THE PHOSPHORYLATION OF RIBOSOMAL PROTEIN S6 ON THE MONORIBOSOMES AND POLYRIBOSOMES OF BABY HAMSTER KIDNEY FIBROBLASTS

David P. LEADER and Amanda A. COIA
Department of Biochemistry, University of Glasgow, Glasgow G12 8QQ, Scotland

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1. Introduction

In normal animal cells in vivo, ribosomal protein S6 (nomenclature from [1]) is the only phosphorylated protein of the 40 S ribosomal subunit [2–5]. The extent to which this protein is phosphorylated can be increased in certain situations, including rapid cellular growth [2,6], the administration of inhibitors of protein synthesis [7,8], and the administration of cyclic AMP [9–11]. The diversity of these stimuli has made it difficult to formulate a consistent biological role for the phosphorylation of S6, but the peculiar multiphosphorylation of this protein [2,6,12] and its evolutionary conservation among eukaryotes [13,14] argue against the phosphorylation being merely gratuitous. One early result that seemed important in this regard was the finding [15] that a protein, which was probably S6, was only phosphorylated on the polyribosomes of reticulocytes and sarcoma cells, the inactive monoribosomes being virtually unphosphorylated. However, because this result was not confirmed with protein S6 from the ribosomes of regenerating rat liver [2], it has generally been disregarded. We report here that in baby hamster kidney fibroblasts (BHK cells) the phosphorylation of S6 is normally confined to polyribosomes, but that in certain circumstances S6 can become phosphorylated on monoribosomes. Our findings make it possible to reconcile the previous conflicting reports and provide a context for the reassessment of the function of the phosphorylation of ribosomal protein S6.

2. Methods

The methods used for growth and labelling of the cells, and preparation of the post-nuclear cell extract are detailed in [6,16]. Cycloheximide (50 μg/ml) was added to cells 1 min before harvesting to prevent any polyribosomes disaggregating during cooling. The post-nuclear cell extract (1.8 ml) was layered onto a 10–50% linear gradient (37 ml) of sucrose in a solution containing 50 mM Tris–HCl (pH 7.6), 200 mM KCl, 5 mM $MgCl_2$ and centrifuged for 2 h at 82 000 × *g* in the SW27 rotor of a Beckman ultracentrifuge at 2°C. The gradients were analysed at 260 nm, using the flow cell of a Gilford Model 240 recording spectrophotometer, and the portions of the gradients indicated in fig.1 were collected

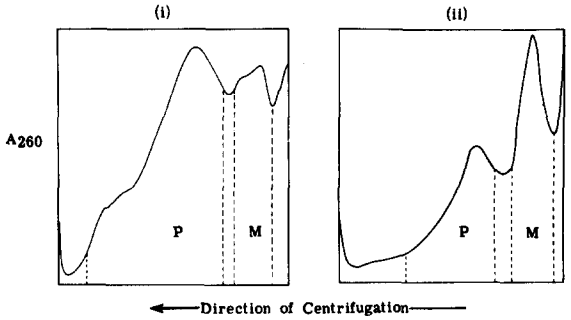

Fig.1. Sucrose density-gradient separation of polyribosomes (P) and monoribosomes (M) from BHK cells incubated with [^{32}P]orthophosphate for 3 h. (i) Untreated cells. (ii) Cells incubated with 2 mM cyclic AMP and 2.5 mM theophylline. The portions of the gradients between the broken lines were collected.

separately for the polyribosome and monoribosome fractions. The ribosomes in these fractions were sedimented at 78 000 X *g* for 16 h in the 30 rotor of a Beckman ultracentrifuge at 2°C. The ribosomes were then dissociated into their subunits and their proteins extracted and isolated as in [17].

The ribosomal proteins were analysed either by one-dimensional electrophoresis in gels containing sodium dodecyl sulphate and having an acrylamide concentration of 12.5% [17], or by two-dimensional gel electrophoresis using minor modifications of the general method [18] as in [19]. In later two-dimensional gel electrophoretic analyses (fig.5) we have used modifications of the first dimension gel composition and interdimensional annealing procedure suggested [20].

3. Results and discussion

The distribution of ^{32}P in 40 S ribosomal proteins from monoribosomes and polyribosomes of confluent BHK cells was analysed by one-dimensional gel electrophoresis in sodium dodecyl sulphate (fig.2).

