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A PROTEIN KINASE SYSTEM FROM PLATELET RICH PLASMA

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SUMMARY - Treatment of platelet rich plasma (PRP) at pH 5 results in the precipitation of a protein kinase system. The protein kinase is associated with the platelet fraction and is capable of phosphorylation of several plasma proteins. Analysis of the ^{32}P-labeled phosphoproteins by two dimensional gel electrophoresis showed the existence of three major phosphoproteins : 72K and 80K proteins with identical isoelectric points (pI) of 6.0 and another 72K protein with a pI of 6.8-7.0. This latter 72K phosphoprotein has recently been identified as the α-chain of fibrinogen. The identity of the other 2 proteins remains to be shown. The activity of the protein kinase is markedly enhanced by Mn^{2+}, it phosphorylates calf thymus histone as an exogenous substrate and is independent of cAMP or cGMP. This protein kinase activity is inhibited competitively by ADP.

Recently, we reported the presence of a protein kinase activity in human plasma platelets which is responsible for the phosphorylation of a 70-72,000 molecular weight protein (72K protein) found in blood plasma (1,2). The protein kinase activity and its substrate bind to poly(G)-Sepharose and can be conveniently assayed in this form (3). The 72K protein has been identified as the α-chain of fibrinogen (4). The interest in this protein kinase system was initiated on the observation that treatment of patients with interferon or inducers of interferon resulted in an enhanced level of such kinase activity (1,5). The relation, if any, of such protein kinase activity with that which is enhanced in interferon-treated human cells in culture, remains to be shown (6,7).

Here, we show that treatment of platelet rich plasma (PRP) at pH 5 results in the precipitation of the protein kinase activity along several substrates besides the α-chain of fibrinogen. This pH 5 pellet, which is referred to as the pH 5 fraction, provides a suitable system to study the identity of the phosphoproteins and the role of the platelet protein kinase activity in blood coagulation. The pH 5 fraction contains fibrinogen and factor XIII (or transglutaminase), two essential factors for fibrin (clot) formation (8,10). Accordingly, addition of thrombin to the pH 5 fraction is sufficient for clot formation (4).

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MATERIALS AND METHODS

Blood from normal volunteers was collected in polystyrene tubes containing heparin (100 u/ml), aprotinin (100 u/ml ; Zymofren, Specia) and ETDA (4 mM) and left 15-30 min at room temperature. PRP was collected after centrifugation (200 X g, 15 min) and stored at -80°C.

The protein kinase activity was assayed either after partial purification on poly(G)-Sepharose (1,3) or after precipitation at pH 5. For the preparation of the pH 5 fractions, different samples of PRP were first diluted in an equal volume of NP40 buffer (10 mM Hepes pH 7.6, 10 mM KCl, 2 mM $Mg(OAc)_2$, 7 mM 2-mercaptoethanol and 2.0 % NP40) before addition of two volumes of 100 mM sodium acetate, pH 5. The samples were then incubated at 4°C for 2-3 hr and the pellets were recovered by centrifugation (1000 X g, 15 min). The pellets were dissolved in a buffer (an equal volume as the original PRP) containing 50 mM Tris-HCl pH 8.0 and 150 mM KCl and are referred to as the pH 5 fraction. The protein kinase activity in the pH 5 fraction could be assayed immediately or within 48 hr if stored at 4°C. Aliquots of the pH 5 fraction (25 µl) were mixed with an equal volume of the kinase reaction buffer (10 mM Hepes pH 7.6, 50 mM KCl, 5 mM $Mg(OAc)_2$, 10 mM $MnCl_2$, 10 mM 2-mercaptoethanol and 20 % glycerol, v/v) and were incubated (30°C, 45 min) in the presence of 50 nM γ-^{32}P-ATP (375 Ci/mmol) ; Amersham, England). The reaction was stopped by the addition of an equal volume of 2 fold concentrated electrophoresis sample buffer. All the samples were heated (95°C, 5 min) and aliquots (35 µl) were analysed on polyacrylamide slab gels (10 or 8.5 %) containing sodium dodecyl sulphate (SDS) as described previously (11).

