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PHOSPHORYLATION OF THE α-CHAIN OF FIBRINOGEN BY A PLATELET KINASE ACTIVITY ENHANCED BY INTERFERON

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SUMMARY - Treatment of patients with interferon or inducers of interferon results in an enhanced level of a protein kinase activity found in platelets (1,3). The kinase activity is responsible for the phosphorylation of a 70-72,000 molecular weight protein (72K protein) found in blood plasma. By the means of a technique based on the precipitation of this protein kinase system (the protein kinase and its substrate), we show here that the 72K protein is the α-chain of fibrinogen. During the coagulation process induced by thrombin, the ^{32}P-labelled 72K protein is recovered in the clot. After incubation in the presence of thrombin, the 72K protein looses a small polypeptide of 2-3000 in molecular weight resulting a shift in its isoelectric point (pI) from 6.8-7.0 to 7.5. At the end of the coagulation process, the ^{32}P-labelled 72K protein becomes undetectable since it gives rise to a covalently linked α-polymer of a high molecular weight. In accord with these results, the 72K protein could be precipitated by antibodies against human fibrinogen.

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 (1,3). 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 (1,2). The relation, if any, of this protein kinase activity with that which is enhanced in interferon-treated human cells in culture, remains to be shown (4,5). Both kinase activities, however, are independent of cAMP and phosphorylate proteins at their serine and threonine residues (1,4).

In all experiments described here, platelet rich plasma (PRP) from normal individuals was used (3). The protein kinase activity was assayed after its partial purification on poly(G)-Sepharose (6) or after precipitation at pH 5 (7). In this latter technique, the protein kinase system (protein kinase activity and the 72K protein) is precipitated by incubation of PRP at pH 5 and it is referred to as 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

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thrombin (9,10). Here, we show that the 72K protein with a pI of 6.8-7.0 is the α-chain of fibrinogen.

MATERIALS AND METHODS

Materials : (γ-^{32}P) ATP was supplied by the Radiochemical Centre (Amersham, England). Human fibrinogen and human thrombin were from Sigma. Aprotinin (Zymofren) was from Specia and heparin was from Choay (Paris, France). Protein-A Sepharose was from Pharmacia. Rabbit anti-human fibrinogen serum was from Institut Behring. Poly(G)-Sepharose was prepared as described before (6).

Preparation of PRP : Blood from normal volunteers was collected in polystyrene tubes containing heparin (100 U/ml), aprotinin (100 U/ml) and EDTA (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 assay : The protein kinase activity was assayed either after partial purification on poly(G)-Sepharose (6) or after precipitation at pH 5 (7). In this latter technique, different samples of PRP were first incubated (4°C, 2-3 hr) in the presence of 50 mM sodium acetate, pH 5 before centrifugation (1000 x g, 15 min). The pellets were then dissolved in a buffer-containing 50 mM Tris-HCl pH 8 and 150 mM KCl and are referred to here as the pH 5 fraction. In a routine assay, 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 (60 Ci/mmol). 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%) containing sodium dodecyl sulphate (SDS) as described previously (6,7).

RESULTS

Action of thrombin on the 72K protein attached to poly(G)-Sepharose : Preliminary results have shown that the 72K protein is not detectable in the serum, thus suggesting that this protein disappears during the coagulation process. For this reason therefore, we investigated the fate of the 72K protein after treatment with thrombin. Fig. 1 shows the ^{32}P-labelled 72K protein phosphorylated by the platelet kinase. The assay was performed after partial purification on poly(G)-Sepharose. This protein has a pI of 6.8-7.0 and seems to consist of several subspecies (7). After treatment with thrombin, it is possible to detect a small shift in the molecular weight of the 72K protein which corresponds to a loss of a 2-3000 molecular weight polypeptide (Fig. 1, lanes 2 and 3).

