The Acidic Ribosomal Phosphoprotein of Eukaryotes and its Relationship to Ribosomal Proteins L7 and L12 of *Escherichia coli*

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(Received 27 April 1978)

The acidic ribosomal phosphoprotein, L_7 , of Krebs II ascites cells was further characterized and compared with proteins L7 and L12 of *Escherichia coli*. Ribosomal protein L_7 was selectively removed from 60S ribosomal subunits by 50% ethanol and 1M-NH₄Cl, and antibodies raised against protein L_7 cross-reacted with *E. coli* protein L12 in immunodiffusion experiments. These and other, previously reported, data support the proposal that the eukaryotic counterpart of *E. coli* proteins L7 and L12 is phosphorylated.

The evolutionary distance between prokaryotes and eukaryotes is reflected in considerable structural differences in ribosomes that catalyse essentially similar overall reactions (see Wool & Stöffler, 1974). The only *Escherichia coli* ribosomal proteins for which corresponding proteins have been demonstrated in eukaryotes are protein L12 and its *N*-acetylated derivative, L7, acidic proteins of the large ribosomal subunit, thought to be involved in the guanosine triphosphatase reactions of the ribosome (see Möller, 1974). Thus immunochemical cross-reaction has been demonstrated between *E. coli* proteins L7 and L12 and two acidic proteins, L40 and L41, of rat liver 60S ribosomal subunits (Stöffler *et al.*, 1974); antisera raised against *E. coli* proteins L7 and L12 inhibited the guanosine triphosphatase reactions of chicken liver ribosomes (Howard *et al.*, 1976), and antisera raised against acidic proteins from yeast ribosomes inhibited the formation of *E. coli* ribosome-elongation factor G-GDP complexes (Wool & Stöffler, 1974); and functional interchangeability has been demonstrated between *E. coli* proteins L7 and L12 and the acidic ribosomal proteins from *Artemia salina* (Möller *et al.*, 1975) or from yeast (Wool & Stöffler, 1974) in restoring the biological activity of 'core' ribosomal particles, depleted of acidic proteins by extraction with ethanol and NH₄Cl.

A structural feature of ribosomes, unique to eukaryotes, is that some of the proteins are phosphoproteins; and considerable interest has been aroused by the demonstration that one of these is an acidic protein located on the large ribosomal subunit (Zinker & Warner, 1976; Leader & Coia, 1977). Although it has generally been assumed (mainly from comparisons of electrophoretic mobility) that these acidic phosphoproteins are equivalent to rat liver proteins L40 and L41, the counterpart to *E. coli*

proteins L7 and L12, this assumption has been called into question by the results of Wool and co-workers, who have been unable to detect the phosphorylation of proteins L40 and L41 (Gressner & Wool, 1974), but find other phosphorylated acidic ribosomal proteins that do not cross-react immunochemically with *E. coli* proteins L7 and L12 (Tsurugi *et al.*, 1978). However, the experiments presented here, which further characterize the acidic ribosomal phosphoprotein (L_7) that we previously described (Rankine *et al.*, 1977; Leader & Coia, 1977, 1978a), support the contention that this protein is related to proteins L7 and L12 of *E. coli*.

Materials and Methods

Preparation of ribosomal protein

Krebs II ascites cells were propagated in Porton-strain mice (in certain experiments they were labelled with [³²P]P_i either intraperitoneally or in suspension in modified Earle's saline), and ribosomes were isolated, as previously described (Rankine & Leader, 1975; Rankine *et al.*, 1977; Leader & Coia, 1978a). Baby-hamster kidney fibroblasts (BHK cells) were maintained in monolayer culture, labelled with [³²P]P_i after 3 days' growth, and their ribosomes isolated as previously described (Leader *et al.*, 1976; Leader & Coia, 1978a). Ribosomes were then dissociated into subunits, and their proteins extracted with 67% (v/v) acetic acid and 33 mM-MgCl₂, dialysed against 1M-acetic acid and freeze-dried (Rankine *et al.*, 1977).

Preparation of 'split' protein and 'core' particles

Ascites-cell 60S ribosomal subunits (approx. 20 mg) were suspended in 3 ml of a buffer containing 1M-NH₄Cl, 10 mM-imidazole/HCl, pH 7.4, 20 mM-MgCl₂ and 1 mM-2-mercaptoethanol at 0°C, 0.5 vol. of

ethanol was added for 10min and then another 0.5vol. of ethanol added for a further 5min, all as described by Reyes *et al.* (1977). The mixture was centrifuged at 30000g for 10min and the pellet re-extracted as before. The protein of the pellet ('core' particles) was then extracted with acetic acid/MgCl₂, as for ribosomal subunits; the supernatant ('split' protein) was dialysed against 1M-acetic acid and then freeze-dried.