The phosphorylation of histones (histone kinase) was done by including 100 µg of calf thymus histone (H-III S; Sigma) in the protein kinase assay of the pH 5 fraction but at 10 µM γ-^{32}P-ATP (25 Ci/mmol). After the incubation (30°C, 45 min), the reaction mix was transferred to a 2.5 cm filter disc (Whatman 3MM paper) and washed (4°C) consecutively with cold 10 % trichloroacetic acid containing 0.5 mM ATP, 5 % trichloroacetic acid containing 0.5 mM NaH_2PO_4 and subsequently with ethanol and acetone at room temperature. The filter discs were dried and the radioactivity was measured by liquid scintillation (2).

RESULTS AND DISCUSSION

We have previously shown that partial purification of PRP on poly(G)-Sepharose provides a convenient method of assay of a specific protein kinase activity which manifests by the phosphorylation of a 72K protein (Fig. 1A, lane 1). The ^{32}P-labeled 72K protein revealed after purification on poly(G)-Sepharose is composed of several subspecies with closely related isoelectric points (pI) of 6.8-7.0 (Fig. 2B, section 1).

To study the physiological role of the protein kinase activity in clot formation, we investigated phosphorylation of proteins in the pH 5 fraction. The interest in this fraction is the fact that besides the precipitation of the protein kinase system, such fractions are known to contain fibrinogen and factor XIII (8), two essential factors for fibrin (clot) formation induced by thrombin (9,10). Incubation of the pH 5 fraction with γ-^{32}P-ATP resulted in the phosphorylation of several proteins as well as the 72K protein (Fig. 1A, lane 3). Analysis of these phosphoproteins by two dimensional gel electrophoresis showed the existence of three major ^{32}P-labeled proteins : 72K (protein *a*) and 80K (protein *b*) proteins with identical pIs of 6.0 and another

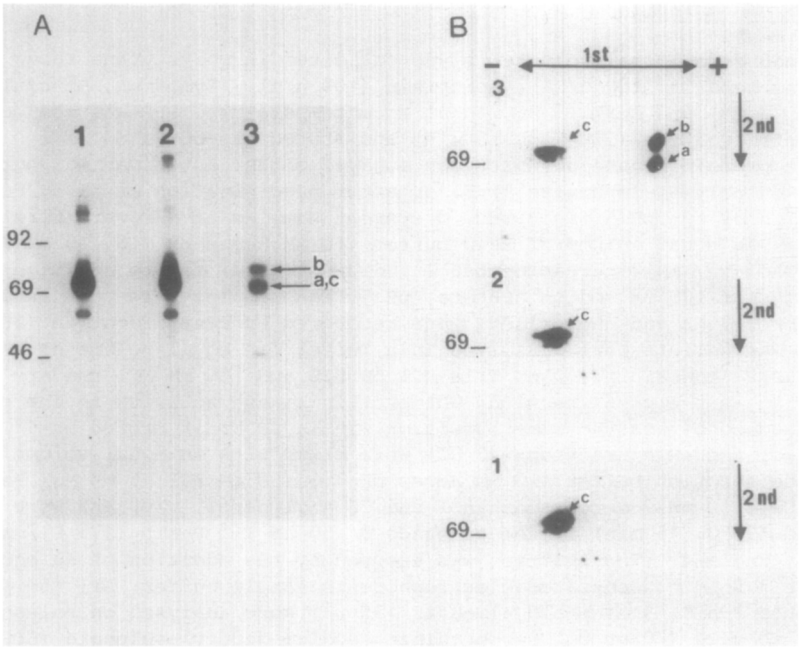

Fig. 1 - The protein kinase activity in PRP after partial purification on poly(G)-Sepharose compared to that of the pH 5 fraction. Analysis of the ^{32}P-labeled proteins by polyacrylamide (10 %) slab gel electrophoresis (A) and by two dimensional gel electrophoresis (B). Assay of the protein kinase activity was as described in "Materials and Methods". In A and B, the different samples represent the ^{32}P-labeled proteins found in 50 µL of PRP (1) or pH 5 fraction (2) after partial purification on poly(G)-Sepharose and in 10 µl of the pH 5 fraction (3) analysed as such. Two dimensional gel electrophoretic analysis was as described by O'Farrel (13) with some modifications (14). The pH gradient obtained by isoelectric focusing (first dimension) was 5.2 to 7.5. Autoradiographs of stained and dried gels are shown. The numbers on the left of each gel in Figs. 1 to 3 give the molecular weight of protein markers in thousands : myosin, 200 ; phosphorylase B, 92 ; bovine plasma albumin, 69 ; ovalbumin, 46 ; chymotrypsin, 30.

