Vol. 116, No. 2, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 31, 1983 Pages 675-681

RESOLUTION AND CHARACTERIZATION OF CALCIUM/PHOSPHOLIPID-DEPENDENT PROTEIN KINASE AND H4-PROTEASE-ACTIVATED PROTEIN KINASE ACTIVITIES IN LYMPHOID CELLS

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Received September 12, 1983

Summary: The calcium/phospholipid-dependent protein kinase (PKC) and the H4 protease-activated protein kinase (H4PK) from lymphosarcoma cells were separated by CM Sephadex chromatography. PKC activity was increased 10-fold in the presence of calcium and phosphatidylserine, but no activation by Mg^{+2}-ATP preincubation or inhibition by NaF was observed. In contrast, H4PK activity was increased 8-fold by preincubation with Mg^{+2}ATP and NaF completely inhibited this enzyme. Activators and inhibitors of PKC did not affect H4PK activity. The substrate specificity of the H4PK and PKC also differed substantially. On the basis of these data it is concluded that PKC and H4PK are not related enzymes.

Protein kinases are characterized by the specific second messengers which activate the phosphotransferases and the unique substrate specificities of the activated enzymes. A protein kinase (PKC) which is activated by calcium and phospholipids has been identified in lymphocytes (1) and purified to homogeneity (2,3). Similarity to a protein kinase in lymphosarcoma cells (4,5) which is activated by a Mg^{+2}ATP-dependent converting enzyme has been noted (1,5). The activation of H4PK isolated from lymphosarcoma cells (4,5) or rabbit reticulocytes (6) appears to involve a proteolytic mechanism. Despite the apparent differences in H4PK and PKC activation mechanisms, the two enzymes are strikingly similar in physical properties and chromatographic behavior (1-6). In this report PKC and H4PK have been isolated from lymphosarcoma cells and the properties of the enzymes systematically investigated in order to establish the unique identity of these two enzymes.

METHODS: Lymphosarcomas (P1798/R) were serially transplanted in BALB/c mice as previously described (4). Tumors (4-6 g) were removed 14-18 days after

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implantation and homogenized in cold 20 mM TRIS, pH 7.5, containing 5 mM EDTA and 15 mM 2-mercaptoethanol (Buffer A). The extract was centrifuged at 105,000 x g for 60 min and the supernatant was applied to a column of DE52 equilibrated with Buffer A. Proteins were eluted with a linear gradient of 0.4 M KCl in Buffer A. Fractions 46-60 from the DE52 effluent (Fig. 1) were combined and solid $(NH_4)_2SO_4$ was added to a final concentration of 60% saturation. The resulting precipitate was collected and dissolved in 20 mM MES, pH 6.8, containing 2 mM EDTA and 15 mM 2-mercaptoethanol (Buffer B). The sample was dialyzed against Buffer B and applied to a column of CM Sephadex which was eluted with a linear gradient of 0.3 M KCl in Buffer B. Further purification of the enzymes is described in the figure legends.

H4PK was assayed as previously described (4,5). Activation was carried out immediately prior to assay by incubation of the enzyme with 10 mM $MgCl_2$ and 100 μM [γ-^{32}P]ATP (20-60 dpm/pmol) in 20 mM MES buffer, pH 6.8. The reaction was initiated by the addition of substrate to the preincubation mix.

PKC was assayed in 20 mM TRIS, pH 7.5, containing 10 mM $MgCl_2$ and 100 μM [γ-^{32}P]ATP (20-60 dpm/pmol). Calcium, diolein, and phosphatidyl serine were 0.3 mM, 3.5 μg/ml and 90 μg/ml, respectively. Phosphoprotein was quantitated by the method of Reimann et al. (7) except when histone H1 or peptides were used as substrates. Phosphotransfer to these substrates was determined by the method of Glass et al. (8).

[γ-^{32}P]ATP and protein and peptide substrates were prepared as previously described (4,5). Phosphatidylserine and diolein were purchased from Supelco and Sigma Chem. Corp., respectively. Protein was determined by the Bradford method (9).

