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Two separate tyrosine protein kinases in human platelets

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Tyrosine protein kinase activities were detected in the cytosolic fraction (PC-TPK) and the particulate fraction (PM-TPK) in human platelets using the synthetic peptide, $E_{11}G_1$ (Glu-Asp-Ala-Glu-Tyr-Ala-Ala-Arg-Arg-Arg-Gly) as a substrate. PC-TPK and PM-TPK were different in substrate specificities, divalent cation requirements and apparent M_r values. These results strongly suggest that in platelets there exist at least two separate tyrosine protein kinases; one is present in cytosol and the other might be associated with membranes.

Tyrosine protein kinase *Human platelet* *Cytosomal enzyme* *Protein phosphorylation*

1. INTRODUCTION

A newly described class of protein kinases that selectively phosphorylate tyrosine residues has been linked to virus transformation [1–7] and growth stimulation with certain growth factors [8–13] and normal cellular proteins homologous to certain virus transforming protein [14–16]. These unique protein kinases are believed to be involved in the regulation of proliferation of normal and malignant cells. Recently it has been found that high tyrosine protein kinase activities are associated with normal hematopoietic cells including platelets [17–20]. Since platelets lack nucleus, tyrosine residue phosphorylation does not seem to be related to the proliferation of cells. So it is of interest to characterize tyrosine protein kinase in platelets and to reveal its role in cellular function.

Here, we found high tyrosine protein kinase activities not only in the particulate fraction (PM-TPK) but in the cytosolic fraction (PC-TPK) using platelets, and these enzyme properties are briefly studied.

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Abbreviations: NP 40, Nonidet P-40; TLCK, *p*-tosyl-L-lysine chloromethyl ketone; TPCK, *p*-tosyl-L-phenylalanine chloromethyl ketone; PC-TPK, platelet cytosolic tyrosine protein kinase; PM-TPK, platelet membrane-associated tyrosine protein kinase; protein kinase C, Ca^{2+}-activated, phospholipid-dependent protein kinase

2. MATERIALS AND METHODS

A synthetic peptide, $E_{11}G_1$ (Glu-Asp-Ala-Glu-Tyr-Ala-Ala-Arg-Arg-Arg-Gly) was prepared as in [21]. (Val^5)Angiotensin II was purchased from Sigma. [γ-^{32}P]ATP was prepared as in [22]. All other chemicals of the highest grade were obtained from commercial sources.

Human platelets were prepared from 40–50 ml of fresh blood as in [23]. Washed platelets were suspended in cold buffer A (50 mM Tris-HCl at pH 7.5, 2 mM $MgCl_2$, 1 mM EDTA, 1 mM TLCK and 1 mM TPCK) and were sonicated on ice with constant stirring. The sonicate was centrifuged at $1000 \times g$ for 10 min to remove unbroken cells. The resultant supernatnt was then centrifuged at 4°C at $48000 \times g$ for 30 min. After this treatment, the supernatant fraction was used as a source of PC-

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TPK. Some of the particulate fractions were resuspended in the buffer A containing 5% NP 40 and stood on ice for 1 h and then centrifuged at 100000 × g for 60 min. The resultant supernatant was applied to a hydroxyapatite column (1 ml) equilibrated with buffer A. The column was washed with 10 ml of buffer A and then washed with 250 mM potassium phosphate buffer at pH 7.0 in buffer A. The detergent-soluble enzyme at this step or particulate fraction were used as sources of PM-TPK as indicated elsewhere. Tyrosine protein kinase activity was measured using the synthetic peptide as a substrate as in [24]. Protein concentration was determined as in [25]. All experiments were carried out with at least 3 preparations and assays were carried out in duplicate.

3. RESULTS

The cytosolic and particulate fractions of human platelets were analyzed for tyrosine protein kinase activity using the peptide, $E_{11}G_1$, having an amino acid sequence similar to that of the tyrosine phosphorylation site of $pp60^{src}$ as a substrate (table 1). In our assay system employed here, the particulate fraction of platelets contained very high tyrosine protein kinase activity (PM-TPK). Under the same conditions the cytosolic fraction also had a substantial amount of tyrosine protein kinase activity (PC-TPK).

To further distinguish PC-TPK from PM-TPK, some of the enzyme properties were characterized and compared. Fig.1 shows different rates of peptide phosphorylation by PC-TPK and PM-TPK. In PC-TPK (Val^5)angiotensin II was a better substrate than $E_{11}G_1$ (fig.1A). On the other hand, PM-TPK phosphorylated $E_{11}G_1$ more efficiently than (Val^5)angiotensin II under the same conditions (fig.1B). By using solubilized instead of non-solubilized PM-TPK we obtained a similar result (not shown).

