PURIFICATION OF A PROTEIN WITH HIGH AFFINITY FOR dRNA

J. M. EGLY*, O. KRIEGER and J. KEMPF
Unité (44) de Recherches Fondamentales sur la Biochimie de la Cellule Cancéreuse de l'INSERM, 11 rue Humann, 67085 Strasbourg Cedex France

Received 24 February 1975

1. Introduction

Recent data have shown that dRNA** and mRNA in eukaryotic cells are associated with specific proteins to form sedimentable nucleoproteins which have been called dRNP-particles or informosomes [1]. Several classes of specific proteins associated with mRNA have been distinguished [2]. We developed a method for the isolation of a set of proteins with a high affinity for dRNA, extracted from free cytoplasmic dRNP particles [3].

The elucidation of the structure of these proteins and of the nature of their binding to mRNA (or dRNA) are of great interest. In the present study, we describe the purification and some of the properties of one of these proteins having a mol. wt of 60 000.

2. Materials and methods

The solid plasma cell tumor RPC_5, transplanted on Balb/c mice, was used. RNA was labelled by intraperitoneal injection of 1 mCi of tritiated uridine, 20 hr before killing. Protein was labelled by 3 intraperitoneal injections of 8 μCi each of ^{14}C-labelled protein hydrolysate 6, 4 and 2 hr before killing. The tumors were homogenized using a glass homogeniser with teflon pestle, in 2.4 vol of ice-cold 0.02 M TEA–HCl buffer (pH 7.6) containing 0.15 M KCl, 0.004 M magnesium acetate, 0.006 M β-mercaptoethanol and 1.1 M sucrose.

2.1. *Isolation of free informosomes*

Ribonucleoprotein particles from a post-mitochondrial supernatant (S_{20}) were fractionated on a discontinuous D_2O-sucrose gradient as described elsewhere [4]. After centrifugation, the heavy D_2O-sucrose layer (density 1.29 g/cm^3) was carefully removed and used as the free informosome-rich ribonucleoprotein fraction. The ribosomal pellet was used for preparation of the protein kinase.

2.2. *Dissociation of ribonucleoprotein complexes and fractionation of informosomal proteins*

The fraction highly enriched in informosomes was first dialysed for 24 hr against 0.010 M TEA–HCl buffer (pH 7.6) containing 0.001 M EDTA and sucrose 0.5 M, and then dialysed for 24 hr against buffer 0.010 M Tris–HCl pH 8, containing 6 M urea, 0.006 M β-mercaptoethanol. DEAE cellulose chromatography was used to obtain a preliminary separation as described by Spitnik-Elson [5].

2.3. *Preparation of 'RNA-agarose'*

RNA was extracted from plasma cell tumors by phenol and phenol–SDS at three temperatures: 0, 45 and 60°C. 'rRNA' (fraction extracted at 0°C) or 'dRNA' (fraction extracted at 60°C) was bound to beaded agarose (Sepharose 2B) following the technique of Wagner et al. [7]: the agarose was activated with CNBr at pH 11 and washed with twice distilled water and 0.2 M morpholine ethane sulfonic acid buffer (pH 6). RNA was coupled by incubation in 0.2 M

* Attaché de Recherche à l'Unstitut National de la Santé et de la Recherche Médicale.

** *Abbreviations:* rRNA: ribosomal RNA; dRNA: DNA-like RNA; mRNA: messenger RNA; dRNP: dRNA-protein particles; SDS: sodium dodecyl sulfate.

North-Holland Publishing Company – Amsterdam

morpholine ethane sulfonic acid buffer (pH 6) for 24 hr at 4°C. Then, the agarose–RNA complex was washed with a solution containing 0.010 M potassium phosphate buffer (pH 7.5). 0.010 M EDTA, 0.2% SDS., 90% formamide until the wash was RNA-free as checked by the absorbance at 260 nm. The RNA agarose could be stored in phenol saturated water at 4°C for several weeks. We have also coupled Poly A, using the same conditions as the RNA.

2.4 *Preparation of the 'ribosomal' protein kinase*

Protein kinase activity was extracted from the polysome pellet with 0.5 M KCl and centrifuged. The supernatant 1.5 hr at 300 000 *g* was dialysed for 12 hr against the following buffer 0.010 M Tris–HCl (pH 7.5), containing 0.12 M KCl, 2×10^{-4} M EDTA, 0.010 M β-mercaptoethanol and chromatographed on DEAE-cellulose as described elsewhere [5].

2.5. *Polyacrylamide gel electrophoresis of proteins*

Proteins were analysed by SDS-polyacrylamide gel electrophoresis using the method of Waehneldt [8] under the same conditions as described in a previous publication [3].