RESULTS: Enzyme activity corresponding to H4PK and PKC coeluted from DE52 at a mean salt concentration of 60 mM (Fig. 1). This fraction also contained cyclic AMP-dependent protein kinase (isozyme I) (10). Analysis of the DE52 effluent by CM Sephadex chromatography resulted in the resolution of the H4PK and PKC activities. The H4PK and endogenous converting enzyme were coeluted

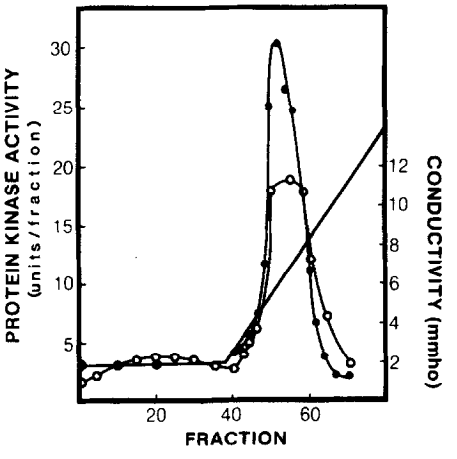

Figure 1. Chromatography of lymphosarcoma extract on DE52. Extract obtained from 38 g of tumor was applied to a column (2.5 x 35 cm) of DE52 equilibrated in Buffer A. Proteins were eluted in 3.2 ml fractions with a 300 ml linear gradient of KCl (0-0.4 M). Protein kinase activity was determined with H4 (–●–; 2 mg/ml) or H1 (–o–; 2 mg/ml) as substrate. H1 assays contained 0.3 M $CaCl_2$, 90 μg/ml phosphatidyl serine and 3.5 μg/ml diolein.

as a broad peak in the KCl gradient. In contrast, the PKC activity did not bind to the CM Sephadex at pH 6.8. Considerable H1 kinase activity was observed in the KCl gradient fractions, however no calcium or lipid dependence was observed. Furthermore, phosphorylation of protamine, an excellent substrate for PKC, was not evident in those fractions (Fig. 2).

Fractions 1-10 and 26-50 in the CM Sephadex effluent were pooled separately, concentrated, and chromatographed independently on S200 Sephacryl (Fig. 3). A single peak of H4PK activity with an apparent M_r 56,000 was observed. Two peaks of calicum/phospholipid dependent activity eluting with apparent M_r 75,000 and 56,000 were observed. Further analyses were carried out using the peak activity fraction from the H4PK S200 preparation (specific activity 116 nmol ^{32}P transferred/min-mg) and from the M_r 75,000 PKC fraction (specific activity 10 nmol ^{32}P transferred/min-mg).

The PKC activity was increased 10-fold by the addition of calcium and lipids, but preincubation with $Mg^{+2}ATP$ did not enhance the observed activity (Table 1). In contrast, H4PK activity increased 8-fold after preincubation with $Mg^{+2}ATP$, but no affect of calcium and/or lipids was observed on either the activated or nonactivated enzyme (Table 1). The calcium chelator EGTA

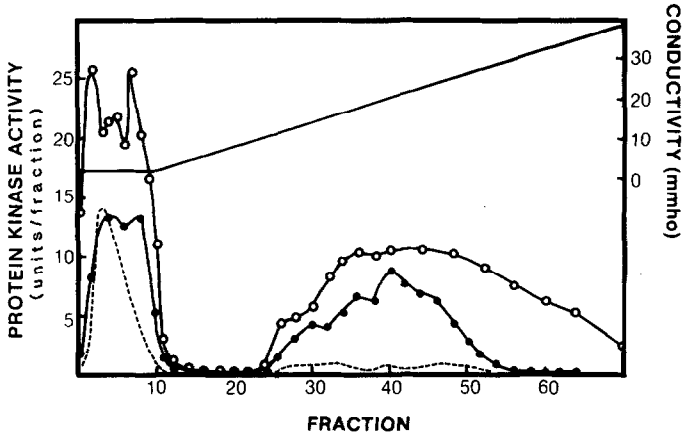

Figure 2. Chromatography of protein kinase activity from DE52 on CM Sephadex. The crude protein kinase fraction (#44-68) from Figure 1 was concentrated by 60% $(NH_4)_2SO_4$ precipitation and applied to a column (0.9 x 8 cm) of (CM Sephadex equilibrated in Buffer B. Proteins were eluted in 2.0 ml fractions with a linear gradient (150 ml) of KCl (0-0.3 M). Protein kinase activity was determined with H4 (-●-) and H1 (-o-) as described in Figure 1. Activity in the presence of protamine (---; 2 mg/ml) from a similar preparation (12 g tumor) is also shown.

