RNA-binding protein kinase from amphibian oocytes is a casein kinase II

K.V. Kandror and A.S. Stepanov

A.N. Bakh Institute of Biochemistry, USSR Academy of Sciences, Leninsky pr. 33, Moscow 117071, USSR

Received 6 March 1984

RNA-binding protein kinase from amphibian oocytes modifies serine and threonine residues in the molecules of substrates and utilizes both ATP and GTP. Low concentrations of heparin inhibit protein kinase. The foregoing suggests that this enzyme is casein kinase II. It is shown that RNA-binding proteins lack active forms of phosphatases and proteases which may affect the results of phosphorylation of both endogenous and exogenous substrates.

Amphibian oocyte *RNA-binding protein* *Cyclic AMP-independent protein kinase* *Phosphatase* *Protease*

1. INTRODUCTION

At present, phosphorylation of components of the protein-synthesizing system is being intensively studied. It has been shown that the translation repressor activated in the lysates of reticulocytes in the absence of hemin or after addition of double-stranded RNA is a cAMP-independent protein kinase phosphorylating a small subunit of initiation factor 2 with the result that the mechanisms of initiation of protein synthesis are upset [1]. Conversely, an increase in the degree of phosphorylation of ribosomal protein S6 correlates with activation of the metabolism and a rise in the level of protein synthesis in eukaryotic cells under hormonal action or when serum or growth factors are added [2,3].

cAMP-independent protein kinase activity is found in informosomes [4–6] and free informosome-forming (RNA-binding) proteins [7,8]. It has been assumed that the phosphorylation of proteins directly interacting with mRNA in vivo may be responsible for the mRNA masking-demasking processes in eukaryotic cells [8,9].

As has been shown, most organisms and tissues contain several types of cAMP-independent protein kinases with different structures, properties and, probably, substrate specificities in vivo. In addition to their endogenous substrates, most protein kinases are capable of phosphorylating casein and phosvitin (casein kinases) and are inactive with histones. Distinction is made between two types of casein kinases: type I includes small monomeric proteins exhibiting exceptional specificity to ATP, modifying the serine residues in the casein molecule and uninhibitable by low concentrations of heparin. Casein kinases of type II are high-molecular mass proteins of a quaternary structure, capable of utilizing GTP along with ATP and attaching phosphate groups both to serine and threonine residues of casein. Low concentrations of heparin [5–20 nmol) completely inhibit casein kinases of type II [10–12].

We have reported that the fraction of RNA-binding proteins of amphibian oocytes contains cAMP-independent protein kinase and phosphorylatable endogenous substrates [8]. We identify here the RNA-binding protein kinase as a casein kinase II. It is shown that RNA-binding proteins lack active forms of phosphatases and proteases capable of affecting the results of phosphorylation of endogenous and exogenous substrates.

Published by Elsevier Science Publishers B.V.
00145793/84/$3.00

2. MATERIALS AND METHODS

The standard buffer was 0.01 M triethanolamine, 0.1 M NaCl, 0.01 M KCl, 0.005 M $MgCl_2$, 0.001 M EDTA, 0.006 M 2-mercaptoethanol, 10% glycerol (pH 7.8).

RNA-binding proteins were isolated by affinity chromatography on poly(U)–Sepharose from ribosome-free extracts of ripening oocytes of *Rana temporaria* as in [13]. The dephosphorylation of commercial casein (Calbiochem) and determination of protein kinase activity were carried out as in [14].

To identify *O*-[^{32}P]phosphoserine and *O*-[^{32}P]phosphothreonine formed in the reaction, ^{32}P-labelled casein was partially hydrolyzed with 6 N HCl at 105–110°C under reduced pressure for 2 h. Hydrolyzed samples were analyzed by high-voltage electrophoresis on Whatman 3 MM paper in 3 N acetic acid (pH 2.11) at 4900 V for 2 h. ^{32}P-containing hydrolyzates were analyzed by autoradiography.

Electrophoresis in the presence of SDS was carried out as in [16]. Standard proteins included bovine serum albumin (68000), glyceraldehyde-phosphate dehydrogenase (39500), TMV protein (17400) and lysozyme (14300). Labelled gels were dried and exposed to X-ray film HS-II (ORWO).

