RESOLUTION OF TWO PROTEIN KINASE MODULATORS FROM LACTATING RAT MAMMARY GLAND

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SUMMARY

Two proteins (modulators I and II) which alter the substrate specificity for cyclic AMP-dependent protein kinase have been partially purified from lactational rat mammary gland. Modulator I stimulates specifically the rate of phosphorylation of F-1 and F2a histones without appreciably altering the rate of phosphorylation of F3 or F2b histones. Modulator II inhibits the activity of protein kinase for the phosphorylation of all histone subfractions. Modulator I was eluted earlier than modulator II during chromatography on DEAE-cellulose and gel filtration through Sephadex G-75, steps which led to their complete resolution. Both proteins are heat-stable.

Previous studies have characterized the cyclic AMP-dependent, cytosol protein kinase of mammary gland and have demonstrated its central role in the hormonal regulation of mammary cells (1-3). The enzyme is rapidly induced in response to prolactin, and serves to phosphorylate specific protein constituents of ribosomes, plasma membranes, and chromatin (3,4). The present study identifies two additional proteins in mammary gland which further regulate protein kinase activity by altering the pattern of phosphorylation of various histone substrates. These proteins have been termed modulators of protein kinase, and their partial purification and separate characteristics are described in this report.

MATERIALS AND METHODS

Chemicals - ATP-γ-^{32}P was obtained from International Chemical and Nuclear Corporation. Calf thymus whole histones, and various purified histone subfractions were purchased from Worthington. Calcium phosphate gel was obtained from Sigma Chemical, and DEAE-cellulsoe was obtained from Bio-Rad.

Assay of protein kinase - Cyclic AMP-dependent protein kinase II was isolated from rat mammary gland by the method described previously (1). The activity of the enzyme was measured in the presence of 5μM cyclic AMP with or without protein kinase modulators by the procedure described previously (3). A unit of enzyme activity was defined as the amount of enzyme which causes the transfer of 1p mole of ^{32}P from ATP-γ-^{32}P to the recovered protein during 30 min under the standard assay conditions. The specific activity of the isolated mammary protein kinase II was 2 units/μg protein when calf thymus whole histones(0.5mg) were used as protein substrate. Total protein was determined by the method of Lowry et al (5) using bovine serum albumin as the standard.

Isolation of protein kinase modulators - Lactating rat mammary gland was homogenized in 4 mM EDTA (pH 7.0), and the homogenate was heated at 90° for 10 min and then chilled. The suspension was centrifuged at 25,000 x g for 10 min and the pellet was discarded (Step 1). The pH of the resulting supernate was adjusted to 4.7 by addition of 1N acetic acid with constant stirring. After 30 min, the suspension was centrifuged at 25,000 x g for 10 min and the precipitate was discarded. The pH of the supernatant fluid was adjusted to 7.0 with sodium bicarbonate and solid ammonium sulfate was added to the above solution (48 g/100 ml) with stirring. After 30 min the precipitate was collected by centrifugation at 25,000 x g for 10 min and the supernate was discarded. The residue was dissolved in 5 mM potassium phosphate buffer (pH 7.0), and the dialyzed fraction was applied to a column of DEAE-cellulose (2.8 x 36 cm) previously equilibrated with 5 mM potassium phosphate buffer (pH 7.0). The column was washed with 180 ml of the equilibration buffer prior to further elution with a linear gradient of potassium phosphate (5 to 300 mM) in a total volume of 350 ml of the buffer. The flow rate was 48 ml per hour and the volume in each fraction was 8 ml (Step 3). Active fractions of modulators were pooled together and concentrated by ultrafiltration

through a UM-10 Amicon filter. The concentrated fractions were dialysed against 5 mM glycerophosphate buffer (pH 6.5) containing 0.2 mM dithiothreitol (Buffer A) and subjected to gel filtration on a column (2.5 x 36 cm) of Sephadex G-75 with buffer A as the eluting solvent. The flow rate was 3 ml per hour and the volume in each fraction was 2.0 ml (Step 4). The active fractions of modulator I were pooled together and treated with a suspension of calcium phosphate gel (1.0 g/25 mg protein) for 30 min. The gel adsorbed the activity of the modulator, which was selectively eluted with 50 mM potassium phosphate buffer, pH 7.0. The gel eluate was concentrated by ultrafiltration through a UM-10 Amicon filter, dialysed against buffer A and subjected to gel filtration on a column (2.8 x 30 cm) of Sephadex G-100 with buffer A as the eluting solvent. The flow rate was 25 ml per hour and the volume in each fraction was 4 ml (Step 5).