Action of thrombin in the pH 5 fraction : The action of thrombin on the 72K protein was further investigated in a partially purified fraction of PRP prepared by precipitation at pH 5. Three major phosphoproteins are present in the pH 5 fraction (Ref. 7 ; Fig. 2A lane 5, 2B Sample) : 72K (protein a) and 80K (protein b) proteins with identical pI of 6.0 and another 72K protein with a pI of 6.8-7.0 (protein c). The protein c is

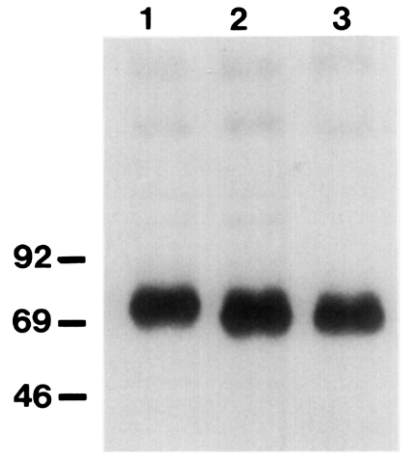

Fig. 1 - Phosphorylation of the 72K protein after partial purification of PRP on poly(G)-Sepharose. The protein kinase activity and the substrate were partially purified on poly(G)-Sepharose before the kinase assay by incubation (30°, 90 min) in the presence of 50 nM (γ-^{32}P)ATP (60 Ci/mmol). The samples were further incubated (37°C, 20 min) with $CaCl_2$ (10 mM) in the absence (lane 1) or presence of 1 (lane 2) and 2 (lane 3) NIH units/ml of human thrombin (Sigma). All the samples were heated (95°C, 5 min) in electrophoresis sample buffer (6) before analysis on polyacrylamide slab gels (10%) containing sodium dodecyl sulfate (SDS). An autoradiograph of a stained and dried gel is shown. The numbers on the left of each gel in Figs. 1 to 4 give the molecular weight of protein markers in thousands : β.galactosidase, 130 ; phosphorylase B, 92 ; bovine plasma albumin, 69 ; ovalbumin, 46 ; carbonic anhydrase, 31. The different samples represent kinase activity from 40 µl of PRP.

the one which binds to poly(G)-Sepharose (7) and whose phosphorylation is enhanced in the PRP of patients treated with interferon (1). Addition of thrombin in the pH 5 fraction led to clot formation. The phosphoproteins *a* and *b* remained in the supernatant (Fig. 2A, lane Sp ; Fig. 2B, Supernatant) while the phosphoprotein *c* was recovered in the washed clot (Fig. 2A, lane P ; Fig. 2B, Pellet) with a shift in its pI from 6.8-7.0 to 7.0-7.5. The shift in the pI of phosphoprotein *c* was probably due to the loss of 2-3000 molecular weight polypeptide by the action of thrombin (Fig. 1).

The fate of the 72K protein c during the coagulation process : The fact that the protein *c* is recovered in the clot after the coagulation process and in view of its molecular weight, suggested to us that it could be the α-subunit of plasma fibrinogen. In the absence of a reducing agent, the protein *c* (in the pH 5 fraction) exists as a complex of high molecular weight (> 300 K) which in the presence of a reducing agent gives rise to three main proteins of molecular weight, 72K, 55K and 45K, among which only the 72K protein (protein *c*) is phosphorylated by the protein kinase activity (data not shown). These three proteins could be seen in the pH 5 fraction shown in Fig. 3A (lane S) just above and below bovine plasma