3. Results and discussion

3.1. *Purification of the protein*

3.1.1. DEAE-cellulose chromatography

Partially purified 'free informosomes' obtained by D_2O-sucrose gradient centrifugation and dialysed as described in Methods, were chromatographed on DEAE cellulose. After dialysis, some protein species are 'free' in solution, others remain more or less loosely 'bound' to the RNA. The elution profile from DEAE cellulose is shown in fig.1. The first peak consist of 'free' basic protein (fraction A); 0.15 M LiCl and 0.30 M LiCl elute 'bound' basic and acidic protein and 'free' acidic protein (fraction B_1 and B_2 respectively). These three fractions are free of RNA, which eluted with some residual protein by the magnesium acetate step (fraction C). They may however contain ribosomal protein of the light ribosomal subunit, which was not quantitatively removed by the density gradient step, in addition to true constitutional protein of dRNP particles with a high affinity for dRNA.

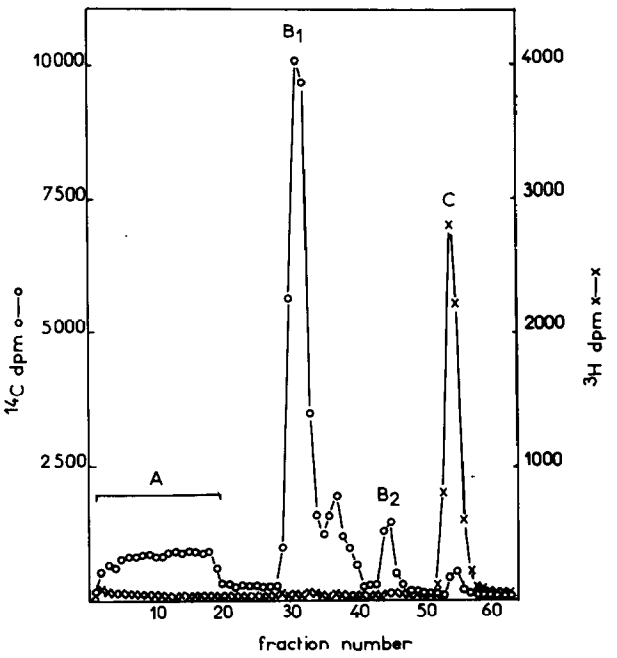

Fig.1. Separation of proteins and nucleic acid from the ribonucleoprotein complexes by chromatography on DEAE-cellulose. The dialysed informosome-rich fraction (approx. 200 mg protein) was applied on a DEAE-cellulose column (12 × 1.6 cm) as indicated in Materials and methods. A: effluent; B_1: fraction eluted with 0.15 M LiCl; B_2: fraction eluted with 0.3 M LiCl; C: fraction eluted with 0.5 M Mg acetate (pH 2). Solutions contained 6 M urea throughout. 5 ml fractions were collected. Radioactivity was measured on 0.3 ml aliquots plated on filter papers, washed with cold 10% TCA, ethanol, ethanol–ether and ether, and counted in the scintillation spectrometer intertechnique ABAC SL 40. (o––o––o) Radioactivity of ^{14}C-labelled protein. (X––X––X) Radioactivity of ^{3}H-labelled RNA.

3.1.2. Affinity chromatography

Fractions B_2 which had been dialysed against a 0.010 M Tris–HCl buffer containing 0.35 M KCl and 1 M urea during 5 hr was applied on a rRNA-agarose column, in order to retain the protein having a high affinity for rRNA. A concentration of 0.35 M KCl was chosen since it has been shown that interactions between ribosomal protein and rRNA are favoured under these conditions [9]. The protein not retained by the rRNA-agarose was dialysed against the column buffer 0.010 M Tris-HCl (pH 7.6); 1 M urea; 0.150 M KCl and then applied on a dRNA-agarose column. A KCl concentration of 0.150 M was found to be most