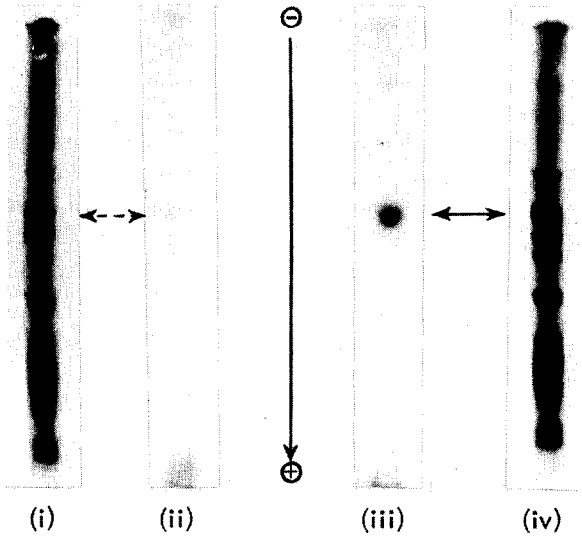

Fig.2. One-dimensional gel electrophoresis of phosphorylated protein extracted from the 40 S ribosomal subunits of untreated BHK cells. (i) Stained gel of protein from monoribosomes. (ii) Autoradiograph of protein from monoribosomes. (iii) Autoradiograph of protein from polyribosomes. (iv) Stained gel of protein from polyribosomes.

It can be seen that the radioactive band present on the polyribosomes was virtually absent from the monoribosomes. A similar pattern of phosphorylation had been observed [15] of their phosphoprotein II in reticulocyte and sarcoma cell ribosomes and although the phosphorylation of II was stimulated by cyclic AMP [9], like that of rat liver S6 [10], the equivalence of phosphoprotein II and S6 was never, in fact, established. However, it is clear from the standard two-dimensional analysis in fig.3 that the phosphoprotein present on the 40 S subunit of BHK polyribosomes is S6. This differential phosphorylation was specific for S6 as, in the same experiment, we found Lγ (the acidic ribosomal phosphoprotein of the 60 S ribosomal subunit) to be equally phosphorylated on monoribosomes and polyribosomes [16].

Other results, however, indicated that the restriction of the phosphorylation of S6 to polyribosomes was not universal. Thus, when BHK cells were treated with cyclic AMP and the monoribosomes examined by one-dimensional gel electrophoresis in sodium dodecyl sulphate, the band corresponding to S6 was found to be labelled, albeit to a lesser extent than in polyribosomes (fig.4). Again, two-dimensional gel electrophoresis confirmed that S6 was the phosphorylated protein (results not shown).

The phosphorylation of S6 on monoribosomes may be due to an overall increase in phosphorylation, for cyclic AMP caused the appearance of phosphorylated derivatives of S6 (fig.5), similar to those originally described [2]. Whether or not this is the correct interpretation of the present results (see below), a dependence of the phosphorylation of S6 in monoribosomes on the overall extent of phosphorylation of the protein would reconcile the results in [2] with those in [15]. Thus in regenerating rat liver the phosphorylation of S6 on monoribosomes would result from the marked stimulation of phosphorylation occurring under those conditions [2].

However in the experiments presented here an alternative explanation is possible for the appearance of phosphorylated S6 on monoribosomes (fig.4). Such phosphorylated S6 may, in fact, be derived originally from polyribosomes which were subsequently converted to monoribosomes by the treatment with cyclic AMP (fig.1). Certainly, the persistence of phosphorylated S6 in monoribosomes derived from polyribosomes is consistent with our previous observa-

