SIMILARITY AND PLEIOTROPIC ACTIONS OF ADENOSINE 3',5'-MONOPHOSPHATE-DEPENDENT PROTEIN KINASES FROM MAMMALIAN TISSUES*

Hirohei Yamamura, Yoshihide Inoue, Reiko Shimomura+
and Yasutomi Nishizuka

From Department of Biochemistry
Kobe University School of Medicine
Kobe, Japan

Received November 8, 1971

Summary Adenosine 3',5'-monophosphate-dependent protein kinases in various mammalian tissues appear to be similar enzymes which show rather broad and same substrate specificities. The enzymes catalyze the incorporation of the terminal phosphate of ATP into the same specific sites of several regulatory enzymes and functional proteins, such as glycogen phosphorylase b kinase, glycogen synthetase, lipase, histone and ribosomal proteins. A plausible evidence seems to relegate a role of crucial importance of the protein kinase to simultaneous control of various biochemical processes in each tissue.

Recent reports from this and other laboratories have provided an evidence that adenosine 3',5'-monophosphate (cyclic AMP)-dependent protein kinase is an inactive form of protein kinase, the activity of which is inhibited by association of regulatory protein (R-protein), and that cyclic AMP dissociates such inactive kinase into R-protein and catalytically active protein kinase (1-6). A preceding paper (7) has shown that protein kinases and R-proteins from homologous as well as heterologous tissues are crosswise reactive. An evidence has been also presented that in many tissues and organs a single protein kinase

* This investigation has been 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 Cancer Research Fund (1971) of the Ministry of Education of Japan.
+ On leave from Tokiwa College, Kobe, Japan.

is present in at least two, presumably multiple, inactive forms which differ from each other in their associated R-proteins, and that all inactive forms are activated by the cyclic nucleotide in a similar manner resulting in the release of an active protein kinase (8). The present communication describes that protein kinases in mammalian tissues appear to be similar enzymes which show rather broad substrate specificities, and that the enzymes phosphorylate the same specific sites of several regulatory enzymes and functional proteins, such as glycogen phosphorylase b kinase, glycogen synthetase, lipase, histone and ribosomal proteins. The evidence may imply, at least in part, a molecular basis of pleiotropic actions of various hormones in controlling several biochemical reactions within the target cells.

Wistar albino rats (150-200 g) and domestic rabbits (3-3.5 kg) maintained ad libitum on CLEA laboratory chows were employed for the present studies. The protein kinase activity was routinely assayed by measuring the radioactivity of ATP-γ-^{32}P incorporated into calf thymus histone as described previously (1). Rat liver protein kinase (cyclic AMP-independent, free of R-protein) and its inactive forms (cyclic AMP-dependent) were purified about 300 and 50 fold, respectively, from the soluble fraction by ammonium sulfate fractionation, followed by DEAE-Sephadex and hydroxylapatite column chromatography as described previously (8).[1/] Active protein kinases (cyclic AMP-independent, free of R-protein) and their inactive forms (cyclic AMP-dependent) of other

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1/ In a preceding paper (8) a method of the purification of active protein kinase free of R-protein (protein kinase B_0) and its two inactive forms (protein kinase B_1 and B_2) from rat liver was described. Both protein kinase B_1 and B_2 were dissociated by cyclic AMP into protein kinase B_0 and different R-proteins (8). For the present studies the active protein kinase (protein kinase B_0) was mainly employed unless otherwise noted.

tissues and organs were purified by the same procedure except that DEAE cellulose was substituted for DEAE-Sephadex.[2] All enzyme preparations employed were practically free of endogeneous phosphate acceptor protei and interfering enzymes.

Cyclic AMP-dependent protein kinases in mammalian tissues appeared to be catalytically similar enzymes and phosphorylate the same specific seryl and threonyl residues of substrate protein molecules. For exampl with a limited amount of calf thymus histone as substrate, the amount of phosphate incorporated by the simultaneous addition of rat liver and rabbit skeletal muscle protein kinases did not exceed the amount by either one of these kinases alone, and the phosphate was saturated at the same level. Acid hydrolysis of the radioactive histone preparation resulted in the formation of mainly phosphoserine and some phosphothreonine. When calf thymus histone was fully phosphorylated by these kinases and subjected to tryptic digestion followed by paper chromatography and high voltage paper electrophoresis, exactly identical radioactive peptide patterns were obtained as shown in Fig. 1. Similar results were also obtained with cyclic AMP-dependent protein kinases partially purified from other tissues and organs including bovine adrenal gland and rat brain (Fig. 1).

