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PROTEIN KINASE ACTIVITIES IN MAMMALIAN BLOOD FLUID

M. Fehst, D. Kübler, W. Pyerin, E. Burow, and V. Kinzel

Institute of Experimental Pathology, German Cancer Research Center,
Im Neuenheimer Feld 280, D-6900 Heidelberg, FRG

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Two protein kinase activities, one specific for phosvitin and another specific for histone, were detected in serum and plasma of calf as well as of human blood after precipitation with ammonium sulfate (40%) and chromatography on DEAE-Sephacel. The enzymes were separated by chromatography on phosphocellulose. The histone kinase is not related to the cyclic AMP-dependent protein kinase; it may derive at least partly from damaged cells. The phosvitin kinase activity carries characteristics of the so called casein kinase type II similar to that present at the surface of cells including blood cells.

Evidence has been presented for protein kinase (PK) activity at the surface of several cultured cell lines (1,2). The catalytic activity is directed towards surface located and/or extracellular protein substrates (1). The enzyme belongs to the family of phosvitin/casein kinases (1,3). The observation that surface protein kinase is also expressed in blood cells (2) may provide insight into the physiology of this enzyme, since substrates for protein kinase have been described in plasma and serum (2,4). Cell surface protein kinase exhibits a unique property as it can be released selectively from intact cells by interaction with its substrate phosvitin (2,5). Therefore we asked in this study whether extracellular protein kinase with specificity for phosvitin could be detected in serum and plasma.

MATERIALS AND METHODS

Calf plasma was obtained from citrated (0.129 M) blood. Calf serum was prepared by routine clotting or was obtained from Gibco-Biocult (Glasgow). Human blood was processed by mixing with ACD-stabilizer (Biotest Pharma, Dreieich, FRG) as anticoagulant; red blood cells were removed by centrifugation (1500xg, 10 min) and the resulting supernatant fluid refered to as "platelet-rich plasma". Centrifugation at 5000xg (10 min) resulted in platelet pellets and supernatants designated as "platelet-poor plasma". Human serum was prepared by routine clotting of freshly taken blood.

Protein kinase inhibitor (PKI) was purified from rat muscle according to (6), heparin (pig mucosa; lipo-hepin; 5000 IU/ml) was obtained from

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Riker-Kettelhak (Borken/Westfalen); NaDodSO$_4$/polyacrylamide (7.5-15% gradient) gel electrophoresis in slab gels was employed (7). Protein stain was with Coomassie Blue or by silver stain. Radioactivity was detected in dried gels by autoradiography with X-ray film. For quantitation of radioactivity, the labeled gel bands were cut out and analyzed by scintillation counting. The protein kinase assay was carried out as described (3,4). The samples were first dialyzed against 100 mM MOPS-buffer (N-morpholino-3-propanesulfonic acid; pH 6.8)/30 mM Mg acetate. Routine assays were performed in a total of 200 µl with histone (1.6 mg/ml) or phosvitin (1 mg/ ml) using 50 µl or 100 µl of the sample for histone and phosvitin phosphorylation, respectively. After 3 min preincubation at 30°C the reaction was started by adding 2 µl [γ-^{32}P]ATP (100 µM final conc.) and terminated usually after 15 min by 3 ml 10% trichloro acetic acid. The material was subsequently analyzed by gel electrophoresis and autoradiography.

