Vol. 117, No. 2, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
December 16, 1983 Pages 631-636

PHOSPHORYLATION OF FIBRINOGEN BY CASEIN KINASE 1

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Received November 10, 1983

Casein kinase 1 phosphorylated human fibrinogen, in a reaction that did not use GTP as phosphoryl donor and was neither stimulated by cyclic AMP or Ca^{2+}, nor inhibited by the cyclic AMP-dependent protein kinase inhibitor protein. Maximal incorporation averaged 4 mol of phosphate per mol of fibrinogen, most of it in the largest CNBr-fragment of the α-chain. Phosphoamino acid analysis revealed that phosphorylation occurred only at seryl residues. The phosphorylation of fibrinogen by casein kinase 1 was reverted by alkaline phosphatase.

Rat liver cytosol contains a cyclic AMP-independent casein kinase whose molecular and kinetic properties correspond with those of other type I casein kinases (or casein kinase 1, CK-1) present in mammalian tissues (1,2). The physiological role of this type of enzymes awaits still confirmation. Nonetheless, studies "in vitro" have shown that CK-1 phosphorylates several biologically active proteins such as glycogen synthase (1,3), phosphorylase kinase, troponin, myosin light chain and myosin light chain kinase (4). Thus, it is concevible that CK-1 may play an important role in the control of a number of physiological events.

Fibrinogen from several mammalian species is an hexameric protein ($\alpha_2\beta_2\gamma_2$) which contains considerable amounts of phosphate bound covalently to serines situated at several locations on the α-chains, including Ser_3 and Ser_{441} in human fibrinogen (5,7).

Previous reports have shown that fibrinogen can be phosphorylated "in vitro" by the cyclic AMP-dependent protein kinase from pig muscle (8), the calcium-activated, phospholipid-dependent protein kinase from rat brain (9) and a protein kinase system from platelet rich plasma (10). In the present report we show that fibrinogen is also phosphorylated by the CK-1 from rat liver in a cyclic AMP and Ca^{2+}-independent manner.

0006-291X/83 $1.50

MATERIALS AND METHODS

Human fibrinogen, thrombin from bovine plasma, alkaline phosphatase from bovine intestinal mucose, phosphoserine and phosphothreonine were obtained from Sigma. Prior to use, samples of fibrinogen were subjected to gel filtration on a (0.9 x 11 cm) Sephadex G-25 column equilibrated with 5 mM Tris-HCl buffer, pH 7.5, containing 150 mM NaCl. Rabbit muscle I-form glycogen synthase was purified to homogeneity as previously described (11). The cyclic AMP-dependent protein kinase inhibitor protein was purified up to the trichloroacetic acid precipitation step by the method of Walsh et al. (12).

CK-1 was purified from rat liver cytosol. The preparation obtained after the second phosphocellulose (1) was further purified by chromatography in a Bio-Gel A-1.5 m column (1 x 90 cm) as indicated in (13). One unit of CK-1 is the amount of enzyme that incorporated one nanomol of ^{32}P from $|\gamma\text{-}^{32}P|ATP$ to casein (4 mg/ml) per min.

The sources of other materials were as indicated previously (1).

Phosphorylation of fibrinogen was carried out at 30°C under the standard assay conditions described for CK-1 (1) except that the reaction mixture contained 60 mM NaCl plus 90 mM KCl and casein was substituted by fibrinogen. At indicated times samples (20 µl) were removed and assayed for ^{32}P-incorporated into protein. To obtain ^{32}P-fibrinogen, the incubated reaction mixture was passed through an AG 1x2 resin equilibrated with 50 mM Tris-HCl buffer, pH 7.5. CK-1 was retained by the column whereas the ^{32}P-fibrinogen was not. The flowthrough fractions were dialyzed extensively against 5 mM Tris-HCl buffer, pH 7.5, containing 150 mM NaCl.

Treatment of ^{32}P-fibrinogen with CNBr was carried out using samples containing 50-150 µg of protein, which were dialyzed against water, freeze-dried and then dissolved in 70% formic. CNBr was added to attain a fibrinogen: CNBr ratio of 1:50 (w/w) and the reaction allowed to proceed at room temperature for 20 hr. At the end of that time, samples were diluted five-fold with ice-cold water and freeze-dried.