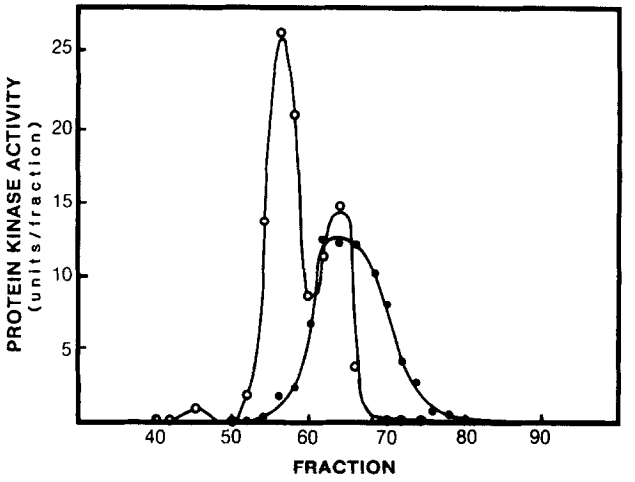

Figure 3. Chromatography of H4PK fractions and PKC fractions on S200 Sephacryl. H4PK-enriched fractions (#26-50; 14 mg protein) and PKC-enriched fractions (#1-10; 45 mg protein) from Figure 2 were concentrated by batch elution with 0.12 M KCl from a column (0.9 x 4 cm) of DE52 equilibrated in Buffer B. The fractions were separately chromatographed on a column (2.5 x 90 cm) of S200 Sephacryl equilibrated with 20 mM MES, pH 6.8, containing 2 mM EDTA, 0.1 mM dithioerythritol, and 0.12 M KCl. Fractions (3.9 ml) were assayed for H4PK (—●—) and PKC (—o—) as described in Figure 1.

and the lipid-binding drug chlorpromazine did not affect the basal activity of either enzyme.

Since PKC activity is apparently lipid-independent with some substrates, i.e. protamine (2), the H4PK fraction was investigated with H1 in place of H4. Similar to results in Table 1, no lipid activation was observed with H1, calcium, and either phosphatidyl serine, phosphatidyl inositol, or phosphatidyl choline (Table 2). The lipid response observed with PKC was comparable to that previously reported (1).

PKC and activated H4PK differed substantially with respect to substrate specificity (Table 3). H4 was the most favorable substrate for the H4PK fraction. In addition, phosphorylation of two synthetic peptide substrates (11) corresponding to phosphorylation sites in H4 (H4-Ser 47) and pyruvate kinase (PKI) was also catalyzed by H4PK. The favored substrate of PKC was protamine and neither synthetic peptide was phosphorylated. Neither PKC nor H4PK catalyzed phosphorylation of a synthetic peptide derived from H1 and associated with a growth-dependent protein kinase (12).

TABLE 1

Effects of $Mg^{+2}ATP$ Activation and Calcium and Lipids on Lymphosarcoma Protein Kinase Activity

	PROTEIN KINASE ACTIVITY (pmol ^{32}P transferred/min)		
	H4PK		PKC
	-ACT	+ACT	
- Activation	10	-	61*
+ Activation	-	80	58*
+ NaF (15 mM)	9.3	7.6	55*
+ $CaCl_2$, diolein	9.1	74	6.5
+ Phosphatidyl serine, diolein	8.7	79	9.3
+ $CaCl_2$, phosphatidyl serine, diolein	7.4	73	64
+ EGTA, chlorpromazine	7.7	83	11*

H4PK (0.9 µg) and PKC (6 µg) were assayed with the denoted modification as described in Methods. Protein substrates were H4 (100 µM) for H4PK and H1 (100 µM) for PKC. The concentrations of $CaCl_2$, phosphatidyl serine, and diolein were 0.3 mM, 90 µg/ml, and 3.5 µg/ml, respectively. The concentrations of EGTA and chlorpromazine were 1 mM and 200 µM, respectively. *$CaCl_2$ and lipids were included in the assay for PKC.

