BBA Report

BBA 61262

Phosphoprotein kinases from rat liver cytosol

SUEO MATSUMURA and MASAO TAKEDA

Department of Biochemistry, Kobe University School of Medicine, Kobe (Japan)

(Received September 18th, 1972)

SUMMARY

Rat liver cytoplasmic protein kinase (casein kinase of the classical type) (ATP: protein phosphotransferase, EC 2.7.1.37) is separable by gel filtration on a Sephadex column into two fractions which specifically phosphorylate either seryl or threonyl residues of casein and phosvitin. These kinases also react with nuclear phosphoproteins and show closely similar kinetic and catalytic properties to the phosphoprotein kinases tightly associated with chromatin. However, the cytoplasmic enzymes appear not to represent simply a leakage of the nuclear enzymes into the cytoplasm during the isolation procedure.

A protein kinase (ATP:protein phosphotransferase, EC 2.7.1.37), which transfers the terminal phosphate of ATP to seryl and threonyl residues of bovine casein and egg yolk phosvitin, has been found by Burnett and Kennedy[1] in rat liver cytoplasm. Subsequently, similar enzymes have been partially purified from various mammalian tissues[2—4], fish roe[5] and brewer's yeast[2]. Naturally occurring phosphate acceptors of these enzymes are phosphoproteins, as yet uncategorized, which are almost ubiquitously distributed in biological systems[6,7]. Such phosphoproteins, containing up to 1.3% phosphorus by weight, have been found to be enriched in rat liver nuclei, and their participation in regulating some nuclear function has been implied[7]. It is also well documented that the phosphate groups bound to these phosphoproteins turn over rapidly[7]. The present paper will briefly describe that the rat liver cytoplasmic protein kinase is separable into two fractions which each phosphorylate specific sites of casein and phosvitin, as well as nuclear phosphoproteins.

Protein kinase activity was routinely assayed by measuring the acid-insoluble radioactivity of [γ-^{32}P] ATP incorporated into bovine casein (Hammarsten, Merk) as described previously[8]. Protein was determined by the method of Lowry *et al.*[9]. [γ-^{32}P] ATP was prepared by the method of Glynn and Chappell[10]. Rat liver nuclear phosphoproteins were

prepared by the method of Langan[7] except that, instead of using Bio-Rex 70, the final preparation (10–20 mg) was passed through a phosphocellulose column (1 cm x 3 cm) equilibrated with 0.2 M NaCl–0.05 M Tris–HCl at pH 7.5, and heated for 2 min at 60 °C to inactivate nuclear protein kinases, present as slight contaminants in the preparation. The nuclear phosphoproteins thus prepared contained more than 1% phosphorous by weight and showed an essentially similar band pattern (more than 10 distinct bands) to Langan's preparation upon sodium lauryl sulfate–polyacrylamide gel electrophoresis[11].

Wistar albino rats, weighing 150–200 g, fed *ad libitum* on Oriental Yeast Company chow were employed for the present studies. All manipulations were carried out at 0–4 °C. The liver (3 g) was quickly removed after decapitation and homogenized with 5 vol. of 0.25 M sucrose, containing 3.3 mM $CaCl_2$, using a Teflon–glass homogenizer. The homogenate was filtered through 4 layers of gauze and was centrifuged for 20 min at 15 000 x *g*, and the cytosol was obtained from the supernatant by centrifugation for an additional 100 min at 105 000 x *g*. To the cytosol (13 ml), solid $(NH_4)_2SO_4$ was added to 60% saturation (5.1 g). After standing for 30 min, the precipitate was collected by centrifugation, dissolved in 0.05 M Tris–HCl at pH 7.5, containing 0.4 M NaCl, to a protein concentration of about 50 mg per ml, and was dialyzed for several hours against a large volume of the same buffer containing 0.4 M NaCl. The $(NH_4)_2SO_4$ fraction was subjected to gel filtration on a Sephadex G-200 column. As shown in Fig. 1, the cytosol protein kinase was resolved into two fractions. These fractions are referred to as protein kinase C_1 and protein kinase C_2, as designated in this figure. Protein kinase C_1 (40 mg protein) was applied directly to a phosphocellulose column (1 cm x 5 cm) equilibrated with 0.05 M Tris–HCl at pH 7.5, containing 0.4 M NaCl. Under these conditions most of the inactive proteins

