# Plasma protein kinase activity enhanced by interferon is found in platelets

J. Galabru, C. Buffet-Janvresse+, Y. Rivière and A.G. Hovanessian

*Unité d'Oncologie Virale, Institut Pasteur, 75724 Paris Cedex 15* and
*+Laboratoire de Virologie, Faculté de Médecine de Rouen, France*

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A protein kinase activity analogous to that found in interferon-treated HeLa cells is detectable in human plasma rich in platelets. This kinase activity is manifested by the phosphorylation of an endogenous $M_r$ 72000 protein which could be conveniently assayed after partial purification on poly(G)–Sepharose. Here, we show that the protein kinase system in the plasma consists of at least 2 components. The protein kinase is found to be localised in the platelets whereas most of the substrate (the $M_r$ 72000 protein) is found free in the plasma and a fraction of it associated with the surface of platelets.

*Protein kinase* *Interferon* *Platelet* *Plasma protein*

## 1. INTRODUCTION

Interferon-treated mouse and human cells show enhanced levels of a protein kinase activity manifested by the phosphorylation of endogenous $M_r$ 67000 (p67K kinase) and 72000 (p72K kinase) proteins, respectively [1]. Besides its presence in interferon-treated mouse cells, the p67K kinase is also detectable in tissues and plasma of mice and its level is enhanced several fold in mice with high levels of circulating interferon [2,3]. These protein kinase activities of mouse and human origin can phosphorylate calf thymus histones and are independent of cyclic AMP [4]. The phosphate is linked to the $M_r$ 67000 or 72000 proteins by their serine and threonine residues [2,5,6]. A protein kinase activity analogous to that found in control and interferon-treated HeLa cells is present in human plasma rich in platelets (PRP). The level of this kinase activity in different normal human plasma varies from one individual to the other. However, treatment of patients with human interferon [6] or an inducer of interferon [7] results in an increased level of p72K kinase activity in the plasma. Therefore, such kinase activity may serve as a marker for the response of an organism towards treatment with interferon.

The p72K kinase activity in human PRP fraction can be conveniently assayed after partial purification on poly(G)–Sepharose and is capable of phosphorylating several plasma proteins, the $M_r$ 72000 protein being the most important. Here, we show the existence of 2 components for this kinase system. A protein kinase activity is isolated in platelets while its substrates are found in the fraction of plasma poor or deficient in platelets (PPP).

## 2. MATERIALS AND METHODS

[$\gamma$-$^{32}$P]ATP was supplied by Amersham International (Bucks). Poly(I)·poly(C)–Sepharose and poly(G)–Sepharose were prepared as in [8]. Blood was collected in polystyrene tubes containing heparin (100 U/ml) and aprotinin (100 U/ml; Zymofren, Specia) and left 15–30 min at room temperature. PRP was collected after centrifugation at $120 \times g$ for 15 min. PPP was prepared by centrifugation of PRP at $1200 \times g$ for 15 min and the pellet (platelets) was suspended in phosphate buffered saline, equal volume as PPP. All the fractions were stored at −80°C. The protein kinase was assayed after its partial purification on poly(G)–



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Sepharose. Samples (100 μl) with or without NP40 treatment (as described) were added to polystyrene tubes containing poly(G)–Sepharose (100 μl) and the binding was carried out at room temperature. The poly(G)–Sepharose-bound kinase fractions were then washed and incubated with [γ-$^{32}$P]ATP as in [6]. The $^{32}$P-labeled proteins were analysed on SDS–polyacrylamide slab gels [4].

The phosphorylation of histones (histone kinase activity) was done by incubation (30°C, 90 min) of 2 μl aliquots of the different plasma or platelet fractions in 50 μl total vol. containing 50 mM Hepes (pH 7.6), 5 mM $Mg(OAc)_2$, 50 mM KCl, 10 mM $MnCl_2$, 7 mM 2-mercaptoethanol, 20% glycerol (v/v), 10 μM [γ-$^{32}$P]ATP (9 Ci/mmol) and 0.1 mg calf thymus histone (H-III S; Sigma). After the incubation, the sample (50 μl) was transferred to 2.5 cm filter discs (Whatman 3 MM paper) and washed (4°C) consecutively with cold 10% trichloroacetic acid containing 0.5 mM ATP, 5% trichloroacetic acid containing $NaH_2PO_4$ and subsequently with ethanol and acetone at room temperature. The filter discs were dried and the radioactivity was measured by liquid scintillation.