Denatured ^{125}I-labelled collagen was kindly provided by Dr A.V. Glinka (A.N. Bakh Institute of Biochemistry, Moscow).

3. RESULTS AND DISCUSSION

Most cAMP-independent protein kinases are clearly distinguished by their capacity to phosphorylate either casein and phosvitin (casein kinases) or histones (histone kinases). As can be seen from table 1 in a standard in vitro system casein is phosphorylated in a more (>20-fold) effective manner than histones. In determining the specific phosphorylation of each substrate we took into account the inevitable self-phosphorylation of RNA-binding proteins.

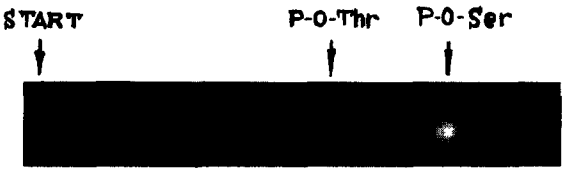

Fig.1. Autoradiographic identification of the products of hydrochloride hydrolysis of ^{32}P-labelled casein phosphorylated by RNA-binding protein kinase. ^{32}P-labelled casein (350 μg, 670 cpm/μg) was precipitated using 3 vols of cooled ethanol and hydrolyzed in 6 M HCl. The hydrolysis products were analyzed by high-voltage paper electrophoresis. The electrophoretogram was exposed with an HS-II X-ray film for 5 days. Arrows indicate the positions of standards.

High-voltage paper electrophoresis of hydrochloride hydrolyzates of ^{32}P-labelled casein phosphorylated by RNA-binding protein kinase has shown that the radioactive phosphate is found predominantly in phosphoserine residues and, to a lesser extent, in phosphothreonine residues (fig.1). Similar results were yielded by analysis of amino acids phosphorylated de novo in the endogenous substrates of the protein kinase, i.e., in RNA-binding proteins themselves [8].

Fig.2 shows that RNA-binding protein kinase is capable of utilizing GTP during phosphorylation

Table 1

Substrate specificity of RNA-binding protein kinase

Reaction mixture components	Composition of the reaction mixture								
[γ-^{32}P]ATP (0.1 mM)	+	+	+	+	+	+	+	+	+
Casein (38 μg)	–	–	–	+	+	+	–	–	–
Histones (50 μg)[a]	–	–	–	–	–	–	+	+	+
RNA-binding proteins (μg)	2.5	3	7	2.5	3	7	2.5	3	7
^{32}P (pmol per μg substrate)	1.6	1.54	1.7	1.12	1.46	2.2	0	0.06	0.02

[a] Use was made of a preparation of total calf thymus histones

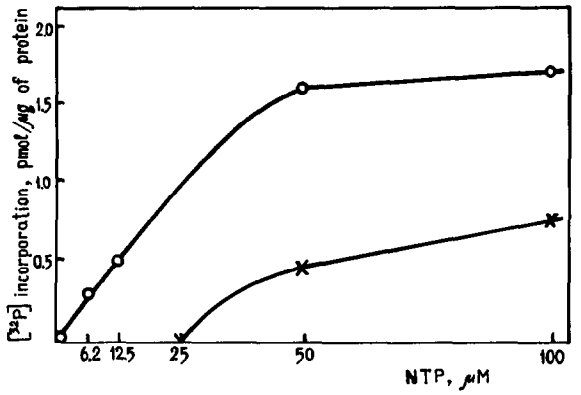

Fig.2. Phosphorylation of casein by RNA-binding protein kinase using [γ-^{32}P]ATP and [γ-^{32}P]GTP. Standard buffer (100 μl) contained 3.2 μg RNA-binding proteins, 38 μg dephosphorylated casein and a specified amount of [γ-^{32}P]ATP (500 cpm/pmol) or [γ-^{32}P]GTP (900 cpm/pmol). The samples were incubated for 1 h at 20°C. [γ-^{32}P]ATP (○), [γ-^{32}P]GTP (×).

of casein. However, it can be seen that ATP is a better substrate for our enzyme than GTP. Perceptible incorporation of the labelled phosphate into casein may occur only at [γ-^{32}P]GTP concentrations exceeding 50 μM, whereas at 6 μM [γ-^{32}P]ATP the degree of casein phosphorylation is prevalent over the background.