Digestion with thrombin (2 NIH units/ml) of ^{32}P-fibrinogen (50 to 100 µg) was carried out in 50 mM Tris-HCl buffer, pH 7.5, overnight at room temperature. The digests were freeze-dried and subjected to electrophoresis.

Gel electrophoresis was carried out in 7 to 16% gradient slab gel containing 0.1% sodium dodecyl sulfate (14). Gels were dried, covered with cellophan and exposed to Mafe RP-C7-film, with the aid of an intensifying screen (Kostix Universal) for autoradiography of the ^{32}P-labelled samples.

Identification of phosphoamino acids was carried out by electrophoresis on cellulose thin layer plates at pH 1.9 for 90 min at 1 KV in glacial acetic acid:formic acid:H_2O (78:25:897, v/v) of samples hydrolyzed in 6 N HCl for 2 hr at 110°C under a nitrogen atmosphere.

The concentration of fibrinogen was determined either by absorbance at 282 nm (5) or by the method of Bradford (15).

RESULTS AND DISCUSSION

It has been shown previously that rat liver cytosol CK-1 phosphorylates casein, phosvitin and glycogen synthase (1). This kinase also phosphorylates human fibrinogen in a time-dependent reaction (Fig. 1). When assayed under the same conditions, the

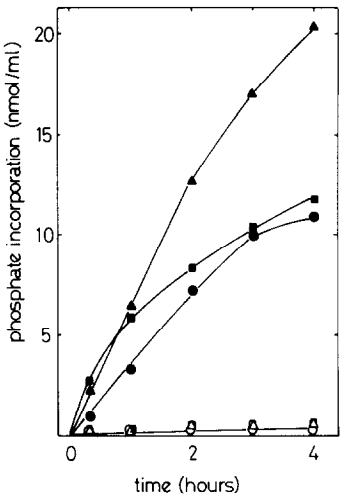

Figure 1 - Phosphorylation of fibrinogen and glycogen synthase by CK-1.
The reactions were carried out as indicated under "Material and Methods" either in the absence (○, △, □) or in the presence of 3 units/ml of CK-1 (●, ▲, ■). The concentration of fibrinogen in the assays was either 1 mg/ml (○, ●) or 2 mg/ml (△, ▲) whereas glycogen synthase was present at 0.5 mg/ml (□, ■).

initial rate of phosphorylation of fibrinogen (2 mg/ml) was similar to that of rabbit muscle glycogen synthase (0.5 mg/ml). The apparent Km value determined for fibrinogen was 11.3 mg/ml, which is about 60-fold higher than the value reported for glycogen synthase (1). As observed with casein and glycogen synthase, the phosphorylation of fibrinogen by CK-1 was neither stimulated by cyclic AMP (20 μM) and Ca^{2+} (0.5 mM) nor inhibited by the cyclic AMP-dependent protein kinase inhibitor protein (0.4 mg/ml). No phosphorylation was observed when GTP was used instead of ATP.

After prolonged incubation, phosphate incorporation into fibrinogen reached a value corresponding to 4 mol of ^{32}P per mol of fibrinogen (Mr 340,000). This value is comparable to the maximal incorporation catalyzed by the catalytic subunit of cyclic AMP-dependent protein kinase from pig muscle (8). Analysis of the amino acid residues in ^{32}P-fibrinogen revealed that CK-1 only phosphorylated serines (Fig. 2).

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate of the ^{32}P-fibrinogen (Fig. 3A) showed that the phosphate was introduced preferentially into the α-chain.