In order to obtain an additional evidence for the similarity of protein kinases, rat liver and rabbit skeletal muscle protein kinases were chosen to examine further whether these kinases phosphorylated some other regulatory enzymes and controlled their enzymic activities. As shown in Fig. 2, both rat liver and rabbit skeletal muscle protein

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2/ In either one of the tissues and organs thus far tested, cyclic AMP-dependent protein kinase was found to be a mixture of at least two inactive forms of a single protein kinase. The inactive forms were dissociated by cyclic AMP into a common active protein kinase and different R-proteins. The detailed experimental results together with the purification procedure will be described elsewhere. For the presen studies the active protein kinases free of R-protein were mainly employ unless otherwise indicated.

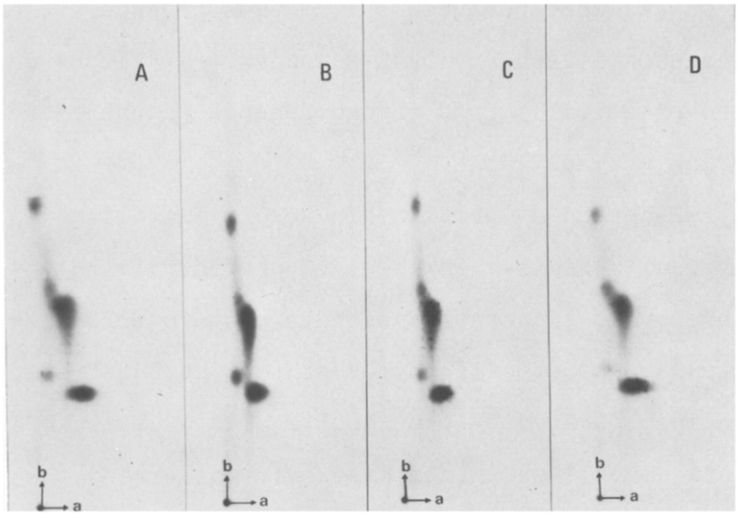

Fig. 1. Autoradiography of the tryptic digests of radioactive histone preparations phosphorylated by various protein kinases. Calf thymus whole histone was fully phosphorylated by the protein kinase indicated, and subjected to tryptic digestion followed by paper chromatography (direction a) with n-butanol-acetic acid-water (4:1:1) and high voltage paper electrophoresis at pH 3.5 (direction b) with Toyo Roshi No. 51 filter paper. The electrophoresis was carried out under the conditions described previously (1). (A) with rat liver protein kinase. (B) with rabbit skeletal muscle protein kinase. (C) with bovine adrenal protein kinase. (D) with rat brain protein kinase (an inactive form) plus cyclic AMP.

kinases reacted with rabbit skeletal muscle glycogen phosphorylase b kinase resulting in the consecutive activation of glycogen phosphorylase b as judged by the phosphorylase activity in the absence of 5'-AMP, and also by the incorporation of the terminal phosphate of ATP into an acid-precipitable material. Some activities observed in the absence of protein kinase in this experiment appeared to be due to autocatalytic phosphorylation of glycogen phosphorylase b kinase as described by Krebs (10), or due to the activated form of glycogen phosphorylase b kinase which slightly contaminated the preparation. Soderling and Hickenbottom (12) and Krebs (13) reported that rabbit skeletal muscle protein kinase phosphorylated glycogen phosphorylase b kinase as well as glycogen synthetase from the same tissue. Consistent with these observations,

