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Identity and substrate specificity of human erythrocyte membrane-bound and cytosolic casein kinases

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The relationship and substrate specificity of the human erythrocyte membrane kinase and casein kinase A were investigated. Based on *Staphylococcus aureus* V8 protease digestion patterns, the 2 kinases appeared to be structurally homologous. These enzymes also exhibited the same substrate specificity and phosphorylated the same synthetic peptides and domains of ankyrin. Both kinases did not utilize GTP effectively as a substrate and were not inhibited by low concentrations of heparin, suggesting that they were type I casein kinases. An analysis of synthetic peptide phosphorylation failed to reveal a specific pattern of recognition of the amino acid sequence surrounding the phosphorylation site.

Erythrocyte; Casein kinase; Membrane; Ankyrin; Phosphorylation

1. INTRODUCTION

Casein kinases are a class of messenger-independent protein serine/threonine kinases that are widely distributed in animal [1] and plant [2] cells. Casein and phosvitin are the 2 substrates commonly used to assay the activity of these enzymes, whence the name casein kinase is derived. Casein kinases can be divided into 2 types, designated as casein kinase I and II [1]. The 2 casein kinases can be easily distinguished by differences in their ability to utilize GTP as a phosphoryl donor and their sensitivities to inhibition by low concentrations of the glycosaminoglycan heparin. Casein kinase II is unique among protein kinases in that it can utilize GTP almost as effectively as ATP as a phosphoryl donor [1,2]. In addition, casein kinase II is strongly inhibited by heparin [2,3]. By contrast, both GTP and heparin are ineffective as substrate and inhibitor, respectively, for casein kinase I [1,3]. Although the role of these kinases in the regulation of cellular processes remains to be established, recent studies suggest that casein kinase II may also be involved in signal transduction [4].

Casein kinase activities have also been detected in human erythrocytes. A casein kinase, designated as membrane kinase, has been purified to apparent homogeneity from the membrane fraction [5]. Two other casein kinases were isolated from the cytosolic fraction. One of these, designated as casein kinase A, was purified by Simkowski and Tao [6], while the other was isolated by Boivin and Galand [7]. This latter cytosolic casein kinase has properties that can be identified with casein kinase II [7,8]. On the other hand, casein kinase A exhibits similar molecular and kinetic properties as the membrane kinase, suggesting that these 2 enzymes may be the same [5,6,9,10]. Both these kinases catalyze the phosphorylation of erythrocyte membrane proteins, including spectrin, ankyrin, band 3, and protein 4.1 [6,11], and may play a role in the regulation of erythrocyte membrane cytoskeletal protein interactions [9,12].

In this report, we further examined the relationship of the membrane kinase and casein kinase A by comparing the peptide maps of these kinases. The substrate specificity of the 2 kinases was also investigated using ankyrin and synthetic peptides. The use of synthetic peptides will allow us to determine whether these kinases recognize a specific sequence motif at the phosphorylation site. The identity of these kinases with casein kinase I was also investigated.

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2. MATERIALS AND METHODS

DDDEESITRR(amide) was custom-synthesized by Multiple Peptide System and further purified by HPLC using a Vydac C18 column. Kemptide (LRRASLG), RKRSRAE, RRREEETEEE, and ACTH fragment 18–39 (RPVKVYPAGAEDESAEAFPLEF) were obtained from Peninsula Laboratories. RRKASGP, LPGLPSAASSEDAGQS(amide), fibrinopeptide A (ADSGEGDFLAEGGGVR), interleukin 1β fragment 163–171 (VQGEESBDK), and the catalytic subunit of bovine cardiac cAMP-dependent protein kinase were purchased from Sigma Chemical Co. [γ-^{32}P]ATP and [γ-^{32}P]GTP were obtained from Amersham. Protein kinase C was purified from bovine brain as described previously [13]. The wheat germ kinase was prepared as described by Yan and Tao [14].

The human erythrocyte membrane casein kinase [5], cytosolic casein kinase A [6], and ankyrin were purified to apparent homogeneity as

described by Lu et al. [9]. Casein kinase activity was assayed according to Tao et al. [5]. One unit of kinase activity was defined as that amount of enzyme that catalyzed the incorporation of 1 nmol of phosphate into casein per min.