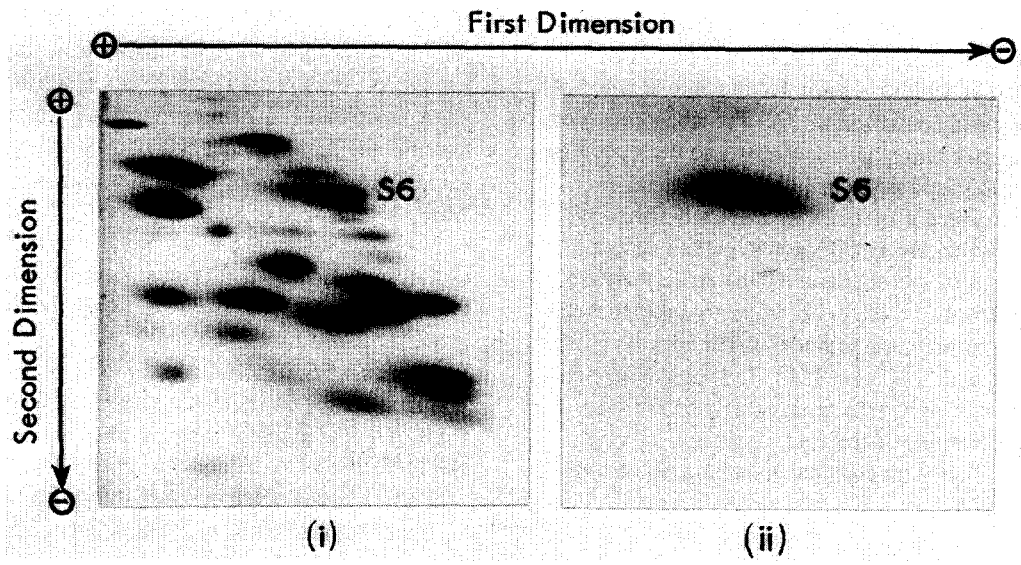

Fig.3. Two-dimensional gel electrophoresis of phosphorylated protein extracted from 40 S subunits derived from the polyribosomes of untreated BHK cells. (i) Stained gel. (ii) Autoradiograph.

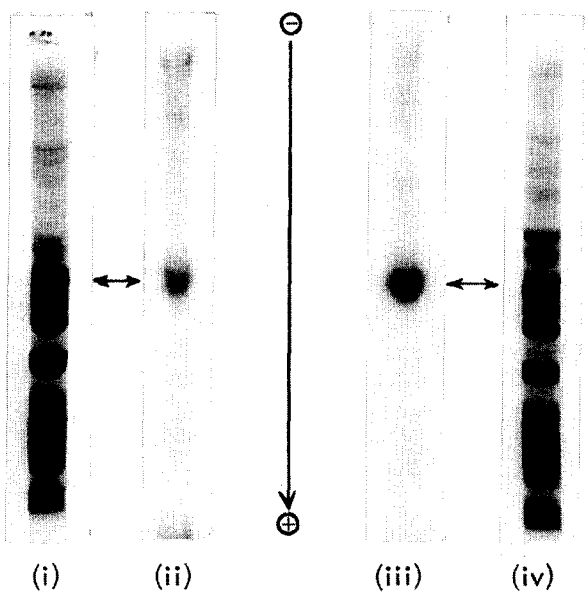

Fig.4. One-dimensional gel electrophoresis of phosphorylated protein extracted from the 40 S ribosomal subunits of BHK cells incubated for 3 h with 2 mM cyclic AMP and 2.5 mM theophylline. (i) Stained gel of protein from monoribosomes. (ii) Autoradiograph of protein from monoribosomes. (iii) Autoradiograph of protein from polyribosomes. (iv) Stained gel of protein from polyribosomes.

tion that rapidly growing BHK cells, subjected to short-term nutritional shift-down, showed no overall reduction in phosphorylation, despite a large increase in the proportion of monoribosomes [6].

Regardless of which of the explanations is correct, it is necessary to consider how these results affect ideas regarding the function of the phosphorylation of S6. We have previously argued that the phosphorylation of S6 has no role in protein biosynthesis [6], and our arguments can be extended to accommodate the present results by assuming that the polyribosomes afford S6 greater protection from dephosphorylation than do monoribosomes. Such a protection might be fortuitous (e.g., if the phosphorylation of S6 were proposed to have a role in the nucleus), or be related to some function of phosphorylated S6 (e.g., protecting active ribosomes from degradation).