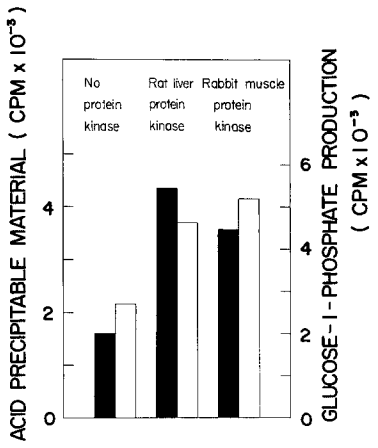

Fig. 2. Phosphorylation and activation of rabbit skeletal muscle glycogen phosphorylase b kinase by rat liver and rabbit skeletal muscle protein kinases. The reaction mixture for the assay of activation of glycogen phosphorylase b kinase contained 5 μmoles of Tris-Cl buffer, pH 7.0, 2 μmoles of $MgCl_2$, 10 mμmoles of ATP, 45 μg of crystalline muscle glycogen phosphorylase b which was prepared and treated with charcoal to remove 5'-AMP by the method of Fischer and Krebs (9), 1 μg of phosphorylase b kinase which was prepared by the method of Krebs (10), and either 1.3 μg of rat liver protein kinase or 5 μg of rabbit skeletal muscle protein kinase in a total volume of 0.135 ml. After incubation for 3 min at 30°, 0.50 ml of a solution containing 200 μg of glycogen and 15 mμmoles of ^{32}Pi (2.2×10^7 cpm/μmole) was added, and the mixture was incubated for additional 2 min at 30°. Then, ^{32}Pi was precipitated as triethylamine-phosphomolybdate complex by the method of Sugino and Miyoshi (11), and the radioactive organic phosphate (glucose 1-phosphate) remaining in the supernatant was determined. The reaction mixture for the assay of phosphorylation reaction contained 10 μmoles of Tris-Cl buffer, pH 7.0, 5 μmoles of $MgCl_2$, 2.5 mμmoles of ATP-γ-^{32}P (6×10^7 cpm/μmole), 140 μg of crystalline phosphorylase b treated as above, 11 μg of phosphorylase b kinase, and either 1.3 μg of rat liver protein kinase or 2.0 μg of rabbit skeletal muscle protein kinase in a total volume of 0.25 ml. After incubation for 5 min at 30°, the acid-precipitable radioactivity was determined using a Millipore filter. ■ radioactivity of acid-precipitable material; □ radioactivity of glucose 1-phosphate produced.

both rat liver and rabbit skeletal muscle protein kinases reacted equally with muscle glycogen synthetase and converted I-form to D-form as shown in Fig. 3. Huttunen et al. (15) and Corbin et al. (16) propos recently that rabbit skeletal muscle protein kinase activated rat adipocyte lipase. Walton et al. (17) and Eil and Wood (18) described that bovine adrenal and rat liver ribosomal proteins also served as substrat for cyclic AMP-dependent protein kinases obtained from the respective

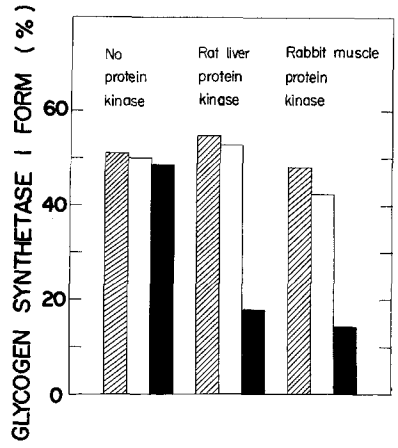

Fig. 3. Conversion of I-form to D-form of rabbit skeletal muscle glycogen synthetase by rat liver and rabbit skeletal muscle protein kinases. The reaction mixture initially contained 2 μmoles of Tris-Cl buffer, pH 7.0, 0.5 μmole of $MgCl_2$, 10 mμmoles of ATP, 5 μg of rabbit skeletal muscle glycogen synthetase prepared by the method of Villar-Palasi et al.(14), and either 78 μg of rat liver protein kinase [an inactive form (protein kinase B_1), see footnote 1] or 98 μg of rabbit skeletal muscle protein kinase [an inactive form, see footnote 2] in a total volume of 0.1 ml. Where indicated cyclic AMP (10^{-5} M) was added. After incubation for 3 min at 30°, 0.05 ml of a solution containing 1 μmole of EDTA, 5 μmoles of Tris-Cl buffer, pH 8.4, 1 mg of glycogen, 1 μmole of UDP-glucose-U-^{14}C (7,500 cpm), and the mixture was incubated for additional 15 min at 30°. The control incubation was made with 1 μmole of glucose 6-phosphate as an additional ingredient to assay the total activity (I- and D-forms) of glycogen synthetase. The reaction was finally stopped by the addition of 10 ml of 75 % ethanol. The precipitate was collected on a glass-filter paper disk (Whatman GF 83) and the radioactivity was determined. ▨ without ATP; ▭ without cyclic AMP; ■ complete system.