72K protein (protein c) with a pI of 6.8-7.0 (Fig. 1B, section 3). Purification of the pH 5 fraction on poly(G)-Sepharose (3) showed the presence of 72K protein, protein c (Fig. 1A, lane 2) ; Fig. 1B, section 2). These results indicate that the phosphoprotein revealed after partial purification of PRP on poly(G)-Sepharose is identical to the phosphoprotein c in the pH 5 fraction. The protein c has recently been identified as the α-chain of fibrinogen (4) while the identity of proteins *a* and *b* remains to be shown.

To localise the protein kinase activity, PRP was separated into a platelet poor plasma (PPP) and a platelet fraction. These fractions were then treated at pH 5 to prepare the pH 5 fractions before the assay of the protein kinase activity. The protein kinase activity was detectable in the PRP fraction but very little if any was detectable in the PPP fraction. No apparent phosphorylation was detectable in the platelet fraction (Fig. 2, sections PRP, PPP and P). Combination of PPP fraction with platelet homogenates resulted in

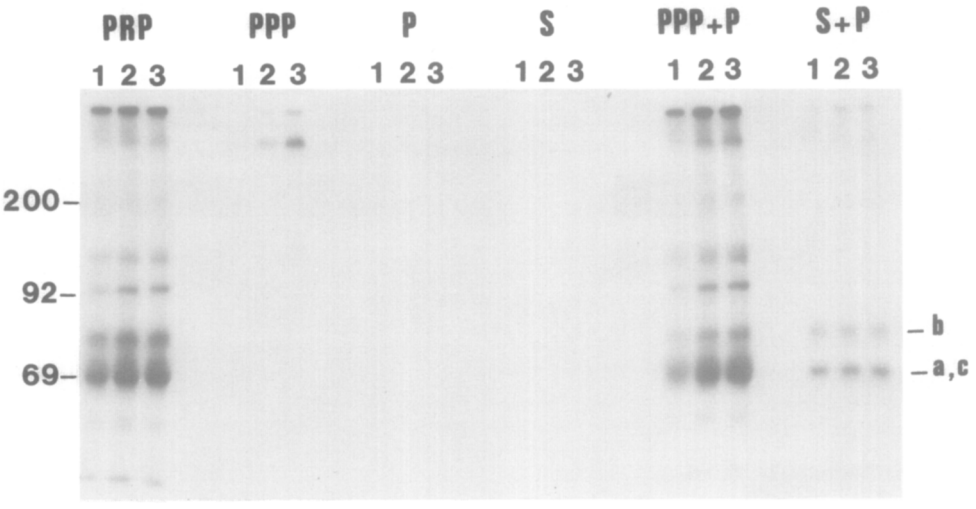

Fig. 2 - Protein kinase activity in different fractions of blood. Blood from a normal volunteer was kept (20 min) at room temperature for the preparation of serum (S) or was collected in a tube containing heparin, aprotinin and EDTA for the preparation of PRP (Materials and Methods). PRP was fractionated into a platelet (P) pellet and a plasma poor in platelets (PPP) supernatant. Platelets were washed and suspended in the washing buffer, equal volume as PRP (2). The different samples were from 10 µl aliquots of PRP, PPP, P, S and equal mixtures (10 µl + 10 µl) of the different samples as indicated. Samples were first treated at pH 5 before assay of the protein kinase activity as described (Materials and Methods). Incubation (30°C) for each case was for 10 (lane 1), 30 (lane 2) and 90 (lane 3) min. Analysis of the ^{32}P-labeled proteins in the pH 5 fractions was on a 8.5 % polyacrylamide gel. An autoradiograph of a stained, dried gel is shown. On the right, the positions of proteins *a*, *b* and *c* are indicated. The proteins *a*, *b* and *c* in samples PRP, PPP + P and S + P were identified by two dimensional gel electrophoretic analysis.

the recovery of the kinase activity (Fig. 2, section PPP + P). Heating the platelet homogenates at 56°C for 5 min completely abolished this kinase activity On the other hand, heating the PPP fraction did not affect the phosphorylation of the different proteins by the platelet extracts (not heated), thus indicating that these proteins are substrates of the platelet protein kinase activity. These results confirm previous observations obtained after partial purification of PRP on poly(G)-Sepharose (2). No protein kinase activity was detectable in the pH 5 fraction from serum (Fig. 2, section S) but mixing experiments with platelet homogenates revealed the presence of low levels of phosphorylated proteins *a*, *b* and *c* (Fig. 2, section S + P).