Electrophoretic analysis of ribosomal proteins

In these studies we have used a modified method of two-dimensional electrophoresis ('sweep' gels), the rationale of which is mentioned in the text, and the full details of which are described elsewhere (Leader & Coia, 1978a). In brief, the first-dimension gel includes 6M-urea and 4% (w/v) acrylamide (0.066% bisacrylamide) at pH5.5, but uses an anodic buffer of pH3.55 and a cathodic buffer of pH7.0; the second-dimension gel is essentially that of Kaltschmidt & Wittmann (1970) and includes 6M-urea and 18% acrylamide (1.4% bisacrylamide) at pH4.5.

Analysis of phosphoserine and phosphothreonine

The hydrolysis of ribosomal protein and the separation of phospho amino acids by paper electrophoresis was as before (Rankine *et al.*, 1977).

Immunization and immunochemical procedures

The ribosomal protein L₇, against which antisera were raised, was separated from total 60S ribosomal-protein of ascites cells by two-dimensional 'sweep' gel electrophoresis. A thin slice of the gel was taken and stained with Coomassie Brilliant Blue, and this stained slice was aligned with the unstained portion of the gel to locate protein L₇, which was then excised with a micro-scalpel (Lin & Wool, 1974). Segments of gel containing the protein (two for each injection) were homogenized in phosphate-buffered saline (170mM-NaCl/3mM-KCl, 12mM-NaH₂PO₄/1.8mM-KH₂PO₄, pH7.6), mixed with Freund's complete adjuvant, and injected intramuscularly into 'half-lop' rabbits (F1 crosses between sandyllops and New Zealand whites). Injections were on days 1, 34 and 83 and the animals were bled on days 0, 42 and every 2 weeks thereafter. The antisera were incubated at 56°C for 30min to inactivate complement, and immunoglobulins were concentrated by precipitation at 33% saturation with (NH₄)₂SO₄. The precipitates were dissolved in phosphate-buffered saline, dialysed against the same buffer, further concentrated in a Minicon B15 concentrator (Amicon, High Wycombe, Bucks., U.K.) to a final concentration of 240mg/ml, and stored at -20°C.

Double-diffusion assays (Ouchterlony, 1958) were performed on a micro-scale on microscope slides

containing 2.5ml of 1% agarose in 20mM-sodium barbitone buffer, pH8.6, containing 0.75M-LiCl, 1.8% poly(ethylene glycol) and 0.01% NaN₃. Circular wells, with centres 4mm apart and capacity 5μl, were cut with a stencil in a hexagonal pattern, and the plates were incubated in a moist container for 24h at 20°C.

Results and Discussion

We had previously found (Leader & Coia, 1977) that acidic eukaryotic phosphoproteins were difficult to detect by the standard system of two-dimensional gel electrophoresis of Kaltschmidt & Wittmann (1970). We therefore devised a separation method ('sweep' gel electrophoresis) in which the first dimension is modified to allow both basic and acidic proteins to migrate in a single direction, the acidic proteins being concentrated in a narrow band (Leader & Coia, 1978a). The second dimension separates the acidic proteins on the basis of both size and charge, and it appeared that a maximum of three stained and two ³²P-labelled species can be resolved, although all of these had not been found together on a single gel. Plate 1 now shows all these species in 60S-ribosomal protein from incubated ascites cells, where the species at the top of the gel is usually lacking. The corresponding (results not shown) sodium dodecyl sulphate/polyacrylamide gel to Plate 1 resolves two labelled bands, consistent with our previous arguments that the three spots contain four species: a 13500-mol.wt. unphosphorylated species of greatest mobility in the second dimension, a phosphorylated 13500-mol.wt. species unresolved from an unphosphorylated 14500-mol.wt. species of similar intermediate mobility, and a phosphorylated 14500-mol.wt. species of least mobility (Leader & Coia, 1978a). In ascites cells labelled intraperitoneally almost all the acidic protein migrated as the phosphorylated 14500-mol.wt. species, and we argued from this and other considerations that the different acidic species are, in fact, different forms of the same protein (L₇), the smaller species most probably being proteolytic degradation products of the larger. The virtual absence of the degraded species from ascites cells labelled intraperitoneally was presumed to be due to the better growth conditions, and this interpretation is consistent with our finding of the species of protein L₇ of least mobility in 60S ribosomes from BHK-cell monolayers (Plate 2).