Kinase purification was done at 4°C. a) Ammonium sulfate treatment: Serum (10 ml) was stirred in presence of 40% (w/v) ammonium sulfate for 30 min. The precipitate was collected by centrifugation (3000 x g, 10 min). The sediment was redissolved in 50 mM Tris-HCl/2 mM EDTA (pH 7.0) and dialyzed against the same buffer containing 100 mM NaCl. b) Anion exchange chromatography: Dialyzed serum fractions from a) were loaded on a DEAE-Sephacel (Pharmacia) column (29x2.5 cm) preequilibrated with the dialysis buffer. The column was washed until the run-through fraction was free of detectable protein. Elution was performed with the buffer (pH 7.0) containing a (0.1-1.0 M NaCl) gradient. 5.5 ml fractions were collected and aliquots assayed for enzymatic activity. c) Phosphocellulose separation: Fractions from b) bearing PK activities were pooled and dialyzed against 50 mM Tris-HCl/ 2 mM EDTA buffer (pH 7.4) containing in addition 1 mM DTE and 0.5 M NaCl. The dialyzed material was applied to the phosphocellulose (P-11 from Whatman) column (18x2 cm) which had been preequilibrated with the dialysis buffer. The run-through fraction was collected until the effluent was free of detectable protein prior to elution with the same buffer (pH 7.4) but containing 0.8 M NaCl. Fractions were collected as in b).

RESULTS

Using histone as well as phosvitin as substrates protein kinase activity could not be detected in unfractionated serum. In contrast, after precipitation with ammonium sulfate and anion exchange chromatography histone-phosphorylating as well as phosvitin-phosphorylating activity was detectable in calf serum fractions (Figure 1 and Table 1). Fractions 21 to 29 eluting from DEAE-Sephacel between 140 mM and 190 mM salt were pooled and used further. Both PK activities could be separated from each other by phosphocellulose chromatography (see also ref. 3). Under these conditions the bulk protein came in the flow-through volume (Table 1) which contained exclusively histone kinase activity (Fig. 2). On elution with 0.8 M NaCl the protein carrying fractions contained exclusively phosvitin kinase activity (Fig. 2).

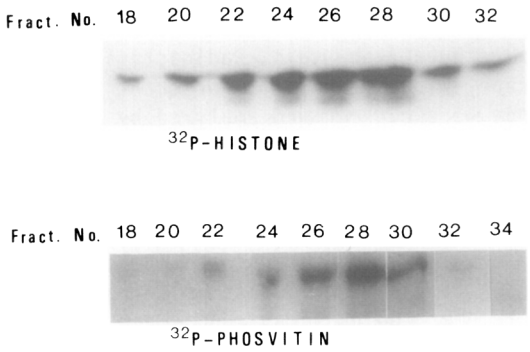

Fig. 1 - Protein kinase activities in calf serum purified via DEAE-cellulose. For details see Materials and Methods. 50 µl aliquots of the eluted fractions (5.5 ml) were assayed for enzyme activity. Shown are autoradiographs of labeled histone and phosvitin.

By use of the same fractionation protocol in addition to calf serum also calf plasma as well as human plasma and human serum were investigated. Figure 3 shows a summary of these results obtained with the fractions from phosphocellulose chromatography. In all cases the flow-through fractions phosphorylated only histone whereas the fractions eluted with 0.8 M NaCl phosphorylated exclusively phosvitin.

In order to evaluate the possibility that PK activities in blood plasma might originate from disintegrated platelets as suggested in ref. 8, "platelet-rich" and "platelet-poor" human plasma were compared. For this purpose, both types of human plasma were fractionated through the DEAE-Sephacel step

Table 1: Protein kinase activity in subsequent purification steps

Fraction	Total volume (ml)	Protein concentration (mg/ml)	PK activity*
calf serum	10	90.0	-
$(NH_4)_2SO_4$ precipitate	15	30.0	-
DEAE eluate	33	3.23	+
P-cellulose flow-through	27.5	3.49	+
P-cellulose eluate	11	0.37	+

*Enzyme assays and detection of activity by autoradiography of SDS gels was as described in Materials and Methods.

