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PARTIAL PURIFICATION AND CHARACTERIZATION OF A NOVEL BOVINE ADRENOCORTICAL PROTEIN KINASE THAT PHOSPHORYLATES ENDOGENOUS 120,000 DALTON PEPTIDE

YOSHIKAZU KURODA and RAMESHWAR K. SHARMA

Department of Biochemistry, University of Tennessee
Center for the Health Sciences, Memphis, Tennessee 38163

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SUMMARY

We describe partial purification of a novel protein kinase (designated PK 380) from bovine adrenal cortex. The enzyme binds neither cyclic AMP or cyclic GMP, nor phosphorylates the known exogenous substrates such as histones, phosphorylase b, casein, phosvitin, or protamine, in the presence or absence of the cyclic nucleotides. The protein kinase activity of the enzyme was not inhibited by the cyclic AMP-dependent protein kinase inhibitor. Addition of exogenous calcium, calmodulin, or EGTA did not affect the process of phosphorylation. It catalyzes, however, the phosphorylation of an endogenous 120,000 dalton polypeptide. We, therefore, conclude that PK 380 is a cyclic nucleotide- and calcium- or calcium-calmodulin independent protein kinase, that phosphorylates the endogenous substrate, a 120,000 dalton polypeptide. Recently, we have found that PK 380 specifically phosphorylates eukaryotic initiation factor 2 α. This enzyme therefore, might play an important role in the translation control of eukaryotic cell.

INTRODUCTION

Investigations by varius laboratories have indicated that protein phosphorylation is an important physiological process that mediates several intracellular metabolic activities of eukaryotes (1-3). The phosphorylation reactions are catalyzed by cyclic nucleotide-dependent or cyclic nucleotide-independent protein kinases. To date cyclic AMP-dependent, cyclic GMP-dependent, calcium-dependent, calcium-calmodulin-dependent, calcium- and cyclic nucleotide-independent, and cyclic AMP-binding autophosphorylating protein kinases have been reported to exist in nature (for a review see ref. 4). The cyclic nucleotide-dependent and independent protein kinases show a broad specificity for their substrates and

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The abbreviation used are: SDS, sodium dodecyl sulfate; EDTA ethylenediaminetetraacetate; EGTA, ethylene glycol bis (ß-aminoethyl ether) N,N,N',N'-tetraacetic acid.

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defining their specific biological role in metabolic reactions is one of the most challenging problems in biology.

This report deals with the partial purification and characterization of a novel adrenocortical protein kinase, designated PK 380, that does not phosphorylate the commonly used exogenous substrates such as histone, protamine, casein, phosvitin, or phosphorylase b, but phosphorylates an endogenous polypeptide of 120,000 dalton. This enzyme is cyclic nucleotides-independent and also is not dependent on either calcium or calmodulin for its catalytic activity.

MATERIALS AND METHODS

Histone (type II-A), cyclic nucleotides, casein, phosvitin, protamine sulfate (grade I), phosphorylase b, cyclic AMP-dependent protein kinase inhibitor, human γ-globulin, beef liver catalase, and horse spleen apoferritin were obtained from Sigma. DEAE-cellulose and Boi-Gel A-1.5m were purchased from Bio-Rad. [γ-^{32}P]ATP (3,000 Ci/mmol), [^{3}H]cyclic AMP (40 Ci/mmol), and [^{3}H]cyclic GMP (40 Ci/mmol) were from Amersham. Calmodulin was a gift from Dr. W.Y. Cheung. Calcium phosphate cellulose gel was prepared as described by Koike and Hamada (5). All other reagents were analytical grade and were obtained commercially.

Protein Kinase Assay - Protein kinase activity was assayed by the incorporation of ^{32}P from [γ-^{32}P]ATP into exogenous or endogenous substrates as previously described (6, 7) with minor modifications as follows.