Heparin inhibits the self-phosphorylation of RNA-binding proteins even at low concentrations (fig.3). We [8] have demonstrated that various

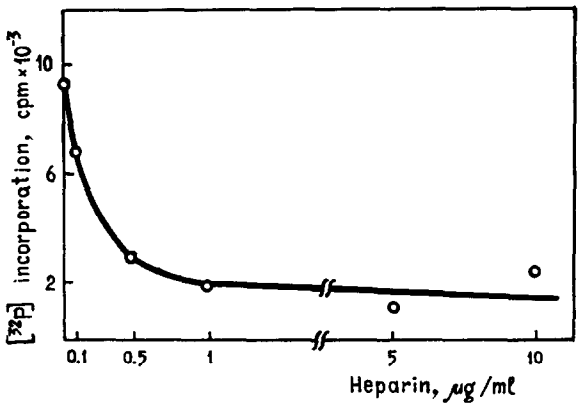

Fig.3. Inhibition of RNA-binding protein kinase by heparin. Standard buffer (100 μl) contained 3 μg RNA-binding proteins, 0.1 mM [γ-^{32}P]ATP (800 cpm/pmol) and a specified amount of heparin. The samples were incubated for 1 h at 20°C.

polynucleotides exhibit the same effect. The effect of heparin on the enzyme must be due to the fact that heparin (as a polyanion) is also capable of immobilizing on itself many RNA-binding proteins (including protein kinase), and in this sense it is an analogue of RNA.

The above suggests that the protein kinase found in RNA-binding proteins of amphibian oocytes is a casein kinase II. As revealed by SDS-polyacrylamide gel electrophoresis the pure enzyme consists of 3 polypeptide chains with M_r ~43000, 41000 and 29000 [17]. The finding that this enzyme is present in informosomes [6,17] and free RNA-binding proteins of animal cells is indicative of direct involvement of casein kinases II in the regulation of protein biosynthesis.

Previously we did not rule out the possibility of RNA-binding proteins exhibiting phosphatase activity that may affect both the specific phosphorylation of these proteins and the set of the polypeptides phosphorylatable in vitro [7,8]. However, attempts to reveal such activity have failed. We added a 100-fold excess of unlabelled ATP (up to 10 mM) to the standard protein kinase system after a 1 h incubation, and continued it for another hour. The phosphorylated proteins were then analysed electrophoretically. Fig.4 is an autoradiograph of the dried gel. It can be seen that the same proteins were phosphorylated in both cases with no perceptible decrease in the label being observed in any of the components.

Incubation of RNA-binding proteins together with ^{32}P-labelled casein carefully separated from the free label does not reduce the radioactivity of the trichloroacetic acid-precipitated material. Addition of 2 mM $MnCl_2$ has no effect on the result. This indicates that no dephosphorylation of ^{32}P-labelled casein takes place. Electrophoretic analysis of ^{32}P-labelled casein before and after incubation with RNA-binding proteins confirms this conclusion (not shown).

While analyzing the complex polypeptide composition of RNA-binding proteins and the set of polypeptide chains phosphorylated in vitro, one must be sure that the sample is free of endogenous protease or any protease admixtures. For this purpose we incubated 17 μg RNA-binding proteins with 1.7 μg denatured ^{125}I-labelled collagen (spec. act. 2 × 10^6 cpm/μg) in a 50 μl volume over different periods of time, and this mixture was ex-

Fig.4. Electrophoresis of RNA-binding proteins phosphorylated in vitro. RNA-binding proteins (150 μg) were incubated in a standard protein kinase system with 0.1 mM [γ-^{32}P]ATP. (a) Phosphorylated RNA-binding proteins, (b) phosphorylated RNA-binding proteins after an additional hour of incubation in the presence of 10 mM unlabelled ATP. The film was exposed for 2 days.

amined by electrophoresis. The position of ^{125}I-labelled collagen was determined by autoradiography of the dried gel (fig.5). This zone was cut out from the gel and the radioactivity counted therein and in the rest of the gel. No decrease in the label was observed in the collagen strip. Incubation of 3.5 μg ^{125}I-labelled collagen with 2 μg pronase (Calbiochem) within the same volume results in complete hydrolysis of the collagen to low molecular mass products within 15 min.