## 3. RESULTS

### 3.1. *The action of non-ionic detergent NP-40 on the detection of p72K kinase activity*

The p67K and p72K kinase activities can be assayed conveniently and efficiently after partial purification on poly(I)·poly(C)–Sepharose or poly(G)–Sepharose. The protein kinase from mouse and human cells in culture and from the different tissues of mice bind specifically to poly(I)·poly(C)–Sepharose [2,3]. However, the protein kinase activity from both mouse and human shows a higher affinity to poly(G)–Sepharose (unpublished). Accordingly in all of these experiments, the p72K kinase activity of the plasma was assayed using poly(G)–Sepharose.

Fig. 1 shows the phosphorylated proteins in a human PRP fraction assayed after purification on poly(G)–Sepharose. The phosphorylation of several proteins of $M_r$ 60000–200000 could be observed in this fraction, the phosphorylation of the $M_r$ 72000 protein being the most striking. The detection of such kinase activity was enhanced in the PRP fraction either by sonication or by freezing at −80°C and thawing before the kinase assay (fig. 1, lane 1 in a–c). Whatever was the initial treatment, however, addition of the non-ionic detergent NP-40 at 0.5–1% final conc. seemed to be essential for the total solubilisation and recovery of the protein kinase activity (fig. 1, lanes 2, 3 in a–c). These results indicate that the protein kinase activity is probably associated with a membrane complex of platelets since lysis of these cells by sonication or freeze–thawing was not sufficient to solubilise all the protein kinase activity. Subsequently, NP-40 at 1% was added to all the protein kinase samples before the kinase assay.

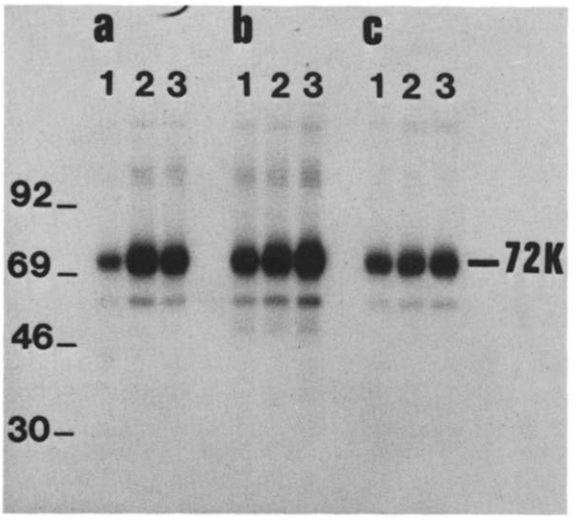


Fig. 1. The effect of sonication, freeze–thawing and non-ionic detergent NP-40 on the detection of the protein kinase activity in PRP fraction. The samples were from fresh PRP (a), sonicated PRP (b) and frozen −80°C and thawed PRP (c). Aliquots (100 μl) of the different PRP preparations (a–c) were assayed as such (lanes 1) or treated with NP-40 at 0.5% (lanes 2) and 1% (lanes 3). The protein kinase was assayed after partial purification on poly(G)–Sepharose and the $^{32}$P-labeled proteins were analysed by polyacrylamide (10%) slab gel electrophoresis. An autoradiograph of a stained, dried gel is shown. On the left, protein markers ($M_r \times 10^{-3}$): phosphorylase B, 92; bovine plasma albumin, 69; ovalbumin, 46; chymotrypsin, 30. On the right, 72 K indicates the position of the $M_r$ 72000 phosphoprotein. Each sample represents kinase activity from 30 μl PRP. The PRP samples (b) were sonicated for 30 s using a MSE sonicator equipped with a 7 mm probe (frequency, 2 kHz).