Figure 3. shows the optimum cation requirements for the assay of the protein kinase activity in the PRP fraction. The amount of phosphorylation of the different proteins increased upon incubation at 30°C (Fig. 2, section PRP, lanes 1-3) and was markedly enhanced in the presence of Mn^{2+} (Fig. 3). Mg^{2+} at 2 to 10 mM slightly enhanced the protein kinase activity but Ca^{2+} had no apparent effect (Fig. 3). Similar requirements were also observed

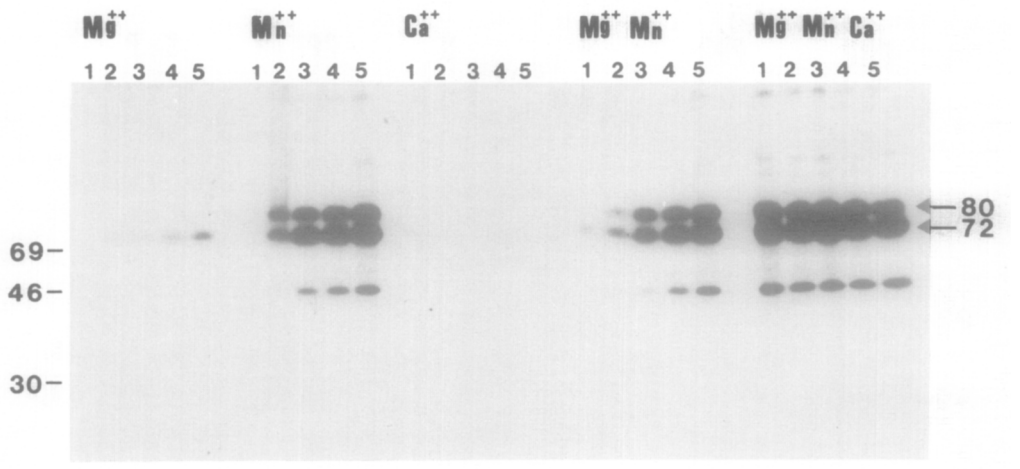

Fig. 3 - The effect of metal ions on the protein kinase activity in the pH 5 fraction. A pH 5 fraction was prepared and assayed as described (Materials and Methods) but at different $Mg(OAc)_2$ (Mg^{++}), $MnCl_2$ (Mn^{++}) and $CaCl_2$ (Ca^{++}) concentrations : 0, 0.5, 1, 2 and 5 mM in lanes 1 to 5 respectively ; Mg^{++} Mn^{++} : protein kinase activity was assayed at 2 mM $Mg(OAc)_2$ but different concentrations of $MnCl_2$, 0, 0.5, 1, 2 and 5 mM in lanes 1 to 5 respectively ; Mg^{++} Mn^{++} Ca^{++} : 2 mM $Mg(OAc)_2$ and 5 mM $MnCl_2$ but different concentrations of $CaCl_2$, 0, 0.5, 1, 2 and 5 mM respectively. The ^{32}P-labeled proteins were analysed on a 10 % polyacrylamide gel. An autoradiograph of a stained, dried gel is shown. The positions of 80K and 72K proteins are indicated on the right.

for the phosphorylation of an exogenous substrate, calf thymus histones (Table 1). For this latter assay, it became necessary to add aprotinin (500 units/ml) to inhibit a potent proteolytic agent(s) in the pH 5 fraction which degraded added histones. The phosphorylation of both endogenous proteins (in the pH 5 fraction) and exogenous histones was not stimulated by cyclic nucleotides such as cAMP and cGMP (Table 1). The strongest inhibition of the protein kinase activity in the pH 5 fraction was found to be ADP (Fig. 4 and Table 1). A complete inhibition of phosphorylation of protein 72K and protein 80K were observed at a concentration of 50 µM ADP. Adenosine, adenine and 5'-AMP showed very little if any inhibitory effect on the protein kinase activity.