250 µl incubation mixture contained 20mM Tris-HCl (pH 7.5), 10 mM magnesium chloride, 1×10^{-7}M [γ-^{32}P]ATP (8 to 20 x 10^{3} cpm/pmol) and an appropriate aliquot of PK 380. When cyclic AMP-dependent protein kinase activity was determined the reaction mixture contained in addition, 1 µM cyclic AMP, and [γ-^{32}P]ATP concentration was 1×10^{-5}M (200 cpm/pmol) in the presence or absence of exogenous substrates.

For the determination of cyclic GMP-dependent protein kinase activity the composition of the reaction mixture was the same except 100 mM magnesium chloride, 1 µM cyclic GMP and 1×10^{-5}M [γ-^{32}P]ATP (200 cpm/pmol) were used. Incubation was

carried out for 1 min at 37°C and the amount of ^{32}P incorporated into trichloroacetic acid precipitable material was measured (6, 7). When protamine was used as an exogenous substrate, ice cold 25% trichloroacetic acid was used to precipitate the protein.

Cyclic Nucleotide Binding Assays - Cyclic nucleotide binding activity was determined by a modification (6) of the Gilman procedure (8). An appropriate aliquot of PK 380 was incubated at 0°C for 60 min in 50mM sodium acetate (pH 4.0), 2mM EDTA and 3 x 10^{-8}M of [^{3}H]cyclic AMP (1 x 10^{-5} cpm/pmol) or [^{3}H] cyclic GMP (1 x 10^5 cpm/pmol). The reaction was initiated by the addition of the enzyme and was terminated with 2 ml of ice cold 70% saturated ammonium sulfate. After 20 min the precipitate was collected on GF/C filters, washed thrice with 25% saturated ammonium sulfate, and the filters were dried, and counted for radioactivity in 5 ml of Omnifluor cocktail in toluene (4 g/l).

Polyacrylamide Gel Electrophoresis - For electrophoresis in a nondenaturing stacking system, polyacrylamide disc gels (0.5 x 6 cm) were prepared as described (9, 10) with some modification (6). Separating gels consisted of 5% polyacrylamide. Samples were adjusted to pH 6.8 and electrophoresed at 2 mA/gel at 4°C. Gels were cut into 1.5mm slices, extracted with 200µl 20mM Tris-HCl (pH 7.5) and assayed for protein kinase activity.

Denaturing polyacrylamide disc gels (0.5 x 12 cm) containing 0.1% SDS were prepared by the method of Favre and Laemmli (11) as modified by Engbaek et al. (12). 10 µg of enzyme isolated by native gel electrophoresis was analyzed. Gels were stained with Coomassie Brilliant Blue, diffusion destained with 7.5% acetic acid and traced with a Gilford gel scanner at 550nm. In a separate experiment, an identical enzyme sample was incubated for 10 min with [γ-^{32}P] ATP under standard conditions prior to electrophoresis. The incubated mixture was dialyzed against distilled water, adjusted to pH 6.8 with the sample buffer of Engbaek (12), and fractionated by SDS gel electrophoresis. The protein band associated with radioactivity was determined by liquid scintillation counting of gel slices, dissolved in 0.5 ml of 30% hydrogen peroxide at 65°C, and mixed with 7 ml Scintiverse.

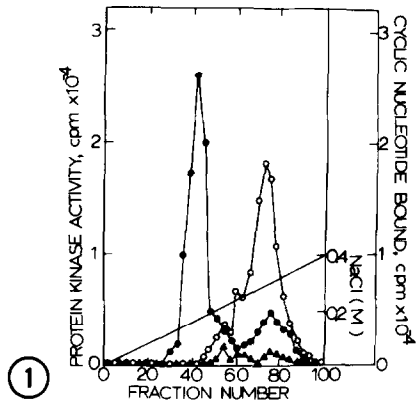
Protein Determination - Proteins were measured by the method of Bradford (13) using the Bio-Rad reagent with bovine serum albumin as a standard.