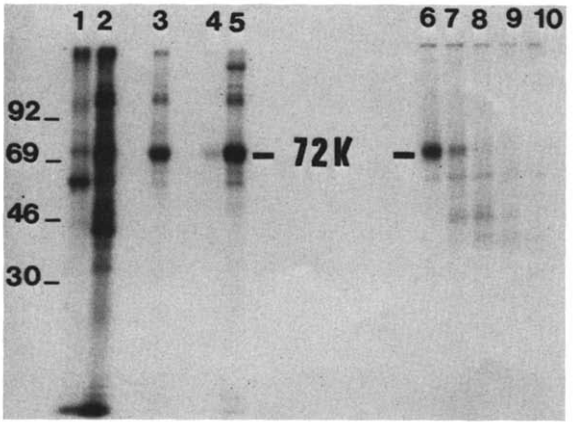


Fig. 2. Protein kinase activity in different fractions of blood lanes 1 and 2 show the protein kinase activity in 100 $\mu$l aliquots of blood (collected with heparin and aprotinin) after partial purification either on poly(I)·poly(C)–Sepharose (lane 1) or poly(G)–Sepharose (lane 2). The rest of the samples (lanes 3–10) were assayed after purification on poly(G)–Sepharose. Lanes 3–5 show the protein kinase activity in 30 $\mu$l PRP, PPP and platelet fractions, respectively; 1 ml blood cells (after the removal of PRP fraction) were washed (120 × $g$, 10 min) consecutively (4 times, lanes 6–9) with 1 ml washing solution (140 mM NaCl, 50 mM KCl and 5 mM $Mg(OAc)_2$). Washed blood cells (lane 10) were then lysed with 1 vol. buffer: 10 mM Hepes (pH 7.6), 10 mM KCl, 2 mM $(MgOAc)_2$, 7 mM 2-mercaptoethanol, 100 U/ml aprotinin and 0.5% NP-40 (v/v). The 4 consecutive washes (1 ml each, 120 × $g$ supernatants) and 100 $\mu$l washed blood cells lysate (lane 10) were assayed for protein kinase activity. Polyacrylamide gel electrophoretic analysis was as in fig. 1.

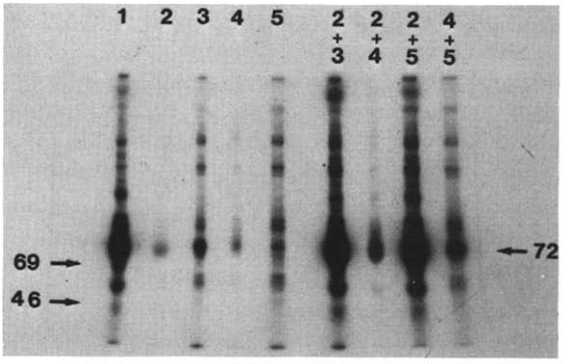


Fig. 3. Separation of the protein kinase activity and the substrate(s). Blood was collected with heparin (100 U/ml), aprotinin (100 U/ml) and EDTA (4 mM). The protein kinase activity was assayed after partial purification on poly(G)–Sepharose. $^{32}P$-Labeled proteins were analysed on a 7.5% polyacrylamide gel. $M_r$ markers were as in Fig. 1. The position of the $M_r$ 72000 protein is indicated on the right. The samples were from 50 $\mu$l aliquots of PRP (lane 1), PPP (lane 2), platelets (lane 3), platelet wash (lane 4), washed platelets (lane 5) and equal mixtures (50 $\mu$l + 50 $\mu$l) of the different samples as indicated. Platelets were washed and suspended in the washing buffer, equal volume as PRP. Washing buffer contained 15 mM Tris–HCl (pH 7.6), 140 mM NaCl, 50 mM KCl, 5.5 mM glucose, 1.5 mM EDTA, 2% bovine plasma albumin, 10 U heparin/ml and 10 U aprotinin/ml.

