THE PHOSPHORYLATION OF ASCITES CELL RIBOSOMES IN VIVO: IDENTIFICATION OF A PHOSPHORYLATED PROTEIN OF THE SMALL RIBOSOMAL SUBUNIT BY TWO-DIMENSIONAL GEL ELECTROPHORESIS

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1. Introduction

Certain ribosomal proteins from rat liver [1,2] rabbit reticulocytes [3,4] and mouse sarcoma cells [5] are phosphorylated in vivo. Of special interest is a protein of the small (40 S) ribosomal subunit, the phosphorylation of which is influenced by various factors, including cyclic AMP [2,4]. In the studies cited, the ribosomal proteins were analysed by one-dimensional polyacrylamide gel electrophoresis, either in urea or sodium dodecyl sulphate. However this technique does not completely resolve the ribosomal proteins; nor does it allow phosphorylated ribosomal proteins to be distinguished, unequivocably, from labelled non-ribosomal contaminants [3,6]. We present here a study of the phosphorylation of ribosomes in Krebs II ascites cells where we have analysed the ribosomal proteins by two-dimensional gel electrophoresis. This method gives much superior resolution of ribosomal proteins and provides a common frame of reference for different workers [7,8]. We report that S6 (nomenclature of Sherton and Wool [8]) is the protein of the 40 S subunit most extensively phosphorylated in incubated ascites cells.

2. Experimental

2.1. *Phosphorylation of ascites cells*

Krebs II ascites cells were propagated in Porton-strain mice, harvested after eight days and washed as described previously [9]. They were resuspended at a concentration of 10^7 cells per ml incubation medium, which was Eagle's medium, buffered with Tris–citrate [10], and containing 10% dialysed calf-serum but without orthophosphate. [^{32}P] orthophosphate (0.05 mCi per ml) was then added and the cell suspension (200 ml) incubated at 37°C for 3 hr in a 1 litre conical flask with constant gentle stirring.

2.2. *Preparation of ribosomal protein*

At the end of the incubation ribosomes were isolated from the cells as previously described [9]. Ribosomal subunits were prepared from these [11] and the ribosomal protein extracted with acetic acid [8]. The ribosomal protein from the 40 S subunit usually had a spec. act. of about 10 000 cpm per mg.

2.3. *Gel electrophoresis*

One-dimensional gel electrophoresis was in sodium dodecyl sulphate and 6 M urea at an acrylamide concentration of 10%, essentially as described by King et al. [12]. The mol. wts of proteins were estimated according to Weber and Osborn [13] from parallel gels with the protein standards: bovine serum albumin, chymotrypsinogen and cytochrome *c*.

Two-dimensional gel electrophoresis was performed essentially as described by Howard and Traut [14]. The first dimension gels contained 4% acrylamide and 6 M urea (pH 8.7) and were run at a current of 6 mA per gel. The second dimension gel slabs (7 × 7 cm) contained 18% acrylamide and 6 M urea (pH 4.5) and were run at a current of 9 mA per gel. Further details are given elsewhere [15]. Autoradiography was with Kodak KD – 54T film and exposure was generally for about one month.

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3. Results and discussion

We first characterised the phosphorylated ribosomal protein from the 40 S subunit by one-dimensional gel electrophoresis in sodium dodecyl sulphate (fig.1). We observed a major phosphorylated band (A), corresponding to a protein of approximate mol. wt 36 000. This is probably the same as the band II which Kabat et al. described and for which they have quoted mol. wts of 33 000 [3] and 27 000 [5]. We also observed a second, much weaker, phosphorylated band (B), corresponding to a protein of approximate mol. wt 46 000 and possibly analagous to band IV (mol. wt: 53 000) of Kabat [4].

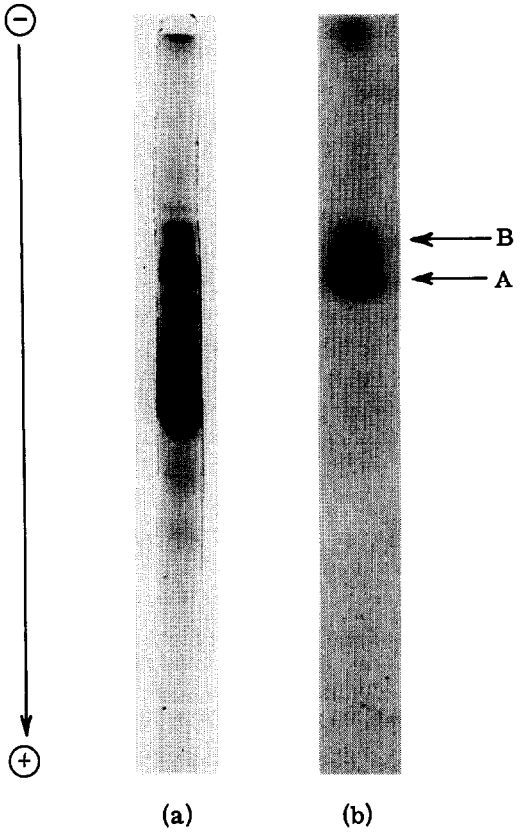

Fig.1. One-dimensional gel electrophoresis of phosphorylated protein from 40 S ribosomal subunits. Ribosomal protein (125 μg) was subjected to electrophoresis as described in Experimental and the gel stained, sliced longitudinally, dried onto filter paper [16] and an autoradiograph made. a) Electropherogram of gel stained with Coomassie Brilliant Blue. b) Autoradiography of the same gel.