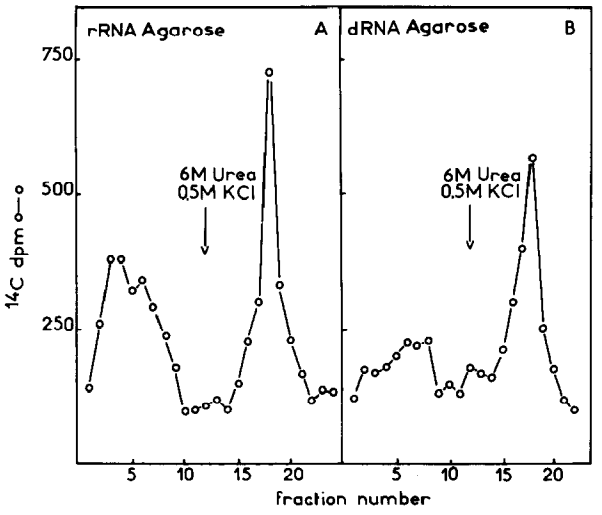

Fig.2. Affinity chromatography of fraction B_2 proteins on rRNA-agarose (panel A), and the effluent not retained on rRNA-agarose is chromatographed on dRNA-agarose (panel B). 10 ml of agarose containing 0.35 mg/ml covalently bound RNA were charged in a glass column of 1 cm internal diameter and equilibrated with a buffered solution 0.020 M Tris–HCl (pH 7.6), 0.150 M KCl, 0.010 M β-mercaptoethanol, 0.5 M sucrose and 1 M urea. Proteins from peak B_2 were slowly filtered at 0–4°C through the rRNA-agarose. The proteins not retained on the rRNA-agarose column were collected and chromatographed on the dRNA-agarose column. Final elution was carried out with the buffered solution containing 1 M LiCl and 6 M urea. (o––o––o) Radioactivity of ^{14}C-labelled proteins, measured on 0.3 ml aliquots plated on filter paper, and washed with 10% TCA, ethanol, ethanol–ether and ether.

favourable for the protein–dRNA interaction. More than 50% of the protein which had not been retained by rRNA-agarose bound to dRNA under our experimental conditions; this protein fraction was eluted with 0.010 M Tris–HCl buffer (pH 7.6) containing 6 M urea and 0.5 M KCl as shown in fig.2.

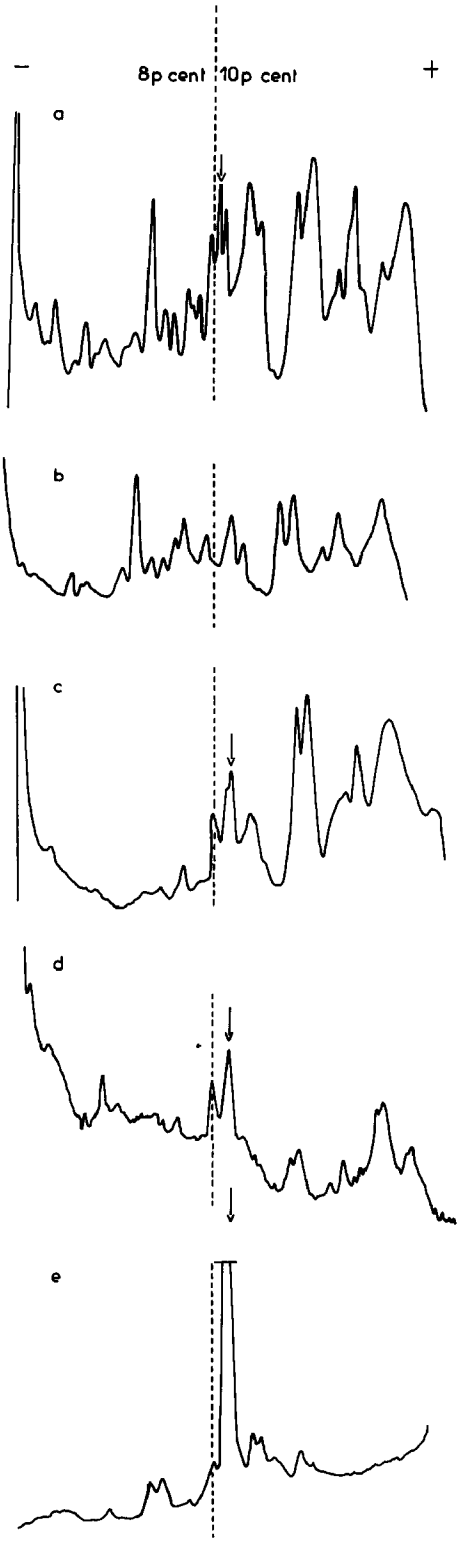

Fig.3. SDS-polyacrylamide gel electrophoresis of proteins obtained from the informosome-rich fraction. Densitogram of the Coomassie Blue stained gels. a) Proteins contained in the heavy D_2O-sucrose layer (d: 1.29 g/cm^3): informosome-rich ribonucleoprotein fraction. b) Proteins from peak B_1, eluted from DEAE cellulose with 0.15 M LiCl. c) Proteins from peak B_2 eluted from DEAE-cellulose with 0.3 M LiCl. d) Proteins from peak B_2 not retained on rRNA-agarose. e) Proteins from this last fraction, retained on dRNA-agarose and eluted by 0.5 M KCl, 6 M urea. The arrow indicates the peak corresponding to protein I 60.

3.2. *Some characteristics of protein I 60*

Using affinity chromatography as a final step of the purification procedure, we obtained a highly purified cytoplasmic protein with high affinity for dRNA. The procedure can be carried out on a preparative scale. The SDS-polyacrylamide-gel profiles of the protein fractions during the different purification steps are shown in fig.3. The fraction retained by dRNA-agarose but not by rRNA-agarose shows a major protein band with few minor components. Compared with standard proteins of known mol. wt, the major protein, called I 60 protein, has a mol. wt of 60 000 ± 2000.