### 3.2. *The p72K kinase activity in different fractions of blood*

Blood (collected with heparin and aprotinin) was separated into different fractions, PRP, PPP, platelets and washed blood cells without platelets. Each fraction was then assayed for the protein kinase activity. The p72K kinase activity was detectable in the blood among the phosphorylation of several other proteins and this activity had a higher affinity to poly(G)–Sepharose (fig. 2, lane 2) than to poly(I)·poly(C)–Sepharose (fig. 2, lane 1). The protein kinase activity detectable in PRP (fig. 2, lane 3) was negligible in PPP (fig. 2, lane 4) but was recovered in the platelet fraction (fig. 2, lane 5). Washed blood cells without platelets showed no phosphorylation (fig. 2, lane 10) since the kinase activity was removed by repeated washings (fig. 2, lanes 6–9). The p72K kinase activity in the PRP, therefore, could be pelleted by high speed centrifugation (1200 × $g$, 20 min) which also results in the sedimentation of platelets. When the PRP fraction is first treated with 1% NP-40 before centrifugation at 1200 × $g$ then the protein kinase activity remains in the supernatant. Similarly, the protein kinase activity which could be detectable in the serum is not centrifugable even at 2500 × $g$, since the coagulation process results in lysis and loss of platelets (not shown).

### 3.3. *Separation of the protein kinase system into a kinase and a substrate fraction*

The results described above suggested that the

p72K kinase activity is associated with platelets. However, it remained possible that this association is simply due to its interaction with the surface membrane of platelets. For this purpose, blood was collected as usual with heparin and aprotinin but in the presence of 4 mM EDTA. Fig. 3 shows that under these conditions, a high level of p72K kinase activity is detectable in the PRP fraction (lane 1) but very little in the PPP (lane 2) or in platelet fractions (lane 3). Washing the platelet fraction with a buffer containing 4 mM EDTA further decreased its p72K kinase activity (lane 5). Combination of PPP fraction with platelet homogenates resulted in the recovery of the kinase activity. 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 $M_r$ 72000 protein by the platelet extracts (not heated), thus indicating that the $M_r$ 72000 protein is the substrate. The different fractions discussed in fig. 3 were further analysed for the phosphorylation of calf thymus histones as an exogenous substrate (table 1). In accord with the localisation of the kinase in platelets, a high level of phosphate was incorporated by the use of PRP or platelet fractions but not by the PPP fraction. These results confirm that the protein kinase activity is in platelets while the substrate (or substrates) is in the plasma (PPP). Early results in which both the kinase and a fraction of substrate were found in the platelet fraction (fig. 2), therefore, were due to the association of the substrate on the platelet membrane. In view of these results, it is worthwhile to mention here, that washed blood cells (without platelets; fig. 2, lane 10) seem to have a protein kinase activity capable of phosphorylating the $M_r$ 72000 protein when mixed with the PPP fraction. The relation of this kinase activity with that of platelets is not clear yet.

Table 1

Histone kinase activity in different fractions of PRP

| Sample | [$^{32}$P]Phosphate incorporated (pmol.mg histone$^{-1}$.assay$^{-1}$) |
|---|---|
| 1. PRP | 55.8 |
| 2. PPP | 5.2 |
| 3. Platelets | 38.2 |
| 4. Platelet wash | 6.6 |
| 5. Washed platelets | 48.2 |
| 2 + 3 | 37.8 |
| 2 + 4 | 3.5 |
| 2 + 5 | 41.1 |
| 4 + 5 | 38.2 |

Samples were as in fig. 2; histone kinase activity was determined as in section 2

## 4. DISCUSSION

These results show that the protein kinase system in the PRP fraction could be dissociated into two components, a kinase in platelets and substrate(s) in the plasma. In the absence of chelating agents a fraction of the substrate is found to be associated with the platelet fraction whereas the rest of the substrate remains soluble in the PPP (shown by mixing experiments with EDTA-washed, platelet lysates). These substrates may have receptor sites on the surface of platelets as demonstrated for plasma fibrinogen [9]. Preliminary results have shown that the level of the protein kinase system is very low in the serum compared with that of the corresponding plasma. Thus, the protein kinase and the substrate(s) in the serum are lost or inactivated during the coagulation process. It is tempting to speculate, therefore, that the protein kinase system may play a role in hemostasis. Lysis of platelets in the coagulation process or during the release reaction of platelets induced under certain conditions [10], may result in the availability of the kinase and ATP to phosphorylate the $M_r$ 72000 protein as well as the other substrates attached on the surface of platelets. Identification of these substrates among the spectrum of plasma proteins will be essential to establish the function of the protein kinase system in the blood and to clarify the role of interferon in enhancing this protein kinase activity [6,7].

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