Studies to establish Lineweaver-Burk plot (data not shown) of the protein kinase activity of the pH 5 fraction in the absence or presence of ADP indicated a Vmax for the phosphorylation of endogenous proteins (proteins *a*, *b* and *c* : 72K and 80K) of 2.5 X 10^{-4} M. The apparent Km for ATP in the absence and presence of ADP (10 µM) was 7.5 X 10^{-6} M and 2.0 X 10^{-5} M, respectively. These results suggest that ADP is a competitive inhibitor of ATP in the phosphorylation reaction. Steiner (12) has previously described a protein kinase activity associated with platelet membrane proteins. This

Table 1. : Phosphorylation of histones by the pH 5 fraction from PRP

Nucleotide	$Mg(OAc)_2$, µM	$MnCl_2$, µM	^{32}P-cpm
None	2.0	–	21751
	–	0.5	35473
	–	2.5	49055
	–	5.0	52679
	2.0	0.5	38714
	5.0	10.0	73342
ADP ; 5 µM	5.0	10.0	53696
25 µM	5.0	10.0	26678
50 µM	5.0	10.0	15860
cAMP ; 50 µM	2.0	0.5	35560
50 µM	5.0	10.0	64678
cGMP ; 50 µM	2.0	0.5	34354
50 µM	5.0	10.0	66056

The histone kinase activity was assayed as described (Materials and Methods). ^{32}P-cpm represent net counts incorporated in histones.

protein kinase activity although has some similarities with the one described here shows stimulation by cAMP.

The results described here show that a platelet protein kinase activity is responsible for the phosphorylation of several blood plasma proteins in

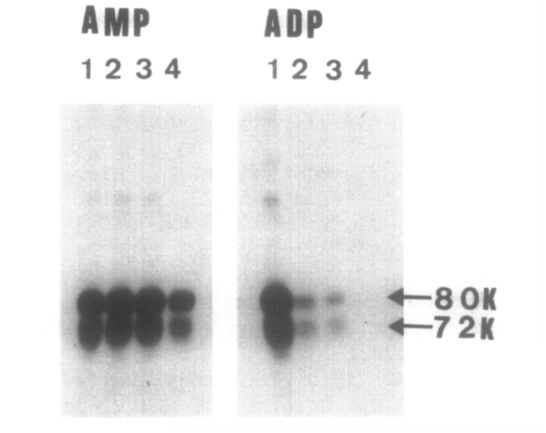

Fig. 4 - Inhibition of the protein kinase activity in the pH 5 fraction by ADP. The preparation of the pH 5 fraction and assay of the protein kinase activity was as described (Materials and Methods). Similar aliquots of the pH 5 fraction were assayed in the presence of different concentrations of AMP (0, 0.01, 0.1 and 1 mM in lanes 1 to 4, respectively) or ADP (0, 0.01, 0.05 and 0.25 mM in lanes 1 to 4, respectively). The ^{32}P-labeled proteins were analysed on a 8.5 % polyacrylamide gel. The positions of 80K and 72K proteins are indicated on the right.

addition to the phosphorylation of an exogenous substrate, calf thymus histone. The three main substrates of the protein kinase activity are the α-chain of fibrinogen (4) and two proteins of identical isoelectric points (pI 6.0) but of different molecular weights (72K and 80K) whose identity remains to be found. All of these proteins are detectable in the serum (Fig. 2) but at very low levels compared to those found in the plasma. Thus, the substrates (proteins *a*, *b* and c) which are normally present in the blood plasma are inactivated or lost during the coagulation process. The pH 5 fraction provides a convenient tool to study the role of phosphorylation, if any, in the last steps of coagulation, i.e., in the clot formation process. It also provides a simple and an efficient method for determination of such kinase activity in the PRP of normal individuals in comparison with patients. For example, recently we have shown that the level of the protein kinase activity in the pH 5 fraction is enhanced in patients with viral and bacterial infections (C. Buffet-Janvresse and A.G. Hovanessian, unpublished results). The level of the protein kinase activity shifts to a normal value in parallel with the disappearance of clinical symptoms.

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