The phosphorylation of synthetic peptides was conducted in a reaction mixture of 50 μl containing 50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 0.2 mM [γ-^{32}P]ATP or [γ-^{32}P]GTP, 0.5 mg/ml synthetic peptide substrates, and 1–2 unit/ml kinase. Incubation was carried out at 37°C for 90 min, and the reaction terminated by the addition of 21 μl glacial acetic acid. The incorporation of ^{32}P into the peptides was analyzed by the Dowex 1 × 8 anion exchange column method [15].

Peptide mapping was conducted as described by Cleveland et al. [16] on a 15% SDS-polyacrylamide gel. The kinase was digested using 15 μg/ml of *Staphylococcus aureus* strain V8 protease. After electrophoresis, the protein bands were visualized by silver staining [17].

Phosphoamino acid analysis was carried out as described by Yan and Tao [18].

3. RESULTS

In order to further examine the relationship between the erythrocyte membrane kinase and casein kinase A, these purified kinases were digested with V8 protease and the resultant peptide fragments separated by SDS-polyacrylamide gel electrophoresis. As shown in Fig. 1 (lanes 2 and 3), the peptide maps of the 2 kinases are identical. The result suggests that the 2 enzymes are structurally homologous and may represent the same enzyme.

The catalytic specificities of the erythrocyte casein kinases were further investigated using ankyrin and synthetic peptides as substrates. Ankyrin is an extrinsic membrane phosphoprotein that binds spectrin and band 3, which is an integral membrane protein. This

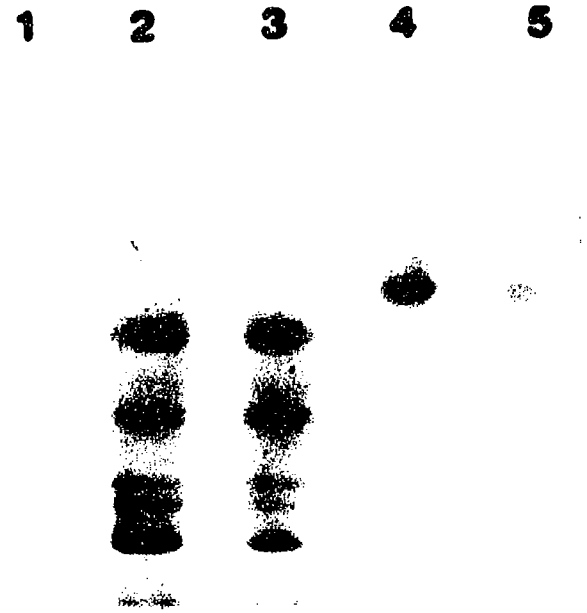

Fig. 1. Peptide maps of human erythrocyte casein kinase A and membrane kinase. About 4 μg each of casein kinase A (lane 2) and membrane kinase (lane 3) was digested with *S. aureus* V8 protease, and the peptide fragments separated as described in section 2. Lane 1, V8 protease alone; lanes 3 and 4 show the electrophoretic profiles of untreated casein kinase A and membrane kinase, respectively.