We have shown previously that dRNP particles contain phosphoproteins [10]. There was incorporation of ^{32}P from ATP[γ ^{32}P] into protein I 60 when incubated in the presence of a ribosomal protein kinase. This ribosomal protein kinase prepared as described above, differs from soluble protein kinase in that it is capable of catalysing the phosphorylation of a specific protein of the ribosomal KCl wash [11]. The phosphorylated I 60 protein can still be retained on a dRNA-agarose column (fig.4), although changes of the interaction strength with RNA cannot be excluded. If such changes were to occur, phosphorylation could modulate the binding of protein to nucleic acid. Work is now in progress to test this hypothesis.

Interaction of cytoplasmic protein with the poly A sequence at the 3′ end of mRNA has been decribed by several authors [12–13]. Protein I 60 however shows no affinity for Poly A (fig.5).

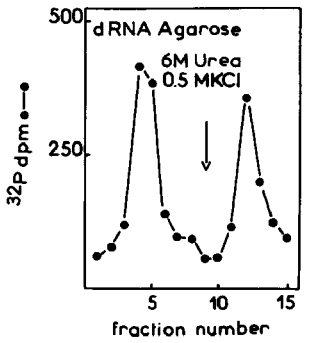

Fig.4. Affinity chromatography on dRNA-agarose of the protein I 60. Protein I 60 was incubated with ATP [γ ^{32}P] $25 \cdot 10^{-4}$ M and 24 μg of the ribosome bound protein kinase in buffer: Tris–HCl, pH 7.6, 0.035 M; AcMg, 0.005 M; β-mercaptoethanol 0.012 M. Protein I 60 was adsorbed and eluted as indicated in fig.2.

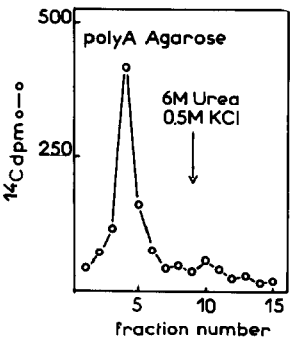

Fig.5. Affinity chromatography on agarose-poly A of the protein I 60. Conditions of adsorption and elution are the same as in fig.2.

We found that elution with 6 M urea without salt, did not elute the bound I 60 protein. Apparently, the interaction with dRNA involves a considerable electrostatic component with coulombic interaction between the charged sites of protein and nucleic acid. Nevertheless, non-coulombic interactions have been shown to play an important role in specific binding [14].

Quantitative analysis of these interactions by determination of binding constants between the I 60 protein and dRNA may lead to a better understanding of the relative importance of the two types of interaction and the specificity of binding.

Acknowledgements

This investigation was supported by a grant (No. 74 703 61) from the Délégation Générale à la Recherche Scientifique et Technique. We are also indebted to N. Pfleger for her skillful technical assistance. Thanks are also due to Dr M. Schmitt for her gift of polysomal protein-kinase and to Dr C. Goridis for his help in reading the manuscript.

References

[1] Spirin, A. S. (1969) Eur. J. Biochem. 10, 20.
[2] Williamson, R. (1973) FEBS Lett. 37.
[3] Egly, J. M., Krieger, O., Mandel, P. and Kempf, J. (1974) FEBS Lett. 40, 101–105.
[4] Kempf, J., Egly, J. M., Stricker, Ch., Schmitt, M. and Mandel, P. (1972) FEBS Lett. 26, 130.
[5] Spitnik-Elson, P. and Greenman, B. (1971) FEBS Lett. 9, 209.
[6] Kempf, J. and Mandel, P. (1966) Bull. Soc. Chim. Biol. 48, 211.
[7] Wagner, A. F., Bugianesi, R. L. and Shen, T. Y. (1971) Biochem. Biophys. Res. Commun. 45, 184.
[8] Waehneldt, T. U. and Mandel, P. (1970) FEBS Lett. 9, 209.
[9] Mizushima, S. and Nomura, M. (1970) Nature 226, 1214.
[10] Egly, J. M., Johnson, B. C., Stricker, C., Mandel, P. and Kempf, J. (1972) FEBS Lett. 22, 181.
[11] Quirin-Stricker, Ch., Schmitt, M., Egly, J. M. and Kempf, J. Eur. J. Biochem. (in press).
[12] Blanchard, J. M., Brissac, C. and Jeanteur, Ph. (1974) Proc. Natl. Acad. Sci. U.S. 71, 1882.
[13] Blobel, G. (1973) Proc. Natl. Acad. Sci. U.S. 70, 924.
[14] Daune, M. (1972) Eur. J. Biochem. 26, 207.