Finally, to exclude the possibility that one fraction contained an inhibitor of PKC, H4PK, or the converting enzyme, enzyme fractions were combined and assayed in the presence and absence of calcium and lipids and $Mg^{+2}ATP$-dependent activation. In all cases the activity of the combined fractions was additive (Table 4).

TABLE 2

Analysis of H4PK and PKC with Histone H1 and Varying Phospholipids

	Protein Kinase Activity (pmol ^{32}P transferred/min)	
	H4PK	PKC
No additions	84	6.5
+ $CaCl_2$	76	6.8
+ Diolein	79	5.1
+ Phosphatidyl serine	86	10
+ $CaCl_2$, phosphatidyl serine, diolein	72	62
+ $CaCl_2$, phosphatidyl inositol, diolein	75	46
+ $CaCl_2$, phosphatidyl choline, diolein	77	10

Preactivated H4PK (0.9 µg) and PKC (6 µg) were assayed using H1 (100 µM) as substrate as described in Methods. Concentration of additions are as described in Table 1.

TABLE 3

Substrate Specificity of Lymphosarcoma H4PK and PKC

Substrate	Protein Kinase Activity (pmol ^{32}P transferred/min)		
	H4PK (+ACT)	PKC -Lipids	PKC +Lipids
H4 (2 mg/ml)	82	14	20
H1 (4 mg/ml)	27	11	70
H4-Ser 47 (100 μM) (V-K-R-I-S-G-L)	33	<1.0	<1.0
PKI (100 μM) (L-R-R-A-S-L-G)	29	3.0	29
GAK-Thr (400 μM) (K-T-P-V-K)	<1.0	<1.0	<1.0
Protamine (2 mg/ml)	19	295	273

Enzyme and lipid concentrations are as described in Table 1. All PKC assays contain 0.3 mM $CaCl_2$. Preparation and phosphorylation of peptides are as described previously (4,5,11,12).

DISCUSSION: The H4PK and PKC separated by CM Sephadex chromatography and further purified by gel filtration chromatography exhibit different activation mechanisms and substrate specificity supporting the hypothesis that these are distinct enzymes. The M_r of H4PK appears to be less than that of PKC based on the gel filtration analysis; however, analysis of the enzyme by SDS gel electrophoresis indicates that the M_r of H4PK is approximately 82,000 (5). No evidence that favors the interpretation that H4PK is a proteolytic product of PKC or *vice versa* has been observed. Flockhart and

TABLE 4

Combined Activity of H4PK and PKC Fractions

	Protein Kinase Activity (pmol ^{32}P transferred/min)			
	$-CaCl_2$, Lipids		$+CaCl_2$, Lipids	
	-ACT	+ACT	-ACT	+ACT
H4PK	6.3	33	5.7	27
PKC	7.1	6.7	66	69
H4PK + PKC	14	37	70	88

Enzyme and lipid concentrations are as described in Table 1. Substrate was H1 (4 mg/ml).

Corbin (13) have observed that proteolysis of several protein phosphotransferases results in loss of regulatory properties but no change in substrate specificity. The observation in this report that H4PK and PKC have substantially different substrate specificities further supports the conclusion that these are unrelated enzymes.

Since calcium and lipid did not affect the activity of either the activated or the nonactivated H4PK fraction when either H1 or H4 was utilized as substrate, the failure to observe calcium/lipid-dependence of H4PK cannot be attributed to a substrate effect similar to that observed with protamine (2). Furthermore, failure of EGTA and chlorpromazine to inhibit H4PK activity indicates that H4PK activity is not derived from endogenous calcium and lipid. Finally, the additivity of the H4PK and PKC activities confirms that PKC is not activated by the H4PK converting reaction. It is concluded that these enzymes are distinct protein kinases, but it is important to note that the similarity in their chromatographic properties may result in enzyme preparations containing both activities.

ACKNOWLEDGEMENTS: This work was supported in part by The Robert A. Welch Foundation Grant #B-864.

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