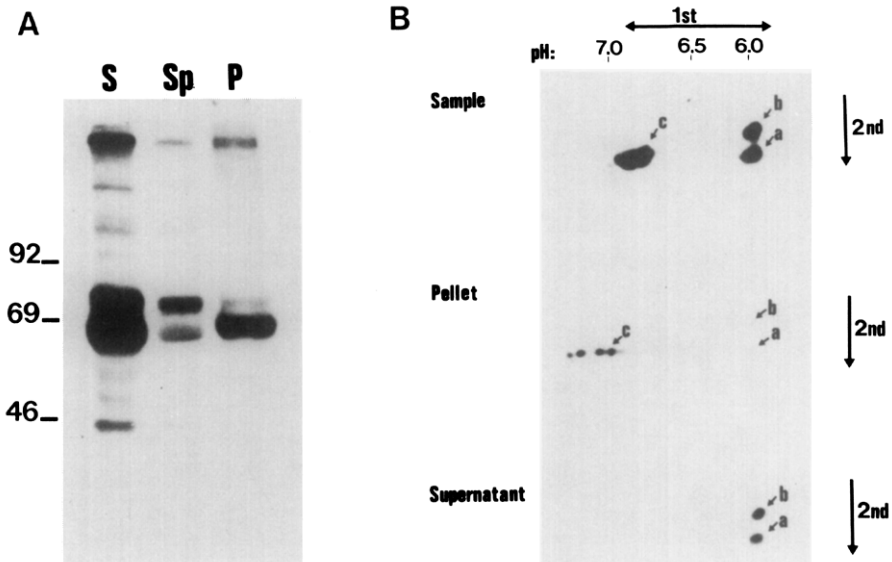

Fig. 2 - The recovery of the ^{32}P-labelled 72K protein c in the clot. The pH 5 fraction (50 µl equivalent to 50 µl of PRP) was incubated (30°C, 45 min) in the presence of 50 nM (γ-^{32}P)ATP (60 Ci/mmol) in a total reaction volume of 100 µl containing 25 mM Tris-HCl pH 8, 75 mM KCl, 2 mM Mg $(OAc)_2$ and 5 mM $MnCl_2$ (Sample, S). Similar samples after the phosphorylation reaction were further incubated (37°C, 20 min) in the presence of thrombin (2 units/ml) and $CaCl_2$ (10 mM) and the clot (Pellet, P) was separated from the supernatant solution (Supernatant, Sp) by centrifugation (1000 x g, 15 min). The sample and supernatant fractions were mixed with an equal volume of 2 fold concentrated electrophoresis sample buffer. The clot (pellet) was washed in buffer containing 50 mM Tris-HCl pH 8, 75 mM KCl, 100 units/ml heparin and 100 units/ml aprotinin, before its solubilisation in the electrophoresis sample buffer containing 5 M urea. All samples were heated (95°C, 10 min) before analysis by polyacrylamide gel electrophoresis (A) or by two-dimensional gel electrophoresis (B), as described by O'Farrel (11) with some modifications (12). The pH gradient obtained by isoelectric focusing (first dimension) was 5.2 to 7.5. Autoradiographs of stained and dried gels are shown. The molecular weight of protein markers were as before. The letters *a*, *b* and *c* designate the three major ^{32}P-labelled proteins.

albumin (69K) and their positions in the polyacrylamide slab gel are the same as a commercially available human fibrinogen (Sigma). Human fibrinogen is composed of two sets of three non-identical chains (α, β, γ) interconnected by disulphide bonds (13). In the presence of thrombin, factor XIII brings about the cross-linking of the fibrinogen molecule by the formation of a covalent linkage between ε-amino group of lysine and γ-carboxyl group of glutamine, thus leading to a cross-linked fibrin consisting of γ-dimers (95K), α-polymers (> 300K) and unchanged β-chains (9,13). All these different species can be resolved by polyacrylamide gel electrophoresis (14). The γ-γ dimers are formed rapidly while the α-α linkage requires approximatively 15 minutes to take place (15). We used these phenomena as markers to monitor the formation of fibrin and to follow the fate of the 72K protein