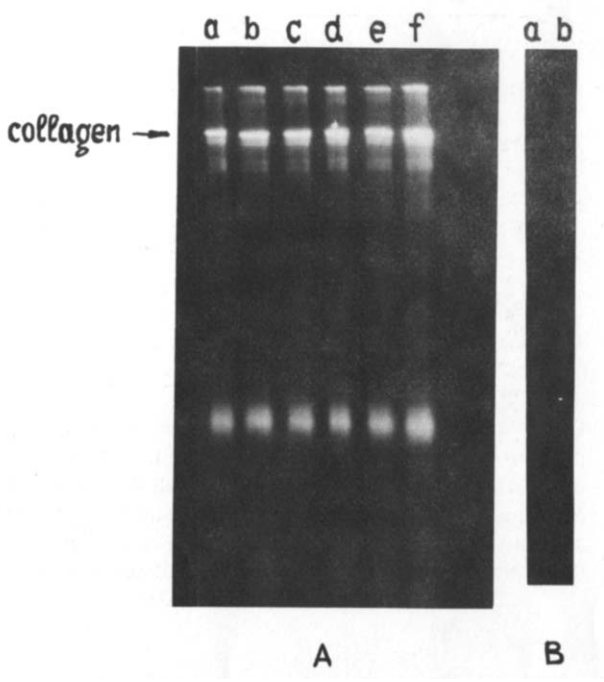

Fig.5. (A) Incubation of ^{125}I-labelled collagen with RNA-binding proteins: (a) immediately after mixing, (b) after 15 min, (c) after 30 min, (d) after 45 min, (e) after 3 h, (f) ^{125}I-labelled collagen without RNA-binding proteins. (B) Incubation of ^{125}I-labelled collagen with pronase: (a) after 15 min, (b) after 30 min. The film was exposed for 12 h (A) and 20 days (B).

The absence of active forms of proteases and phosphatases in RNA-binding proteins rules out the possibility that the results of phosphorylation of both endogenous and exogenous substrates are affected.

ACKNOWLEDGEMENTS

The authors express their deep gratitude to Professor A.S. Spirin for his constant interest and discussion of the results.

REFERENCES

[1] Ochoa, S., DeHaro, C., Siekierka, J. and Grosfeld, H. (1981) Curr. Top. Cell. Regul. 18, 421–435.
[2] Lastic, S.M. and McConcy, E.H. (1980) Biochem. Biophys. Res. Commun. 95, 917–923.
[3] Decker, S. (1981) Proc. Natl. Acad. Sci. USA 79, 4112–4115.

[4] Bag, J. and Sells, B.H. (1979) J. Biol. Chem. 254, 3137–3140.
[5] Egly, J.-M., Schmitt, M., Elkaim, R. and Kempf, J. (1981) Eur. J. Biochem. 118, 379–387.
[6] Rittschof, D. and Traugh, J.A. (1982) Eur. J. Biochem. 123, 333–336.
[7] Bag, J. and Sells, B.H. (1980) Eur. J. Biochem. 106, 411–424.
[8] Stepanov, A.S., Kandror, K.V. and Elizarov, S.M. (1982) FEBS Lett. 141, 157–160.
[9] Sinclair, G.D. and Dixon, G.H. (1982) Biochemistry 21, 1869–1877.
[10] Hathaway, G.M., Lubben, T.H. and Traugh, J.A. (1980) J. Biol. Chem. 255, 8038–8041.
[11] Feige, J.J., Pirolett, F., Cochet, C. and Chambas, E.M. (1980) FEBS Lett. 121, 139–142.
[12] Meggio, F., Donella Deana, A., Brunati, A.M. and Pinna, L.A. (1982) FEBS Lett. 141, 257–262.
[13] Elizarov, S.M. and Stepanov, A.S. (1978) Biokhimiya 43, 1347–1356.
[14] Kandror, K.V. and Stepanov, A.S. (1983) Biokhimiya 48, 1674–1679.
[15] Schaffner, W. and Weisman, C. (1973) Anal. Biochem. 56, 502–514.
[16] Laemmli, U.K. (1970) Nature 227, 680–685.
[17] Kandror, K.V. and Stepanov, A.S. (1984) Biokhimiya, in press.