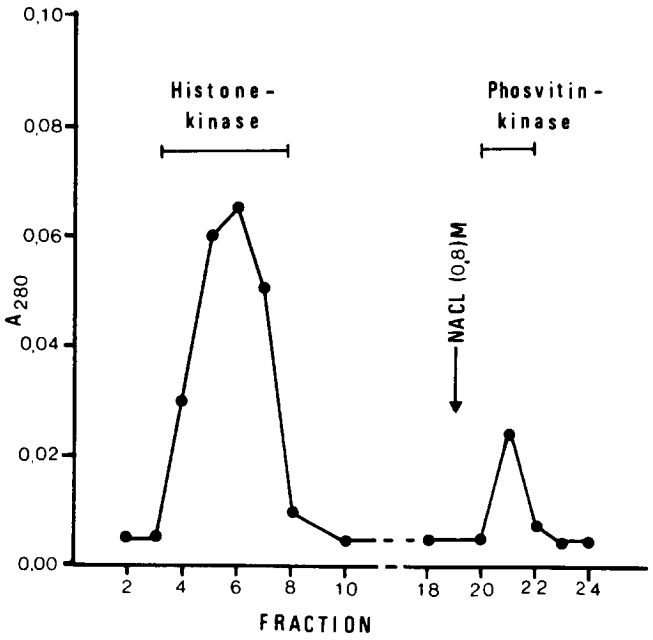

Fig. 2 - Separation of serum protein kinase activities by phosphocellulose. The pool of DEAE-fractions (33 ml) bearing PK activities was subjected to phosphocellulose chromatography. Shown are the profiles (A_{280}) prior to and after increase of salt concentration applied to the phosphocellulose column. PK assay was performed with dialyzed samples. Enzyme-positive fractions (indicated by bars) were pooled.

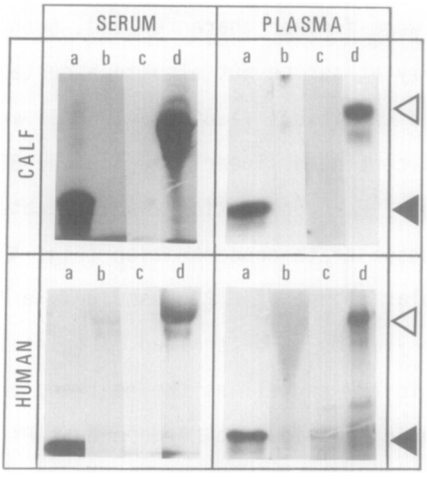

Fig. 3 - Protein kinase activities in blood fluids from calf and human after separation by phosphocellulose. Shown are autoradiographs of gels. For substrate phosphorylation either 50 µl of the flow-through fraction (lanes a,b) or 100 µl of the eluate (lanes c,d) were assayed. Lanes a and c show the results obtained with histone; lanes b and d show the results obtained with phosvitin. The position of histone is indicated by ◀ , that of phosvitin by ◁ .

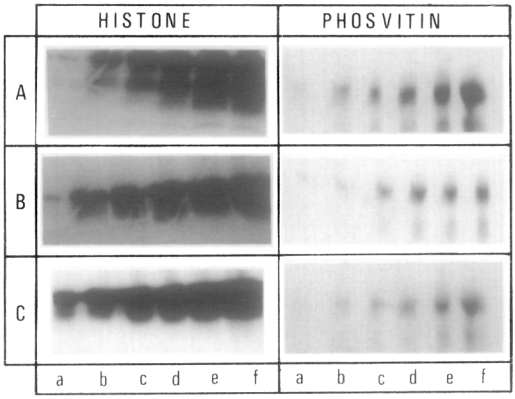

Fig. 4 - Characteristics of histone and phosvitin kinase activities from calf serum after phosphocellulose fractionation. Phosphorylation was assayed under standard conditions as given in Materials and Methods with the modifications indicated below. In A the phosphorylation was carried out for (a) 1 min, (b) 5 min, (c) 10 min, (d) 20 min, (e) 30 min, (f) 60 min. In B the phosphorylation was carried out with various ATP concentrations: (a) 0.25 µM, (b) 0.5 µM, (c) 1 µM, (d) 1.5 µM, (e) 2 µM, (f) 4 µM. In C the phosphorylation was carried out in presence of various substrate concentrations, histone: (a) 3.2 µg, (b) 8 µg, (c) 16 µg, (d) 32 µg, (e) 64 µg, (f) 128 µg; phosvitin: (a) 2 µg, (b) 5 µg, (c) 10 µg, (d) 20 µg, (e) 40 µg, (f) 80 µg.

and analyzed for PK activity. In both types of plasma similar levels of phosvitin kinase were detectable. However, histone kinase activity appeared to be enriched at least by the factor of 2 in the case of "platelet-rich" plasma.