RESULTS AND DISCUSSION

Purification of PK 380 - All procedures were carried out at 0-4°C.

Step I: Homogenization, ammonium sulfate precipitation and calcium phosphate cellulose gel chromatography - Bovine adrenocortical tissue (400 g) was dissected out of the adrenal glands and homogenized with 800 ml of 20 mM potassium phosphate buffer containing 6 mM 2-mercaptoethanol and 2 mM EDTA (buffer A, pH 7.0) in a Waring blender for 2 min. To the postmitochondrial supernatant (750 ml) solid ammonium sulfate was added to 60% saturation. The precipitate was dissolved in buffer A and dialyzed overnight against the same buffer. The dialyzed fraction was applied to a calcium phosphate cellulose column (5 x 20 cm) previously equilibrated with buffer A, washed with 2 l of the same buffer, and eluted with a 3 l of linear potassium phosphate gradient, 20-400 mM, containing 6 mM 2-mercaptoethanol and 2 mM EDTA (pH 7.0). The PK 380 peak was obtained at approximately 120 mM potassium phosphate buffer. Some resolution of PK 380 peak from cyclic AMP-dependent protein kinase peak (200 mM potassium phosphate) was obtained but the complete separation of these enzymes was not achieved.

Step II: DEAE - cellulose chromatography - To the pooled PK 380 fractions, solid ammonium sulfate was added to a final concentration of 60% saturation. The precipitate was dissolved in 20 mM Tris-HCl buffer containing 6 mM 2-mercaptoethanol, 2 mM EDTA and 10% glycerol (buffer B, pH 7.5) and dialyzed overnight against the same buffer. The dialyzed fraction was applied to a DEAE-cellulose column (2.8 x 16 cm) preequilibrated with the same buffer. The column was washed with 500 ml of buffer B and eluted with 1 l of the linear NaCl gradient (0 - 400mM) in buffer B. PK 380 peak emerged at 0.17 M NaCl and was clearly resolved from cyclic AMP-binding and cyclic GMP-binding proteins (Fig. 1).



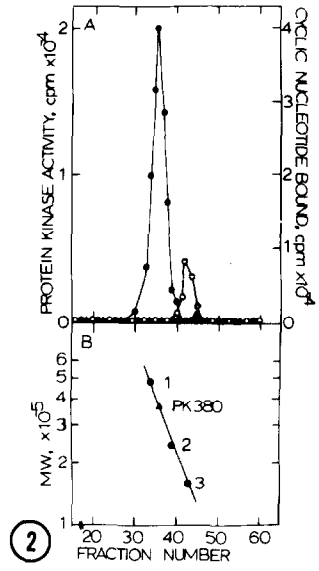


Fig. 1 DEAE-cellulose column chromatography of PK 380 from bovine adrenal cortex. Approximately 450 mg protein obtained by elution from calcium phosphate cellulose gel was applied to a column of DEAE-cellulose (2.8 x 16 cm) equilibrated with 20 mM Tris HCl, 6 mM 2-mercaptoethanol, 2 mM EDTA and 10% glycerol, pH 7.5. The column was washed with 5 column volume (500 ml) of the equilibration buffer and eluted with 1l of the linear gradient of NaCl (0 - 0.4M) in the same buffer. Fractions (6ml) were analyzed for protein kinase (●), cyclic AMP binding (o) and cyclic GMP binding activity (▲) as described under "Materials and Methods".

Fig. 2 (A) Bio-Gel A-1.5m column chromatography of PK 380 from bovine adrenal cortex. About 29mg protein obtained by elution from DEAE-cellulose was applied to Bio-Gel A-1.5m (2.5 x 60cm) equilibrated with 20 mM Tris-HCl, 2-mercaptoethanol, 2mM EDTA and 10% glycerol, pH 7.5. Elution was performed with the same buffer at flow rate of 25 ml/h. Fractions (5 ml) were analyzed for protein kinase (●), cyclic AMP binding (o) and cyclic GMP binding activity (▲) as described under "Materials and Methods". (B) Estimation of molecular weight of PK 380 by gel filtration. 1. horse spleen apoferritin (Mr = 480,000), 2. beef liver catalase (Mr = 248,000) and 3. human γ-globulin (Mr = 160,000) were used as standard proteins.