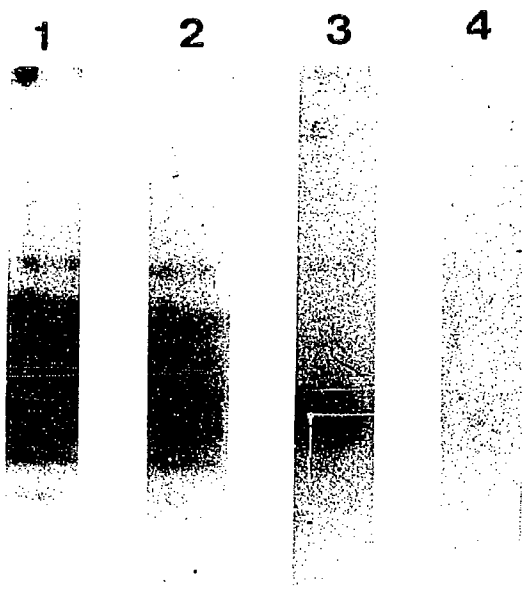

Fig. 2. Phosphopeptide maps of ankyrin phosphorylated by casein kinase A and membrane kinase. The phosphorylation of ankyrin (8 μg) by casein kinase A (lane 1), membrane kinase (lane 2), or the catalytic subunit of cAMP-dependent protein kinase (lane 3) was carried out at 37°C for 90 min in a reaction mixture (100 μl) containing 50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 0.2 mM [γ-^{32}P]ATP (150 cpm/pmol), 40 mM KCl, 10 μg/ml each of the protease inhibitors, leupeptin, pepstatin and aprotinin, and 3 units/ml of casein kinase A or membrane kinase, or 5 units/ml of the catalytic subunit of cAMP-dependent protein kinase. Phosphorylation by protein C (lane 4) was conducted essentially as described by Chao and Tao [13]. The reaction mixtures were electrophoresed on an 8% SDS-polyacrylamide gel; and the ^{32}P-labeled ankyrin was excised from the gel, digested with V8 protease, and the cleavage products analyzed as described in section 2. The autoradiogram was developed from a Kodak X-Omat film that had been exposed to the gel for 12 h.

binding property of ankyrin provides an attachment site for the erythrocyte membrane cytoskeletal network to the lipid bilayer [19]. Ankyrin is a substrate for both the membrane-bound and cytosolic casein kinases as well as for the cAMP-dependent protein kinase [9,20]. Whether these kinases phosphorylate the same or different domains within ankyrin was analyzed by comparing the electrophoretic patterns of phosphopeptides obtained from V8 protease cleavage. Figure 2 shows that the electrophoretic patterns of the phosphopeptides derived from ankyrin phosphorylated by the membrane-bound (lane 2) and cytosolic (lane 1) casein kinases are the same. By contrast, a different pattern of phosphorylation (lane 3) was obtained with the bovine cardiac muscle cAMP-dependent protein kinase. The results presented in Fig. 2 (lane 4) further show that ankyrin is not a substrate of protein kinase C.

The use of synthetic peptides of known amino acid sequences as substrates will provide a clue as to whether phosphorylation involves the recognition by the kinases of a specific sequence motif at the phosphorylation site.

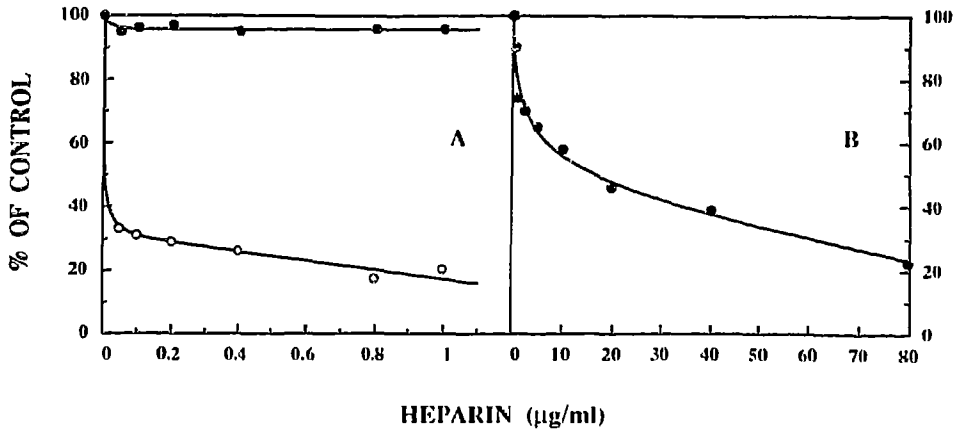

Fig. 3. Effect of heparin on the activities of casein kinase A and wheat germ kinase. Phosphorylation of casein by casein kinase A (●) and wheat germ kinase (○) was carried out at 37°C for 5 min in 200 μl of reaction mixtures containing 50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 150 mM KCl, 2 mg/ml casein, 0.2 mM [γ-^{32}P]ATP (31 cpm/pmol), 0.2 unit/ml of the kinase, and varying amounts of heparin.

Some of these synthetic peptides are model substrates for specific protein kinases. Table I shows the activities of casein kinase A and membrane kinase toward various synthetic peptides. For comparison, the phosphorylation of these peptides by the cAMP-dependent protein kinase and a wheat germ casein kinase [14] was also examined. The synthetic peptide, DDDEESITRR, was the most effective substrate for both casein kinase A and membrane kinase, and was not significantly phosphorylated by the cAMP-dependent protein kinase and the wheat germ kinase. Phosphoamino acid analysis indicated that phosphorylation occurred primarily on the seryl residue, and that the threonyl residue was not phosphorylated.