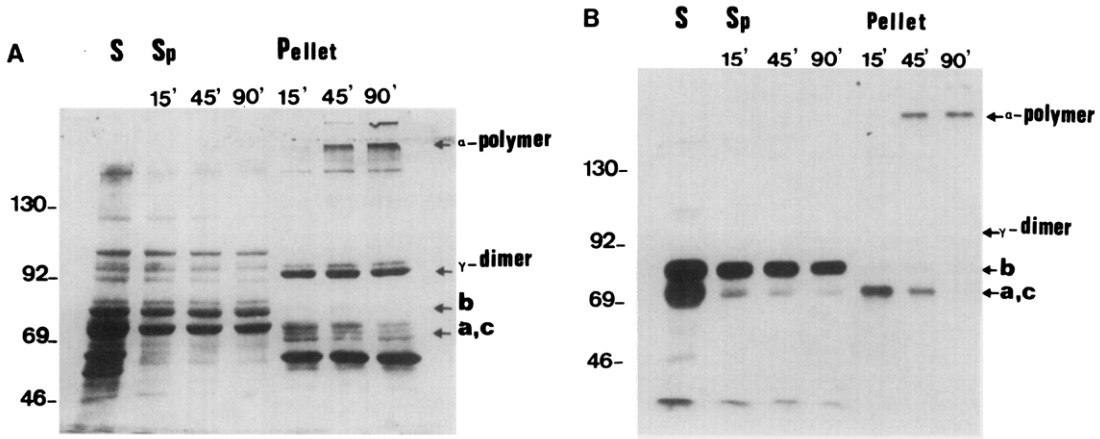

Fig. 3 - The fate of the ^{32}P-labelled protein C in the coagulation process. Polyacrylamide gel (8.5 %) electrophoretic analysis of the pH 5 fraction. A : gel stained for protein; B : an autoradiograph of the stained and dried gel. Similar aliquots of the pH 5 fraction were incubated with (γ-^{32}P)ATP (as in Fig. 2) before analysis as such (S) or after incubation in the presence of thrombin (2 units/ml) and $CaCl_2$ (10 mM) for 15, 45 and 90 min. At each time interval, the clot (pellet) and the supernatant (Sp) were analysed as described in the legend of Fig. 2. On the right of each gel, the position of α-polymer, γ-dimer and proteins *a*, *b* and *c* are indicated. The ^{32}P-labelled 72K protein observed in the pellet at 15 min is protein *c* since it has a pI of 6.8-7.0.

(protein *c*) during the process of clot formation. Fig. 3 shows the pattern of proteins (A) in parallel with that of ^{32}P-labelled proteins (B) in the pH 5 fraction incubated in the presence of thrombin at different time intervals (15, 45 and 90 min). At each point, the clot and the supernatant were separated by centrifugation and analysed on polyacrylamide gels. It is apparent from this figure that addition of thrombin in the pH 5 fraction led to the formation of the γ-dimer and the α-polymer with similar kinetics to those reported previously (Fig. 3A, Pellet). The ^{32}P-labelled 72K protein (protein *c*) which was recovered in the clot at 15 min, disappeared with time since it gave rise to a covalently linked high molecular weight protein complex at the same position as the α-polymer (Fig. 3B, Pellet).

Immunoprecipitation of the ^{32}P-labelled 72K protein c by antibodies against human fibrinogen : The pH 5 fraction was first phosphorylated in the absence of 2-mercaptoethanol before incubation with control rabbit serum or rabbit anti-human fibrinogen serum. The immune complexes were then separated by protein-A Sepharose and the samples were analysed by polyacrylamide gel electrophoresis in the presence of 2-mercaptoethanol (as usual). Fig. 4 shows that a ^{32}P-labelled 72K protein is precipitated by anti-human