Step III: Gel-filtration chromatography - The DEAE-cellulose PK 380 peak fractions were combined and solid ammonium sulfate was added to 60% saturation. The precipitate was dissolved in buffer B and dialyzed overnight against the same buffer. The dialyzed preparation was applied to a Bio-gel A-1.5m preparative column (2.5 x 60 cm) previously equilibrated with buffer B, and eluted with the same buffer. The PK 380 peak obtained corresponded to a molecular weight of 380,000 (Fig. 2). The enzyme thus isolated showed neither cyclic AMP- nor cyclic GMP-binding activity nor cyclic AMP- or cyclic

Table 1

Purification of PK 380 from Bovine Adrenal Cortex

Purification of protein kinase was carried out as described in the text. The assay procedure used is described under "Materials and Methods".

Fraction	Total Protein	Specific Activity	Total Unit a)	Purification
	mg	Units/mg	Units	fold
60% Ammonium sulfate of postmitochondrial supernatant b)	9,690	0.11	1030	1
Calcium phosphate cellulose gel	449	2.3	1030	23
DEAE cellulose	29	18	522	164
Bio-Gel A-1.5m	2.9	80	230	727

a) One unit was defined as that amount of enzyme which catalyzed the incorporation of one pmole of inorganic phosphate from [γ-^{32}P]ATP into endogenous substrate per min at 37°C.

b) The 1 fold purification was arbitarily taken as 1 based on 1030 units activity mentioned under calcium phosphate cellulose gel purification step.

GMP-dependent kinase activities. All kinase properties of the enzyme were conducted on this fraction. Table I shows the summary of purification procedures.

Table II shows that PK 380 did not phosphorylate exogenous substrates such as histone, protamine, phosvitin, casein or phosphorylase b. This characteristic distinguished PK 380 from other cyclic nucleotide-independent kinases which utilize at least one of the above mentioned substrates. Curiously, it was observed that phosphorylase b inhibited the phosphorylation of PK 380. The reason for this is yet undetermined.

In order to differentiate the phosphorylation of PK 380 from that of cyclic AMP-, cyclic GMP-, calcium, and calmodulin-dependent protein kinase, the isolated enzyme was incubated with the cyclic nucleotides, calcium chloride, calmodulin, or EGTA. Table III shows that none of these agents affected the phosphorylation of PK 380. Further evidence that PK 380 is different from cyclic AMP-dependent

Table II

Substrate Specificity of PK 380

The enzyme (26µg) was assayed under standard conditions as described under "Materials and Methods" except that 1 x 10^{-5}M [γ-^{32}P]ATP (5,000 cpm/pmol) was used and 100µg each of various protein substrates was added as indicated.

Substrate	Acid Precipitable radioactivity
	(cpm)
PK 380	13,600
+Histone	12,500
+Protamine	11,200
+Phosvitin	13,000
+Casein	14,000
+Phosphorylase b	1,600

Table III

Effect of cyclic nucleotides, calcium and calmodulin on the phosphorylation of PK 380

Protein kinase from Bio-Gel A-1.5m was dialyzed against 20 mM Tris HCl (pH 7.5) buffer containing 10% glycerol. The enzyme (15µg) was assayed under standard conditions as described under "Materials and Methods" except that each compound described below was added as indicated.