RESULTS AND DISCUSSION

The mammary protein kinase modulator preparation was resolved by DEAE-cellulose chromatography into two distinct peaks, I and II (Fig. 1). Modulator I stimulated considerably the rate of phosphorylation of F-1 histone by mammary cytosol cyclic AMP-dependent protein kinase. Modulator II markedly inhibited the phosphorylation of whole histones by the mammary protein kinase. Fig. 2 shows the Sephadex G-75 gel filtration patterns of each of these active fractions. Modulator I was eluted as a single peak of activity, and the activity of modulator II was undetectable in this preparation. The modulator II activity as obtained by DEAE-cellulose chromatography was contaminated by modulator I which was completely removed by Sephadex gel filtration. The rate of elution of modulator I was much faster than that of modulator II, indicating its larger molecular size.

The activities of both the protein kinase modulators were destroyed by incubation with trypsin but not by incubation with DNase or RNase,

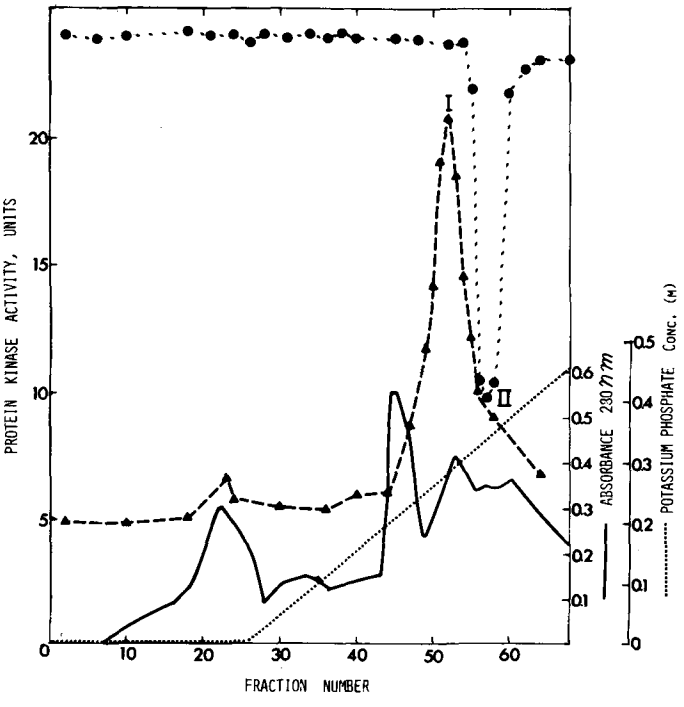

Fig. 1. Separation of two rat mammary protein kinase modulators (I and II) by chromatography on DEAE-cellulose column. Modulator preparation (780 mg protein) obtained from 58 g lactating mammary gland was used for chromatographic separation. The effect of an aliquot of 50 μl of each fraction on the activity of the cyclic AMP-dependent mammary protein kinase II (15 μg) was determined in presence of 5 μM cyclic AMP by using 0.5 mg of total histones (●-----●) or 0.25 mg of F-1 histone (▲--▲) as protein substrates under the standard assay conditions.

indicating that they are protein in nature. The modulator activities were stable to heat treatment at 100° for 10 min. Table I shows the effect of modulators I and II on the substrate specificity of mammary cyclic AMP-dependent protein kinase for the phosphorylation of various histones. The pattern of phosphorylation of specific histones by mammary protein kinase was greatly altered by the modulators. Modulator I stimulated the phosphorylation of F2b and F-1 histones by protein kinase, and the stimulatory effect was maximal when F-1 histone was the protein substrate. Modulator I had no significant effect on the rate of phosphorylation of F2a and F-3 histones by protein kinase. When whole histones