Somewhat surprisingly, both membrane kinase and casein kinase A also phosphorylate LPGLPSAASSEDAGQS. This peptide is derived from the carboxyl terminal of porcine prepro-galanin [21]. The peptide contains several seryl residues that could potentially serve as phosphorylation sites. Which of these seryl residues was phosphorylated by the red cell casein kinases, however, remains to be determined. The cAMP-dependent protein kinase and wheat germ kinase, however, were unable to phosphorylate the prepro-galanin peptide fragment.

Table I

Phosphorylation of peptides by cAMP-dependent protein kinase and erythrocyte and wheat germ casein kinases

Peptide	^{32}P Incorporated (pmol)			
	PKA	CKA	MK	WGK
RRKASGP	6 314	48	19	7
RRREEETEEE	73	27	9	1518
DDDEESITRR	6	341	303	46
RKRSRAE	4 663	61	37	9
LRRASLG	4 700	61	23	54
LPGLPSAASSEDAGQS	0	262	212	31

PKA, catalytic subunit of cAMP-dependent protein kinase; CKA, casein kinase A; MK, membrane kinase; WGK, wheat germ kinase.

Neither of the erythrocyte kinases catalyzed phosphorylation of the cAMP-dependent protein kinase substrates, RRKASGP, RKRSRAE, and kemptide (LRRASLG). RRREEETEEE, a casein kinase II substrate [22], was phosphorylated by the wheat germ kinase but not by the kinases from erythrocytes. In addition to the peptides listed in Table I, we have also examined a number of commercially available peptides as possible substrates for the membrane kinase and casein kinase A. Our results indicate that neither of these kinases can utilize ACTH fragment 18–39, interleukin 1β fragment 163–171, and fibrinopeptide A as substrates.

Since DDDEESITRR has been identified as a casein kinase I substrate [15] and is phosphorylated by both membrane kinase and casein kinase A, the possibility that these erythrocyte kinases may represent the casein kinase I enzyme has been examined. Confirming an earlier observation [6], we have also found that both kinases exhibit little activity with GTP as the phosphoryl donor (data not shown). Figure 3A shows that, unlike the wheat germ kinase, casein kinase A is not inhibited by low concentrations of heparin. Higher concentrations of heparin, however, were found to inhibit casein kinase A activity (Fig. 3B). The effect of heparin on the membrane kinase was essentially the same as that on casein kinase A. The wheat germ kinase can also effectively utilize GTP as a phosphoryl donor [14], and thus has the properties of casein kinase II.

4. DISCUSSION

The evidence presented in this report supports the notion that the membrane kinase and casein kinase A are either the same enzyme or closely related isozymes. The electrophoretic patterns of the peptide fragments of the 2 kinases generated by V8 protease digestion are

identical. However, whether they have completely identical primary structures remains to be determined.

Studies with ankyrin and synthetic peptides indicate that the membrane kinase and casein kinase A have substrate specificity that is clearly distinct from that of the cAMP-dependent protein kinase and casein kinase II. The membrane kinase and casein kinase A can effectively phosphorylate the seryl residue of the synthetic peptide DDDEESITRR, which is derived from the phosphorylation site of casein kinase I in β-casein [15]. This together with the inability to utilize GTP as a phosphoryl donor and to be inhibited by low concentrations of heparin indicate that these kinases may be classified as casein kinase I. Unlike casein kinase II, which recognizes a cluster of acidic amino acid residues at the C-terminal side of serine or threonine, casein kinase I has been suggested to prefer sites downstream from acidic sequences [23,24]. However, this requirement is not absolute. As shown in Table I, both membrane kinase and casein kinase A exhibit significant activities toward LPGLPSAASSEDAGQS, which does not contain strategically positioned acidic residues. Likewise, Kuret et al. [25] have identified specific sites in skeletal muscle glycogen synthetase phosphorylated by casein kinase I despite their lack of acidic amino acids in the surrounding sequence. The results suggest that other factors may be involved in the phosphorylation site selectivity by casein kinase I.

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