Addition	Acid precipitable radioactivity
	(cpm)
PK 380	10,400
+cyclic AMP (1µM)	10,500
+cyclic GMP (1µM)	9,800
+EGTA (0.8mM)	10,500
+Ca^{2+} (0.4 mM)	10,100
+Calmodulin (80µg/ml)	9,900
+Ca^{2+} (0.4 mM) and Calmodulin (80µg/ml)	10,200

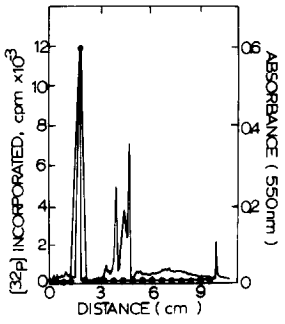


Fig. 3. SDS disc polyacrylamide gel electrophoresis of PK 380. PK 380 (10µg) from native gel (5%) was subjected to SDS disc polyacrylamide gel electrophoresis. The gel was stained, destained and scanned at 550nm (⟵). In a parallel experiment, PK 380 (10µg) was preincubated with [γ-^{32}P]ATP prior to electrophoresis. The protein band associated with radioactivity (●⟵●) was determined by counting individual gel slices dissolved in hydrogen peroxide. Myosin (Mr = 200,000), β-galactosidase (Mr = 116,500), phosphorylase b (Mr = 94,000), bovine serum albumin (Mr = 68,000) and ovalbumin (Mr = 43,000) were used as standard proteins.

protein kinase was provided by the following experiment. PK 380 was incubated with cyclic AMP-dependent protein kinase inhibitor or the regulatory subunit of adrenal cyclic AMP-dependent protein kinase. Neither the inhibitor nor the regulatory subunit inhibited the kinase activity of PK 380 (data not shown). In contrast, a cyclic AMP-responsive holoenzyme could be readily inhibited by cyclic AMP dependent protein kinase inhibitor.

Fig. 3 shows that when PK 380 is phosphorylated with [γ-^{32}P] ATP and analyzed by polyacrylamide gel electrophoresis under denaturing conditions, it phosphorylates an endogenous single 120,000 dalton polypeptide.

The peptide nature of the 120,000 dalton substrate was further substantiated as follows. The phosphorylated endogenous substrate, 120,000 dalton, was treated with chloroform-methanol, DNAse, RNAse, phospholipase C, or pronase before analysis of gel electrophoresis. Only pronase digestion eliminated the phosphorylation of 120,000 dalton band indicating that the phosphate was originally incorporated into the polypeptide.

It is therefore obvious that PK 380 is a cyclic nucleotide-independent protein kinase and can be differentiated from the cyclic nucleotide-dependent protein kinases by the following criteria. First, it has no binding affinity for cyclic AMP or cyclic GMP nor do these nucleotides activate the phosphorylation of

exogenous substrates such as histone, protamine, casein, phosvitin, or phosphorylase b. Second, the cyclic AMP-dependent protein kinase inhibitor or the regulatory subunit of adrenocortical cyclic AMP-dependent protein kinase are unable to inhibit the endogenous phosphorylation. Third, the molecular weight of PK 380 is far greater than any of the known cyclic AMP-dependent or cyclic GMP-dependent protein kinases. Furthermore, the enzyme activity is not affected by either calcium alone or by calcium in the presence of calmodulin (14). This property differentiates it from the calmodulin-dependent (15-17) and calmodulin-independent but calcium-dependent protein kinases (18). In addition, PK 380 does not phosphorylate casein or phosvitin indicating that it is different from the reported casein kinase (19-21).

At present, we cannot assign any physiological role to this enzyme. Recently[1], we have found that PK 380 *in vitro*, specifically phosphorylates eukaryotic initiation factor-2α. It is therefore a possibility that this enzyme might have some role in the translational control of eukaryotic cell. Future studies designed to correlate the relationships of the phosphorylation of 120,000 dalton polypeptide and the phosphorylation of eukaryotic initiation factor-2α with the *in vitro* protein translation in cell free system will define such a role of this enzyme. But, before such studies are conducted, it is a prerequisite that the enzyme is purified to homogeneity and further characterized kinetically.

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