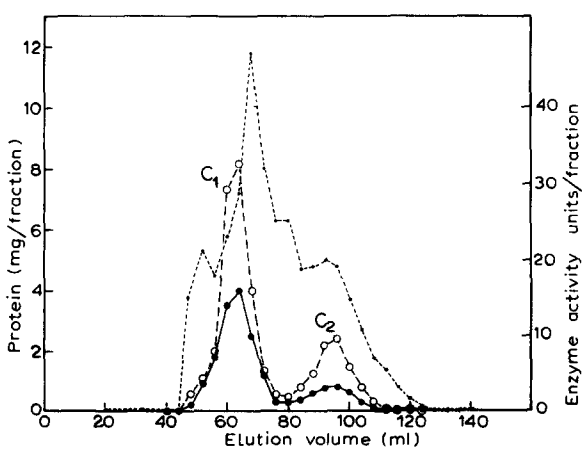

Fig. 1. Separation of two protein kinases from cytosol by gel filtration on a Sephadex G-200 column. An enzyme preparation (112 mg protein) was applied to a Sephadex G-200 column (1.2 cm x 124 cm) and both the gel filtration and enzyme assay were carried out with 80 μg of nuclear phosphoproteins or 200 μg of casein as substrate under the conditions previously described[8]. ●- - -●, protein; ○- - -○, activity with casein; ●——●, activity with nuclear phosphoproteins.

were not adsorbed on the column. After the column was washed with 30 ml of the equilibration buffer, the enzyme was quantitatively eluted from the column with 12 ml of the buffer containing 0.6 M NaCl at a flow rate of 0.4 ml/min. Protein kinase C_2 (25 mg protein) in Fig. 1 was first diluted with an equal volume of 0.05 M Tris–HCl at pH 7.5, and similarly applied to a phosphocellulose column (1 cm x 5 cm) equilibrated with the buffer containing 0.2 M NaCl. After washing the column with 30 ml of the equilibration buffer, the enzyme was eluted with 12 ml of the buffer containing 0.5 M NaCl with a recovery of about 70%. By these procedures protein kinases C_1 and C_2 were purified about 2000- and 500-fold, respectively, starting from the crude cytosol with an overall yield of more than 90% of the original total activity. This better recovery appeared to be due to the removal of some endogenous inhibitory materials.

Protein kinases C_1 and C_2 phosphorylated bovine casein and egg yolk phosvitin (Mann), as well as nuclear phosphoproteins prepared from rat liver (Table I). Calf thymus

TABLE I

SUBSTRATE SPECIFICITIES OF PROTEIN KINASES A_1, A_2, C_1 AND C_2

The incubation was carried out under the standard assay conditions previously described[8] except using 100 μg of each of the substrate proteins. Instead of 10% trichloroacetic acid, 5% trichloroacetic acid containing 0.25% sodium tungstate at pH 2.0 (ref. 12) was employed to precipitate histone and protamine. Each number is expressed as a percentage of the initial velocity, with that for casein being 100%.

Substrate	*Initial velocity (%)*			
	A_1	A_2	C_1	C_2
Casein	100	100	100	100
Phosvitin	75	55	79	59
Nuclear phosphoproteins	50	11	53	17
Histone	5	2	5	3
Protamine	4	5	3	5
Albumin	0	0	0	0

histone (prepared by the method of Johns[13]) and salmon sperm protamine (Sigma) were only slightly active as phosphate acceptors. These protein kinases were not stimulated by cyclic AMP, and were clearly distinguishable from the cyclic AMP-dependent protein kinases which favor histone and protamine as substrates[14]. In order to ascertain whether these protein kinases each phosphorylated specific sites of the substrate molecules, casein was fully phosphorylated with each of these kinases separately and radioactive casein preparations were subjected to acid hydrolysis followed by high voltage paper electrophoresis under the conditions specified earlier[8]. Protein kinase C_1 was found to phosphorylate preferentially threonyl residues (phosphoserine/phosphothreonine = 0.2), whereas protein kinase C_2 phosphorylated mainly seryl residues (phosphoserine/phosphothreonine = 7.8). In another set of experiments, similarly prepared radioactive casein preparations were subjected to tryptic digestion followed by electrophoresis on a cellulose

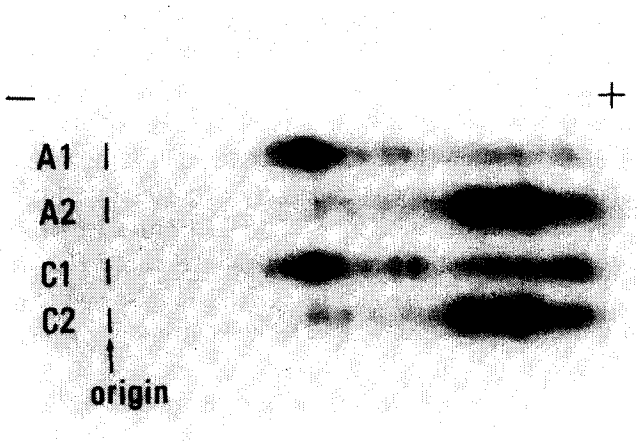

Fig. 2. Autoradiography of the electropherogram of the tryptic digests of casein phosphorylated by protein kinases A_1, A_2, C_1 and C_2. Casein was phosphorylated for 6 h at 30 °C with an excess amount of each protein kinase under the conditions previously described[8], except that 50 μg of casein was employed. The amounts of phosphate incorporated by protein kinases A_1, A_2, C_1 and C_2 were 32, 17, 32 and 17 nmoles per mg casein, respectively. After washing with 10% trichloroacetic acid followed by acetone, the radioactive casein was digested with trypsin at pH 8.0 for 24 h at 37 °C (trypsin:casein = 1:20). The digests were concentrated and subjected to Cellogel R.S. (Chemetron) electrophoresis at 15 V/cm for 2 h with 0.05 M veronal buffer at pH 9.0 containing 5 M urea.

acetate strip and autoradiography. As illustrated in Fig. 2, the radioactive peptide patterns obtained with protein kinases C_1 and C_2 were clearly distinguishable from each other. These results indicate that protein kinases C_1 and C_2 phosphorylate specific and different seryl and threonyl residues of the substrate protein.