This interpretation of the 'sweep'-gel analysis of ribosomal protein L₇ is most consistent with there being only a single site of phosphorylation. However, in previous studies of the phosphoproteins of ascites 60S ribosomal subunits we had found ³²P incorporated into both phosphoserine and phosphothreonine residues (Rankine *et al.*, 1977). We have therefore repeated the analysis of phosphorylated

EXPLANATION OF PLATE I

Two-dimensional 'sweep' gel electrophoresis of protein (200 μ g) extracted from the 60S ribosomal subunits of ascites cells incubated with [32 P] P_i for 3 h

(a) Stained gel; (b) radioautograph. The three arrows indicate corresponding positions on the two frames, the parenthesis being used to indicate the absence of a radioautographic spot corresponding to the lower stained spot.

EXPLANATION OF PLATE 2

Two-dimensional 'sweep' gel electrophoresis of protein (200 μ g) extracted from the 60S ribosomal subunits of BHK cells labelled with [32 P] P_i for 3 h

(a) Stained gel; (b) radioautograph. The purpose of the arrows is as for Plate 1, the broken arrow here being used to denote a relatively faint spot. The two arrows in this Plate correspond to the upper two spots in Plate 1.

EXPLANATION OF PLATE 3

Two-dimensional 'sweep' gel electrophoresis of 'split' and 'core' protein from 60S ribosomal subunits of ascites cells
(a) 'Split' protein (5 μ g); (b) 'core' protein (200 μ g). The 'split' and 'core' protein fractions were isolated as described in the Materials and Methods section.

EXPLANATION OF PLATE 4

Double-diffusion analysis

The left-hand well (Ag) contained *E. coli* protein L12 (0.1 μ g) and the contents (5 μ l) of the other wells were: (1) concentrated non-immune serum (from rabbit RD 903 at day 0); (2) concentrated anti-(protein L₇) serum (from rabbit RD 903 at day 77); (3) anti-(*E. coli* protein L12) serum. The *E. coli* protein L12 and antiserum (80 mg of protein/ml) were gifts from Dr. N. Brot (Roche Institute of Molecular Biology, Nutley, NJ, U.S.A.). Similar results were obtained with concentrated antisera which we raised against *E. coli* proteins L7 and L12 (10 μ g per injection), provided by Dr. G. Stöffler (Max-Planck-Institut für Molekulare Genetik, W. Berlin, Germany).

Fig. 1. High-voltage paper electrophoresis of acid hydrolysates of protein extracted from the 60S ribosomal subunits of ascites cells of mice labelled with [^{32}P]P $_i$ intraperitoneally for 1 h

The Figure shows a radioautograph of the electrophoretogram, with the positions of migration of standards electrophoresed in parallel.

amino acids from ribosomes in which protein L $_y$ was the sole 60S-ribosomal phosphoprotein, and now find only phosphoserine in 60S ribosomal subunits from ascites cells (Fig. 1) and BHK cells (D. P. Leader & A. A. Coia, unpublished work). These findings agree with those of Horak & Schiffmann (1977) for the recently described acidic phosphoprotein of HeLa cells, and the phosphothreonine that we previously detected was presumably derived from one of the minor 60S-ribosomal phosphoproteins that we had occasionally observed (Leader & Rankine, 1976; Rankine *et al.*, 1977). The results do not, of course, exclude the phosphorylation of two serine residues per molecule of L $_x$ protein, but none of our other results indicate this possibility.

The acidic ribosomal proteins L7 and L12 of *E. coli* are detached from ribosomes by ethanol and NH $_4$ Cl (Hamel *et al.*, 1972), as are the functionally similar proteins EL7 and EL12 of *A. salina* and the functionally similar and immunochemically cross-reacting proteins L40/L41 of rat liver (Möller *et al.*, 1975; Wool & Stöffler, 1974). We therefore performed a similar extraction of 60S ribosomal subunits from ascites cells growing intraperitoneally, and subjected the extracted 'split' protein and the residual 'cores' to 'sweep' gel electrophoresis. Plate 3(a) shows that the major component of the 'split' protein migrates to the position of L $_y$ protein (cf. Plate 2), whereas the acidic protein is absent from the 'cores' (Plate 3b). Although not conclusive proof, this result is consistent with the proposition that ascites L $_y$ protein is equivalent to rat liver proteins L40 and L41, *A. salina* proteins EL7 and EL12, and *E. coli* proteins L7 and L12. Indeed evidence has been presented

indicating that *A. salina* proteins EL7 and EL12 are also phosphorylated (van Agthoven *et al.*, 1977).

