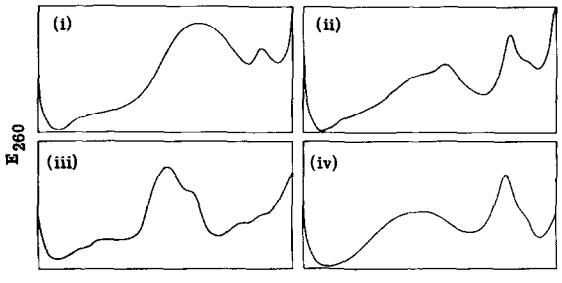

Figure 3. Sucrose density gradient analysis of polysomes and monosomes from BHK cells. Sedimentation was from right to left, as described in the text. (i) pre-confluent cells (2 days of growth) (ii) post-confluent cells (7 days of growth) (iii) post-confluent cells incubated 1 hr in fresh, complete, medium (iv) pre-confluent cells incubated 3 hr in medium lacking tryptose phosphate broth.

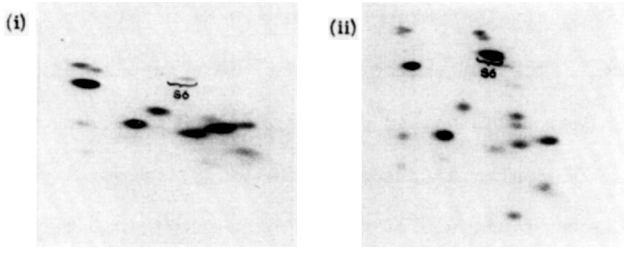

Figure 4. Effect of fresh medium on the phosphorylation of ribosomal protein S6 in post-confluent BHK cells. The frames show stained two-dimensional gels of ribosomal proteins from the 40S subunits of unlabelled post-confluent BHK cells (i) No addition of fresh medium before harvesting cells. (ii) Fresh medium added 1 hr before harvesting cells.

similar to those with orthophosphate, we have used the ratio of polysomes to monosomes as an indication of the rates of protein synthesis. Fig. 3, (i)-(iii), shows that the proportion of polysomes was lower in the post-confluent than in pre-confluent cells, but that this difference was abolished when fresh medium was added to the former. However this addition of fresh medium did not increase the extent of phosphorylation of S6 in post-confluent cells (Fig. 4), suggesting that the phosphorylation of S6 was not related to the rate of protein synthesis. This conclusion was supported by other considerations. Thus, replacement of the medium of pre-confluent cells with a medium lacking tryptose phosphate broth results in a decrease in the proportion of polysomes (Fig. 3, iv). However the pre-confluent cells of Fig. 1 were labelled in just such a medium but have S6 no less phosphorylated than the pre-confluent cells of Fig. 2, i. Finally, a similar difference in extent of phosphorylation of S6 was seen in pre-confluent and post-confluent cells of the more

rapidly growing, polyoma-transformed, PyY line (not shown).

Our findings that extensive phosphorylation of ribosomal protein S6 can occur without an elevation of the concentration of cellular cyclic AMP and independently of the rate of protein synthesis call into question previous ideas that the biological role of this phosphorylation is related to the function of ribosomes in the cytoplasm. Although we have no direct evidence to support it, we are attracted to the idea (5) that the phosphorylation might, instead, be important for the formation of ribosomes in the nucleolus. This is because our results can be reconciled with previous ones (2 - 7) by assuming that ribosomal protein S6 is normally dephosphorylated after leaving the nucleus, but is susceptible to (non-functional) rephosphorylation under certain conditions. If this were so, the greater phosphorylation of S6 in pre-confluent BHK cells could reflect either a higher proportion of newly synthesised ribosomes or a lower cytoplasmic phosphoprotein phosphatase activity than in post-confluent cells.

However there are other conceivable roles for the phosphorylation of S6, for example in preventing the degradation of ribosomes, which is increased in resting cells (26). Clearly, further experiments are required to distinguish between these and other possibilities.

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