The recent finding by Ogata et al. (quoted in [21]) that ribosomal protein S6 can be chemically cross-linked to mRNA is quite consistent with the suggestion that S6 is more protected from dephosphorylation on polyribosomes than on monoribosomes. However, considered alongside the results presented here, it also allows for an alternative possibility in which the phosphorylation of S6 might promote the binding of mRNA to the ribosome. We do not feel that the

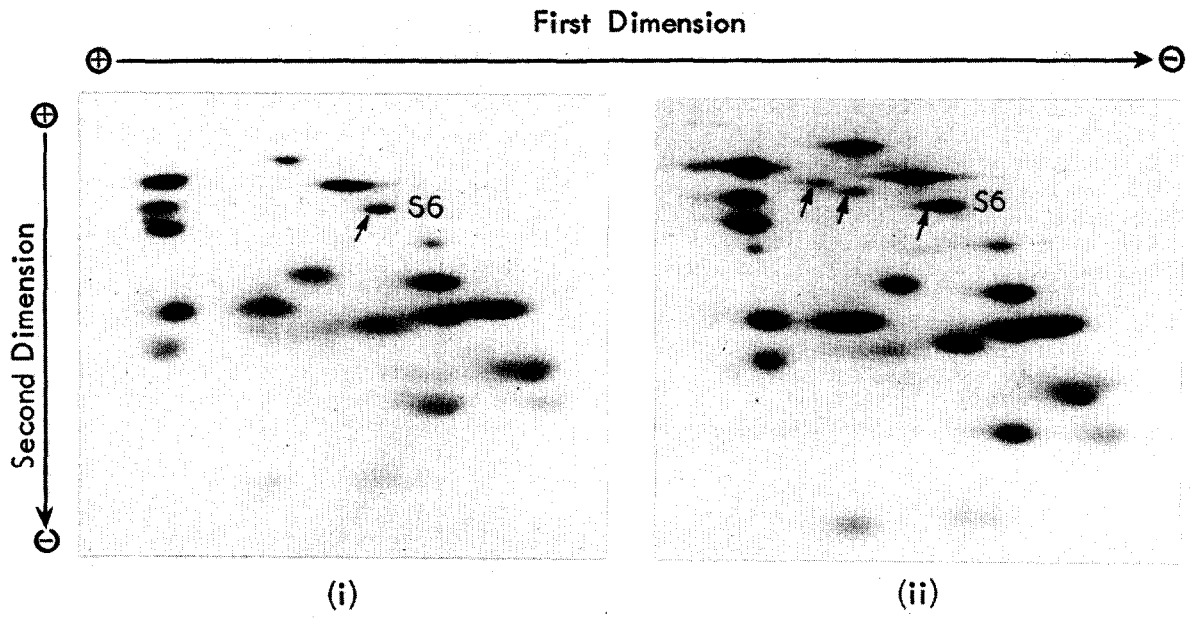

Fig.5. Two-dimensional gel electrophoresis of protein extracted from the 40 S subunit of BHK cells. (i) Stained gel of protein from untreated cells. (ii) Stained gel of protein from cells incubated for 3 h with 2 mM cyclic AMP and 2.5 mM theophylline. The arrows indicate the 'parent' S6 spot and the anodic phosphorylated derivatives of this. The overall increase in incorporation of [^{32}P]orthophosphate into 40 S ribosomal protein following treatment with cyclic AMP was, on average, about 100%.

published data are consistent with an obligatory role for the phosphorylation of S6 in protein biosynthesis, but we do find it conceivable that the phosphorylation might be one of a number of means of increasing the efficiency of initiation. This idea would account for the phosphorylation of S6 in rapidly growing cells where a high rate of protein synthesis is appropriate, but encounters more difficulty in accomodating those examples of phosphorylation associated with the inhibition of protein biosynthesis. However it can be postulated that in these instances the phosphorylation is an attempt to compensate for the inhibition of protein synthesis. In both types of circumstances where S6 is phosphorylated the rate of protein biosynthesis might become limited by other components (mRNA, initiation factors, etc.) so that monoribosomes bearing phosphorylated S6 could accumulate.

In conclusion we emphasise that the results presented here firmly reassert the preferential phosphorylation of S6 in polyribosomes, while demonstrating that circumstances exist in which S6 becomes phosphorylated in monoribosomes. While the results themselves do not confirm any particular hypothesis regarding the function of the phosphorylation of S6, they provide relevant information which any valid hypothesis must be able to accommodate.

Acknowledgement

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