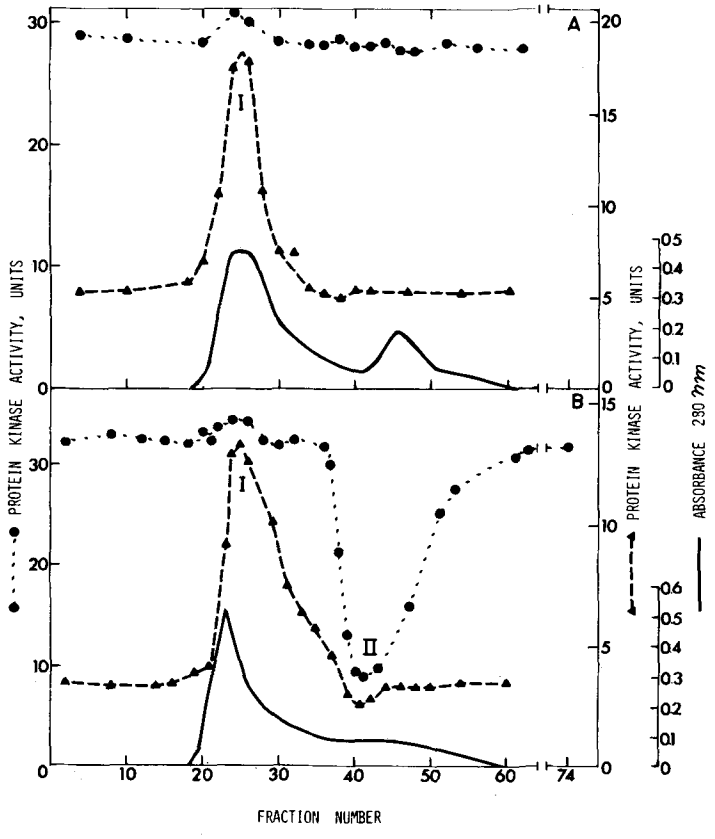

Fig. 2. Sephadex G-75 gel filtration patterns of rat mammary protein kinase modulators I (▲- - -▲) and II (●----●). The following amounts of the modulators obtained by DEAE-cellulose chromatography were applied to the column: A, modulator I, 45 mg protein (Fractions 47 to 54, Fig. 1); B, modulator II, 40 mg protein (Fractions 55 to 60, Fig. 1). The activities of modulators were monitored by determining the effects of an aliquot of 50μl of each fraction on the activity of the cyclic AMP-dependent mammary protein kinase II (15μg protein) by using F-1 histone (A, 80 μg; B, 0.25 mg) as substrate with modulator I and total histones (0.5 mg) as substrate with modulator II. All the assays were done in the presence of 5μM cyclic AMP and under the standard conditions.

were used as protein substrate of protein kinase, modulator I stimulated slightly (approximately 8%) the activity of the enzyme. In contrast, modulator II exhibited an inhibitory effect on protein kinase activity when total histones or histone subfractions were used as substrates, and the degree of inhibition was substrate specific. The rates of phosphorylation of the endogenous proteins of the partially purified preparations

TABLE I

Effect of modulators on the phosphorylation of whole histones and histone subfractions by the cyclic AMP-dependent mammary protein kinase.

The activity of cytosol mammary protein kinase (15 μg) was measured by using 250 μg of each of the various histone fractions as protein substrates in presence of 5 μM cyclic AMP with or without added mammary modulators: I (37 μg protein) and II (66 μg protein). Preparation of modulator II (Fractions 40 to 43) as obtained by Sephadex G-75 gel filtration was used for these studies. Modulator I (Sephadex G-75 fraction) was further purified by a combination of calcium phosphate gel adsorption and Sephadex G-100 gel filtration. The activity of modulator I was eluted as a single peak (Fractions 16 to 24), the active fractions were pooled together and this preparation of modulator I was completely free of modulator II activity. This preparation of modulator I was used for these studies.

Histone substrates	Incorporation of ^{32}P into protein		
	Without modulator	Modulator I	Modulator II
	p moles/30 min		
F-1	4.8	18.1 (277)*	3.8 (21)**
F-3	12.7	13.0 (N.S.)*	4.9 (61)**
F-2a	6.5	6.5 (N.S.)*	2.8 (57)**
F-2b	43.4	57.1 (32)*	26.4 (39)**
Total	19.8	21.3 (8)*	5.4 (73)**

* Figures within parenthesis show per cent stimulation.
** Figures within parenthesis show per cent inhibition.

of modulators by the exogenous protein kinase were insignificant, indicating that the actions of modulators represent alterations in the rate of phosphorylation of histones. The modulators do not function by inhibiting or stimulating the dephosphorylation of phosphorylated histones. The inhibitory action of modulator II on cyclic AMP-dependent mammary protein kinase is noncompetitive with respect to whole histones as protein the substrate.

Protein kinase modulator (6,7) and inhibitor (8,9) activities have been demonstrated in several other sources. A modulator from lobster tail muscle has been shown to act by altering the substrate specificity

of both cyclic GMP-dependent and cyclic AMP-dependent protein kinases, increasing the rate of phosphorylatin of some protein substrates and decreasing that of others (6,7). The present study demonstrates the presence of two protein kinase modulators (I and II) in lactating mammary gland. Modulators I and II have substrate-specific stimulatory and inhibitory actions, respectively, on the cyclic AMP-dependent mammary protein kinase for the phosphorylation of various histones. It is thus possible that the process of cell differentiation confers upon cells a tissue-specific pattern of protein kinase modulator distribution. During cell differentiation the tissue-specific protein kinase modulator(s) may interact with protein kinases to achieve a high degree of specificity in the phosphorylation of sites in the chromatin proteins. Studies are currently in progress to characterize further the mammary protein kinase modulators and to determine their functional role in intracellular regulation.

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