For further characterization of PC-TPK and PM-TPK, divalent cation requirements were tested (fig.2). Both Mg^{2+} and Mn^{2+} could serve as

Table 1

Intracellular distribution of tyrosine protein kinase activity in human platelets

Preparation	Protein (mg)	Total activity (pmol/min)	Specific activity (pmol/min per mg)
Cytosolic	11.9	262	22
Particulate	6.3	790	126

The cytosolic and the particulate fractions were assayed for tyrosine protein kinase activity by estimating the phosphorylation of $E_{11}G_1$ (1 mg/ml) in the presence of 60 μM [γ-^{32}P]ATP, 50 mM $MgCl_2$, 0.05% NP 40 and 10 μM vanadate as in [24]. The amount of protein used was 20 μg/tube for cytosolic enzyme and 10 μg/tube for particulate enzyme

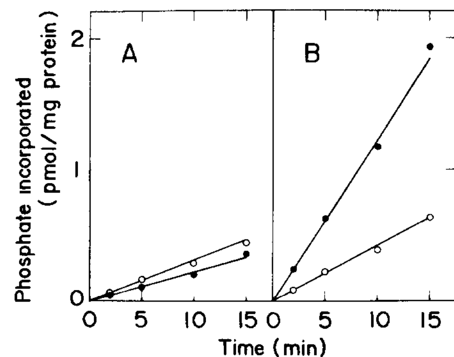

Fig.1. Peptide substrate specificity of PC-TPK and PM-TPK in platelets. Tyrosine protein kinase activity with each peptide was determined at 1 mg/ml concentration for the various time periods indicated. Other conditions as in table 1. (A) With PC-TPK, (B) with PM-TPK. (●) $E_{11}G_1$ phosphorylation; (○) (Val^5)angiotensin II phosphorylation.

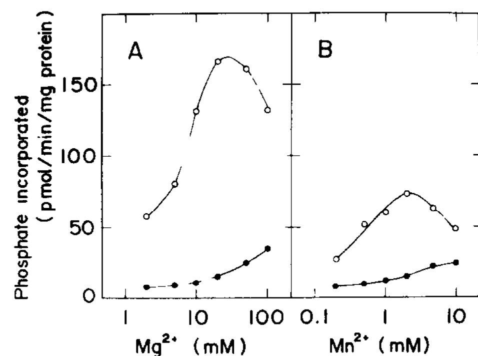

Fig.2. Effects of divalent cations on the phosphorylation of $E_{11}G_1$ by PC-TPK and PM-TPK in platelets. Tyrosine protein kinase activities of both enzymes were measured as a function of various concentrations of divalent cations. Assay conditions as in table 1. (A) With Mg^{2+}, (B) with Mn^{2+}. (●) With PC-TPK, (○) with PM-TPK.

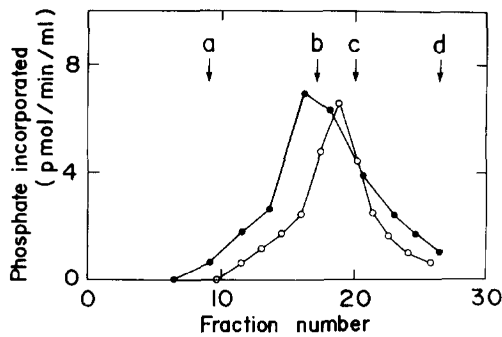

Fig.3. Molecular masses of PC-TPK and PM-TPK from sucrose density gradient sedimentation. 100 μl of either PC-TPK or PM-TPK was applied to a 5–20% sucrose density gradient in buffer A containing 0.4 M KCl. After centrifugation at 37700 rpm for 35 h in a Hitachi SW 50 rotor, 30 fractions were collected from the bottom of the tube (0.18 ml/fraction) and an aliquot (30 μl) was assayed for $E_{11}G_1$ phosphorylation as in table 1. (●) With PC-TPK, (○) with PM-TPK. Standards of human γ-globulin (a), bovine serum albumin (b), ovalbumin (c) and cytochrome *c* (d) were run on parallel gradients and located by UV absorption.

divalent cations in both kinases, but these enzymes showed apparently different concentrations of each metal ion requirement for maximal activity. PM-TPK required 20–50 mM Mg^{2+} and 1–2 mM Mn^{2+} for maximal activity, whereas PC-TPK required much higher concentrations of both metal ions. By using solubilized PM-TPK the same results were obtained as with non-solubilized PM-TPK (not shown).

To show the difference between PC-TPK and PM-TPK more clearly, the apparent M_r values of both enzymes were estimated by sucrose density gradient sedimentation (fig.3). The apparent M_r values of PC-TPK and solubilized PM-TPK in this system were about 70000 and 52000, respectively. These results strongly suggest that there exist at least two tyrosine protein kinases in platelets; one is present in the cytosol and the other is presumably associated with membranes.

4. DISCUSSION

Two tyrosine protein kinases were found to be present in the cytosolic (PC-TPK) and particulate fractions (PM-TPK). PM-TPK may probably be related to or identical with a 60-kDa protein previously reported in [17]. PC-TPK may be a novel type of tyrosine protein kinase that has not yet been reported or be related to the cytosolic tyrosine protein kinase in bone marrow cells [26]. The different enzyme properties of PC-TPK and PM-TPK, such as substrate specificity, divalent cation requirement and apparent molecular mass strongly suggest that the tyrosine protein kinase in cytosol, if not completely exclusive, is separate from that associated with membranes. Recently, several lines of evidence have suggested that high tyrosine protein kinase activities are associated with normal non-proliferating cells [17,27,28]. To discover the role of tyrosine protein kinases in such cells, the study of tyrosine residue phosphorylation in platelets should supply us with a lot of information about cellular regulation. Particularly, it has recently been postulated that protein phosphorylation by protein kinase C plays an important role in platelet activation by thrombin [29]. It is very attractive to assume that either of the tyrosine protein kinases we described in this paper might be involved in the cellular function of platelets along with protein kinase C.

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