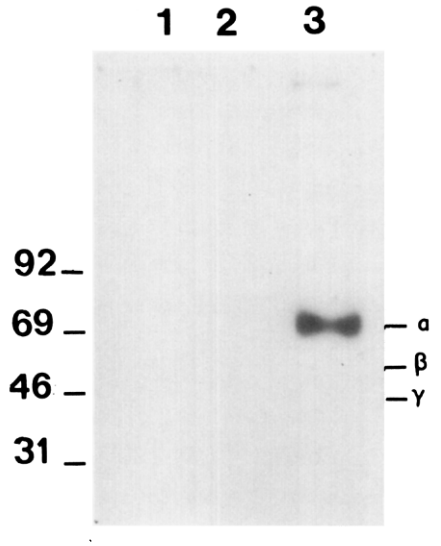

Fig. 4 - Immunoprecipitation of the ^{32}P-labelled 72K protein c by antibodies against human fibrinogen. The pH 5 fraction was first phosphorylated as before in the presence of (γ-^{32}P)ATP before incubation (37°C, 30 min) as such (lane 1) and in the presence of 20 µl of rabbit control serum (lane 2) or rabbit anti-human fibrinogen containing serum (lane 3). Protein-A Sepharose (25 µl) was then added and further incubated overnight at 4°C with gentle agitation. The different samples were washed successively with 2 ml of buffer A (50 mM Tris-HCl pH 8, 50 mM KCl, 2.5 mM Mg $(OAc)_2$, 5 mM $MnCl_2$, 10% glycerol, 10 units/ml heparin, 200 units/ml aprotinin and 2 ml of buffer B (0.1 mM Tris-HCl pH 8, 0.2 M LiCl, 0.1% 2-mercaptoethanol, 0.5 N NaCl). All the samples were then suspended in electrophoresis sample buffer, heated and analysed by polyacrylamide (10%) slab gel electrophoresis. On the right, the positions of the α, β and γ chains of fibrinogen as detected on the stained gel. The positions of the molecular weight protein markers were as mentioned before.

fibrinogen serum (Fig. 4, lane 3). This protein was shown to have a pI of 6.8-7.0 by two dimensional gel electrophoretic analysis (data not shown) and thus indicating that it is the phosphoprotein c.

DISCUSSION

Human fibrinogen is composed of two sets of three non-identical chains (α, β, γ) interconnected by disulphide bonds (13). Its molecular weight is 340,000 daltons. In the presence of thrombin, factor XIII brings about the conversion of fibrinogen to a cross-linked fibrin which consists of α-dimers, γ-polymers and unchanged β-chains (10). In accord with these facts, several results mentioned here confirm that the 72K protein with a pI of 6.8-7.0 (phosphoprotein c) is the α-chain of fibrinogen molecule. (1) In the absence of a reducing agent, the protein c exists a a complex of a high molecular weight (> 300K) which in the presence of a reducing agent gives rise to three main proteins of molecular weight, 72K, 55K and 45K. The 72K protein has a pI of 6.8-7.0, similar to that of phosphoprotein c.

(2) The 72K, 55K and 45K proteins bind to poly(G)-Sepharose as the commercially available human fibrinogen. Analysis of the proteins attached to poly(G)-Sepharose or those in the pH 5 fraction by polyacrylamide slab gel electrophoresis indicates that 72K, 55K and 45K proteins migrate at positions comparable to that of α, β and γ subunits of human fibrinogen.
(3) Addition of thrombin to the pH 5 fraction is sufficient for clot formation. The ^{32}P-labelled protein c (72K) is recovered in the clot.
(4) The phosphoprotein c looses a 2-3000 molecular weight polypeptide after treatment with thrombin. This loss of a small peptide is comparable to the release of fibrinopeptide A from α-chain of fibrinogen by the action of thrombin (16,17).
(5) At the end of the coagulation process, the ^{32}P-labelled protein c becomes undetectable, since it gives rise to a covalently linked polymer of a high molecular weight at the same position on a polyacrylamide slab gel as the α-polymer of fibrin.
(6) The ^{32}P-labelled protein c could be precipitated by antibodies against fibrinogen.

In view of these results, it is possible to suggest that a protein kinase system may be implicated in the blood coagulation process. It is tempting to speculate, therefore, that lysis of platelets in the coagulation process or during the release reaction of platelets induced under certain conditions, may result in the availability of the kinase and ATP to phosphorylate the α-chain of fibrinogen. The possible physiological effects of α-chain phosphorylation by a platelet kinase whose activity can be modified by interferon (1) or inducers of interferon (2) remain to be shown.

Several workers have shown the phosphorylated state of fibrinogen in its native form (18,19). Besides a preliminary report by Steiner (20), however, this is the first description of a platelet protein kinase activity responsible for the phosphorylation of α-chain of the fibrinogen molecule.

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