tissues. These observations were confirmed with rat liver as well as rabbit skeletal muscle protein kinases as exemplified by the experiment given in Fig. 4 where both kinases were shown to be capable of phosphorylating rat liver ribosomal proteins.

Protein kinases obtained from various tissues showed closely similar kinetic properties, and were not distinguishable from each other by their substrate specificities, Km values for ATP, heat stability, Mg requirement and pH optima. Either one of these kinases was inhibited by R-protein from the homologous as well as from the heterologous tissue and the inhibition was completely overcome by the addition of cyclic AMP. The exact similarity or identity of the protein kinases may be

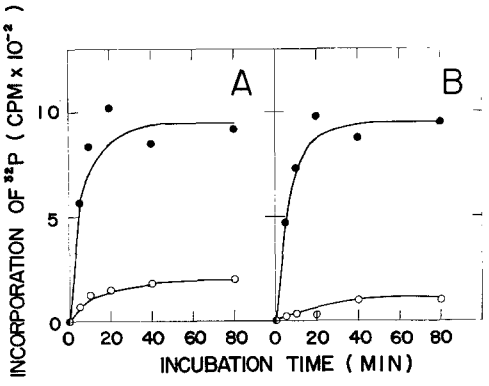

Fig. 4. Phosphorylation of rat liver ribosomal proteins by rat liver and rabbit skeletal muscle protein kinases. The reaction mixture (0.25 ml) contained 12 μmoles of Tris-Cl buffer, pH 7.0, 3 μmoles of $MgCl_2$, 2.5 mμmoles of ATP-γ-^{32}P (6 x 10^7 cpm/μmole), rat liver ribosomes (400 μg protein) which were prepared by the method of Honjo et al. (19) and washed 5 times with 0.5 M sucrose containing 0.5 M NH_4Cl and 2 mM $MgCl_2$, and either 52 μg of rat liver protein kinase [an inactive form (protein kinase B_1), see footnote 1] in Fig. 4A or rabbit skeletal muscle protein kinase [an inactive form, see footnote 2] in Fig. 4B. Where indicated cyclic AMP (10^{-6} M) was added. Incubation was carried out at 30°. The acid precipitable radioactivity was determined using a Millipore filter. The radioactive phosphate was shown to be covalently linked to seryl and threonyl residues as judged by acid hydrolysis followed by paper electrophoresis with authentic samples of phosphoserine and phosphothreonine. Under these conditions neither ribosomes nor protein kinase alone produced acid-precipitable radioactivity. ●——●, with cyclic AMP; O——O; without cyclic AMP.

explored by further investigations.

A vast array of evidence now available in the literature indicates that cyclic AMP acts as an intracellular mediator of various hormones and regulates a variety of biochemical processes. The experimental results presented in this paper suggest that cyclic AMP-dependent protein kinases in most tissues and organs from various mammalian species appear to be closely similar enzymes which show rather broad and identical spectra of phosphate acceptor proteins. Therefore, a single protein kinase activated by the cyclic nucleotide may possibly phosphorylate several regulatory enzymes and functional proteins simultaneously, although the exact substrate proteins in each tissue has not yet been completely elucidated. Nevertheless, a plausible evidence may suggest that the action of hormone seems to be amplified by the succesive

activation of two consecutive enzymes, adenyl cyclase and protein kinase, resulting in the control of various biochemical reactions in each target cell. However, this assumption may not necessarily exclude additional actions of cyclic AMP which are not mediated by protein kinases. Further studies are underway to explore the detailed properties as well as the precise role of protein kinases distributed in mammalian tissues.

Acknowledgment The authors are grateful to Misses S. Egami and K. Kazumasu for their skillful technical assistance.

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