Subsequent to their separation on phosphocellulose the PK activities obtained from calf serum were further characterized. Both phosphorylated their substrates preferentially at serine but also to a certain degree at threonine residues. Under standard conditions (1.66 µM ATP; 3.2 mg/ml histone) the histone kinase reaction continued linearly during the 30 min period studied (Fig. 4). An increase of ^{32}P incorporation was also obtained either with increasing substrate or cosubstrate concentration (Fig. 4). The addition to [γ-^{32}P]ATP of an equimolar amount of GTP caused only a slight reduction (by 17%) of histone phosphorylation as determined by scintillation counting of excised gel bands. Cyclic AMP as well as the heat-and acid-stable inhibitor specific for the catalytic subunit of cAMP-dependent PK did not alter the rate of histone phosphorylation. (The amount of inhibitor applied completely blocked histone phosphorylation at much higher activities of the catalytic subunit prepared from bovine heart or rat muscle; 11).

Phosvitin phosphorylation by the eluate from phosphocellulose chromatography was dependent on incubation time, substrate and ATP concentration

(Fig. 4). The addition to [γ-^{32}P]ATP of an equimolar amount of GTP resulted in a significant reduction (by 36%) of phosvitin radiolabeling. A 10fold and 100fold excess of GTP over ATP caused further reduction by 68% and 81%, respectively. Heparin, (known to inhibit preferentially type II casein kinases, at concentrations below 1 µg/ml; 2), caused a 50% inhibition of the phosvitin kinase activity in serum fractions at a concentration of 0.05 µg/ml.

DISCUSSION

We describe the detection of two seryl/threonyl type protein kinase activities in bovine and human blood fluids; one with a specificity for histone, the other for phosvitin. These enzyme activities are observed after fractionation of ammonium sulfate precipitates from serum or plasma by DEAE-chromatography. In contrast, the enzyme activities are not detectable in non-fractionated blood, thus indicating the presence of activities suppressing the enzyme reactions.

The histone kinase activity is of the cyclic AMP-independent type and does not represent to the catalytic subunit of the cAMP-dependent PK (9,10) as indicated by the ineffectiveness of cyclic AMP and the specific inhibitor protein to influence kinase activity. This enzyme has been also described by others (8). Its origin is unknown but it has been obtained from platelet-rich plasma under conditions through which the platelets are disrupted. Platelet-poor plasma appears to contain less of this enzyme. In view of earlier data (11) this observation might indicate that the histone kinase has been released during cell disruption.

The phosvitin kinase activity appears to be related to type II casein kinases (12) by two criteria; (i) heparin inhibited the activity at concentrations below 0.1 µg/ml, (ii) GTP was able to displace ATP indicating that the enzyme is able to use GTP efficiently as cosubstrate. In these respects the enzyme resembles the ecto-phosvitin kinase expressed at the surface of several cells including blood cells (2). Since the ecto-kinase can be released from intact cells under physiological conditions (2,5) one is tempted to speculate that phosvitin kinase present in blood fluid may at least partly be derived from intact blood cells.

With respect to the physiological role of PK activity in blood fluid, several substrates including fibrinogen have been shown to be present as well (10, 13-18). Previous studies in this laboratory (1,2,4) have demonstrated a 135 kD

polypeptide in serum with a > 300 kD precursor in plasma which were selectively phosphorylated by the ecto-kinase. Remarkably, the 135 kD protein in serum also represents a substrate for the blood fluid-derived phosvitin kinase (unpublished). Hence we may deal here with natural members of an extracellular protein phosphorylation system which appears to meet physiological requirements in that the supply of cosubstrate does not seem to be a problem (19).

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