Despite finding functional relationships between *A. salina* proteins EL7 and EL12 and *E. coli* proteins L7 and L12 (Möller *et al.*, 1975), Möller and co-workers have been unable to find any immunochemical cross-reaction between these proteins (van Agthoven *et al.*, 1977). We had not purified ascites L $_y$ protein to homogeneity [other weak spots are present in Plate 3(a) and, more clearly, on one-dimensional gels containing sodium dodecyl sulphate (D. P. Leader & A. A. Coia, unpublished work)] and therefore decided to attempt to raise antibodies against pieces of gel containing ascites L $_y$ protein, as Stöffler *et al.* (1974) had done for rat liver proteins L40 and L41. The L $_y$ protein used was predominantly the undegraded phosphorylated form (that of least mobility in Plate 1), and, when antisera against this were tested in Ouchterlony immunodiffusion experiments for cross-reaction with *E. coli* protein L12, a precipitin line was obtained that fused with that between anti-(protein L12) serum and *E. coli* protein L12 (Plate 4). Though the former precipitin line was much weaker than the latter, it was completely unambiguous and obtained repeatedly with anti-(L $_y$ protein) from six different 'bleeds' from two separate rabbits.

This last result indicates that L $_y$ protein shares immunochemical determinants with *E. coli* proteins L7 and L12 and that the eukaryotic counterpart of *E. coli* proteins L7 and L12 is a phosphoprotein. Further, it now seems to us that the similarities between the different eukaryotic acidic ribosomal proteins strongly suggest that these proteins are equivalent, despite some apparent discrepancies in their properties. Thus ascites L $_y$ protein is similar to rat liver proteins L40 and L41 in bearing an immunochemical relationship to *E. coli* proteins L7 and L12; L $_y$ protein is similar to *A. salina* proteins EL7 and EL12, in being a phosphorylated 'split' protein; and proteins L40 and L41 are similar proteins to EL7 and EL12 in their functional implication in the ribosomal guanosine triphosphatase activity. We suggest that the difficulty of labelling liver phosphoproteins with [^{32}P]P $_i$ *in vivo* and the relatively short labelling time used (30 min, compared with 3 h in our work) could explain why Gressner & Wool (1974) did not detect ^{32}P labelling of rat liver proteins L40 and L41. It is more difficult to explain why no cross-reaction was found between *E. coli* proteins L7 and L12 and *A. salina* proteins EL7 and EL12 (van Agthoven *et al.*, 1977), although negative immunochemical results need not be regarded as conclusive. Perhaps differences between the amino acid sequences of the *A. salina* and the rodent proteins are a key factor. Alternatively, the purification of proteins EL7 and EL12 may have resulted in the generation of proteolytically cleaved forms (cf. Plate 1) lacking

the determinants common to the prokaryotic proteins. By using L_1 protein excised from polyacrylamide gels of total 60S-ribosomal protein we would have avoided such a problem.

A final point of similarity between prokaryotic and eukaryotic acidic proteins that it seems pertinent to mention is the apparent location of these proteins at the interface of the two ribosomal subunits. This is clearly established for *E. coli* proteins L7 and L12 (Tischendorf *et al.*, 1975), and was previously suggested by us for a eukaryotic phosphoprotein, the acidic nature of which we did not at the time appreciate (Rankine & Leader, 1976). This suggestion was based on the finding of the 13500-/14500-mol.wt. phosphoprotein on the 40S subunit of BHK cells, in contrast with the 60S-subunit location in ascites cells. Plate 2 shows that we now find L_1 protein (which corresponds to these proteins) on the 60S subunit of BHK cells, probably as a result of modifications in the method used to isolate the ribosomes. Our original suggestion has, however, received independent support from the studies Horak by Schiffmann (1977) on the acidic phosphoprotein of HeLa-cell 60S ribosomal subunits. They found that when this protein was on 80S ribosomes it was inaccessible to protein kinase, consistent with a location at the subunit interface.

In conclusion we should stress that, although marked alteration in the extent of phosphorylation of L_1 protein can be produced *in vivo* (Leader & Coia, 1978b), it is not known whether the phosphorylation has any functional significance. Further studies of ribosomes, in which L_1 protein is in different states of phosphorylation, may help to clarify this.

We thank Dr. N. Brot for generous gifts of L12 protein and anti-(L12 protein) serum and Dr. G. Stöffler for a generous gift of proteins L7 and L12. This work would not have been performed without the patient immunochemical instruction and advice of Professor A. R. Williamson and his co-workers, especially Dr. T. Mosmann and Mrs. E. Blakely, who also performed the immunizations. We also gratefully acknowledge the skilled technical assistance of Mr. G. Mosson, the interest and support of Professor R. M. S. Smellie, and the financial support of the Medical Research Council, through a project grant to D. P. L.

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