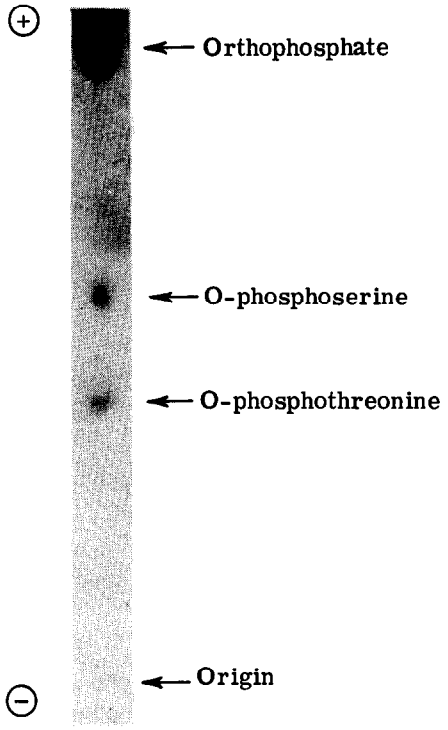

Fig.2. High-voltage paper electrophoresis of acid hydrolysate of protein extracted from 40 S ribosomal subunits. The protein was hydrolysed in 2 N HCl for 16 hr at 105°C, subjected to electrophoresis at pH 1.85 for 2 hr at 2.5 kV, essentially as described by Langan [17] and an autoradiograph made. The positions of authentic marker compounds are indicated.

It was necessary to determine whether the radioactivity we had found associated with the ribosomal protein was, in fact, incorporated into the phosphoryl groups of phosphoprotein. When the protein was heated at 90°C for 20 min in 10% trichloroacetic acid only 13% of the radioactivity was released. However when it was incubated for 60 min at 37°C with alkaline phosphatase, 75% of the radioactivity was liberated (results not shown). When the protein was hydrolysed in acid and analysed by paper electrophoresis (fig.2) both phosphoserine and phosphothreonine were found. (There was also a considerable amount of radioactive orthophosphate which may have arisen from acid hydrolysis of the phosphoserine and phosphothreonine [18]). We therefore felt reasonably

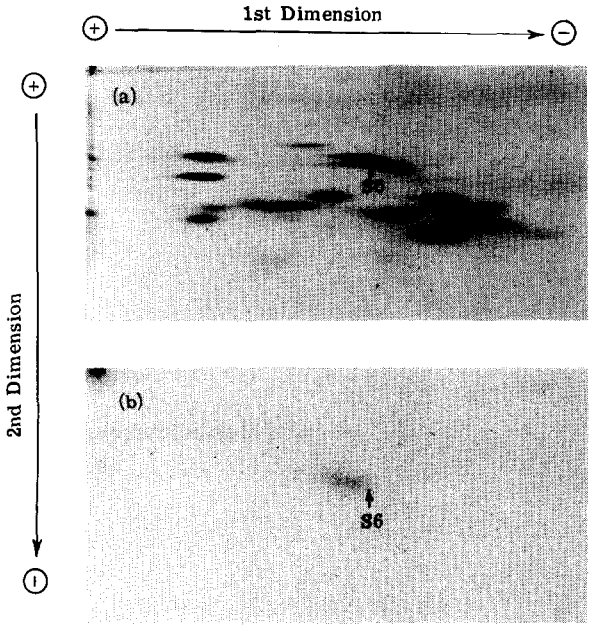

Fig.3. Two-dimensional gel electrophoresis of phosphorylated protein from 40 S ribosomal subunits. Ribosomal protein (100 μg) was subjected to electrophoresis as indicated in the text. The gel was sealed in a polythene bag and an autoradiograph made. a) Electropherogram of gel stained with Coomassie Brilliant Blue. (The numbering of proteins other than S6 is described elsewhere [8,15]). b) Autoradiograph of the same gel. The arrow indicating the 'tail' of protein S6 is at the same position in both a) and b).

confident that we were studying phosphoprotein and, as the protein had been extracted from 40 S subunits prepared at 880 mM KCl, we thought it likely that this was ribosomal protein.

Next we attempted to analyse the phosphorylated protein by two-dimensional gel electrophoresis. Initially we had difficulty detecting any labelled ribosomal protein, probably because of the dispersion of the radioactivity over a larger area in the two-dimensional gel. When we reduced the running time of the second dimension from sixteen to six hr (with the time of the first dimension also reduced from three to two hr) we obtained much sharper, elongated spots, with quite good resolution (fig.3a). When the gel was subjected to autoradiography a single spot was found (fig.3b) which corresponded, unequivocably, to the 'tail' of the protein S6 (nomenclature of Sherton and Wool [8].) This is a clearly resolved and very distinct protein which also corresponds, we think, to protein S9 of Bielka et al. [19] and protein S13 of Howard and Traut [20]. The 'tail' could conceivably be a protein other than S6, which had not previously been detected. However it seems most likely that it is the phosphorylated derivative of S6 which, having a reduced positive charge, has a lower mobility in the first dimension. It has been variously estimated that the mol. wt of this protein is 32 000 [20] or 38 500 [21] and hence it probably corresponds to band A of fig.1.

It is of interest that S6 is also the protein most extensively phosphorylated by protein kinase in vitro [21,22]. Furthermore, while we were preparing this manuscript, Gressner and Wool reported that S6 is phosphorylated in rat liver in vivo [23].

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