It was reported in a preceding paper[8] that two protein kinases (protein kinases A_1 and A_2), tightly associated with rat liver chromatin, phosphorylated separate sites of casein, phosvitin and nuclear phosphoproteins. Therefore studies were undertaken to compare the cytoplasmic and nuclear protein kinases. Protein kinases A_1 and A_2 were extracted from rat liver chromatin as described previously[8], and then purified further by phosphocellulose column chromatography under the conditions employed for protein kinases C_1 and C_2, respectively (see above). The purified nuclear enzyme preparations were essentially free of endogenous phosphate acceptor proteins. As shown in Fig. 2, protein kinases C_1 and A_1 phosphorylated the same specific sites of casein, and similarly protein kinases C_2 and A_2 produced identical radioactive tryptic peptides. Further analysis revealed that protein kinases C_1 and A_1, as well as protein kinases C_2 and A_2, showed closely similar substrate specificities (Table I) and were indistinguishable from each other in their kinetic properties; protein kinases C_1, A_1, C_2 and A_2 showed K_m values for ATP of $6 \cdot 10^{-6}$, $6 \cdot 10^{-6}$, $1 \cdot 10^{-5}$ and $1 \cdot 10^{-5}$ M, respectively and pH optima of 6.8–7.5, 6.8–7.5, 7.5–8.5 and 7.5–8.5, respectively. These results suggest that the cytoplasmic protein kinases are probably identical with the respective nuclear protein kinases. However, the cytoplasmic enzymes appeared not to represent simply a leakage of the nuclear enzymes into cytosol during the

isolation procedure, since using the tissue fractionation procedure with non-aqueous medium[15] it was confirmed that these protein kinases were equally distributed in both cytoplasm and nucleus. Nevertheless, the physiological roles of the protein kinases presented in this paper still remain unexplored.

The authors wish to thank Prof. Yasutomi Nishizuka for valuable discussion and encouragement. Thanks are also due to Mrs S. Nishiyama and Miss K. Kazumasu for their skilful technical assistance. This research was supported in part by the research grants from the Jane Coffin Childs Memorial Fund for Medical Research, the Waksman Foundation of Japan, the Mitsubishi Foundation, and the Scientific Research Fund (1971–1972) of the Ministry of Education of Japan.

REFERENCES

1 Burnett, G. and Kennedy, E.P. (1954) *J. Biol. Chem.* 211, 969–980
2 Rabinowitz, M. and Lipmann, F. (1960) *J. Biol. Chem.* 235, 1043–1050
3 Sundararajan, T.A., Sampath-Kumar, K.S.V. and Sarma, P.S. (1958) *Biochim. Biophys. Acta* 29, 449–450
4 Baggio, B. and Moret, V. (1971) *Biochim. Biophys. Acta* 250, 346–350
5 Mano, Y. (1970) *Biochim. Biophys. Acta* 201, 284–294
6 Pinna, L.A., Clari, G. and Moret, V. (1971) *Biochim. Biophys. Acta* 236, 270–278
7 Langan, T.A. (1967) in *Regulation of Nucleic Acids and Protein Biosynthesis* (Koningsberger, V.V. and Bosch, L., eds), pp. 233–242, Elsevier, Amsterdam
8 Takeda, M., Yamamura, H. and Ohga, Y. (1971) *Biochem. Biophys. Res. Commun.* 42, 103–110
9 Lowry, O.H., Rosenbrough, N.J., Farr, A.L. and Randall, R.J. (1951) *J. Biol. Chem.* 193, 265–275
10 Glynn, I.M. and Chappell, J.B. (1964) *Biochem. J.* 90, 147–149
11 Weber, K. and Osborn, M. (1969) *J. Biol. Chem.* 244, 4406–4412
12 Gardner, R.S., Wahba, A.J., Basilio, C., Miller, R.S., Lengyel, P. and Speyer, J.F. (1962) *Proc. Natl. Acad. Sci. U.S.* 48, 2087–2094
13 Johns, E.W. (1967) *Biochem. J.* 104, 78–82
14 Langan, T.A. (1968) *Science* 162, 579–580
15 Siebert, G. (1967) in *Methods in Cancer Research* (Busch, H. ed.), Vol. 2, pp. 287–301, Academic Press, New York, London