Analysis of the ^{32}P-fibrinogen digested with thrombin (Fig. 3B) indicated that most of the radiactivity was associated with the largest fragment of the α-chain, with only a small a-

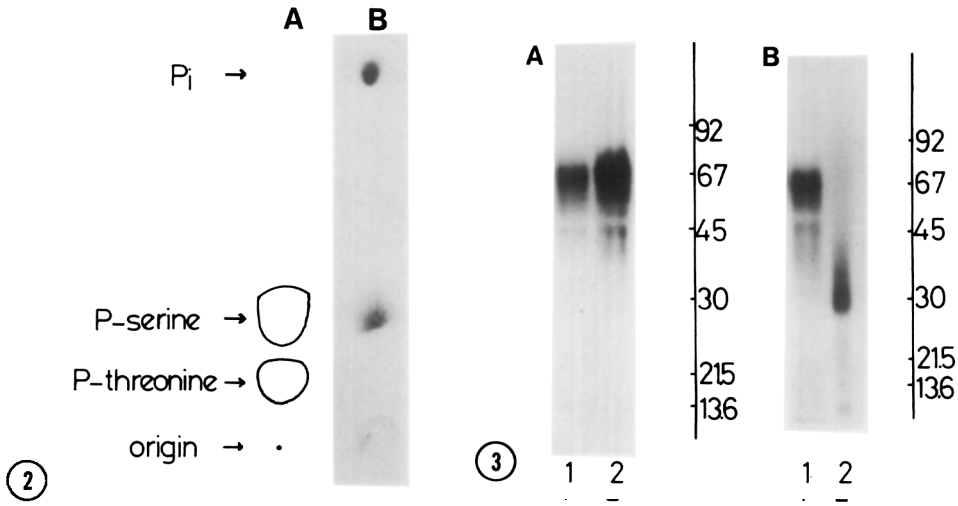

Figure 2 - Phosphoamino acid analysis of human fibrinogen phosphorylated by CK-1.
100 µg of fibrinogen phosphorylated by CK-1 was hidrolyzed and subjected to high voltage electrophoresis on cellulose thin-layer plates. (A) Location of marker phosphoamino acids and inorganic phosphate; (B) autoradiogram.

Figure 3 - Polyacrylamide gel electrophoresis of human fibrinogen phosphorylated by CK-1.
(A) Samples of fibrinogen incubated with [γ-^{32}P]ATP and CK-1 were subjected to SDS-gel electrophoresis and autoradiography. ^{32}P incorporation into fibrinogen was 1.4 mol/mol (line 1) and 3.6 mol/mol (line 2). (B) Phosphorylated fibrinogen (3.6 mol of ^{32}P/mol) was digested either with thrombin (line 1) or CNBr (line 2) and the digests analyzed by SDS-gel electrophoresis and autoradiography. Numbers to the right are the Mr in kilodaltons.

mount of ^{32}P migrating with the fibrinopeptides. Digestion of ^{32}P-fibrinogen with CNBr lead to the appearence of a main phosphopeptide which migrates as having an apparent Mr of 30,000 and could be tentatively identified as the largest CNBr fragment derived from the α-chain (16). A minor phosphorylated CNBr-fragment with an apparent Mr<10,000, was also consistenly observed. Wether this fragment corresponds to the amino terminal region of the α-chain or represents another phosphorylation site is not known yet.

These data indicates the existence in the α-chains of fibrinogen of multiple sites phosphorylated by CK-1. Most of the phosphate would be located in the largest CNBr fragment which has a high content of Ser residues, including Ser_{441} which is one of the sites phosphorylated "in vivo" (6).

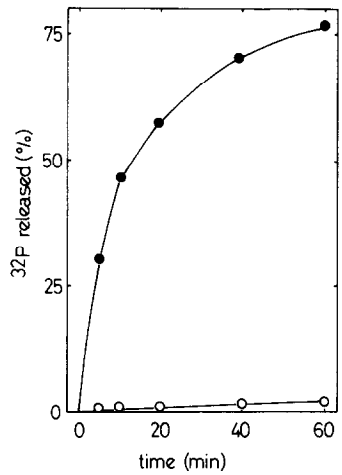

Figure 4 - Dephosphorylation of ^{32}P-fibrinogen. Fibrinogen (0.5 mg/ml) phosphorylated by CK-1 up to 1.9 mol ^{32}P/mol was incubated alone (○) or with alkaline phosphatase (50 units/ml) (●). At the indicated times samples were removed and assayed for the ^{32}P released, as in (17).

Incubation of ^{32}P-fibrinogen with alkaline phosphatase lead to a rapid release of ^{32}P from the phosphoprotein (Fig. 4) whereas no release of ^{32}P was observed in its absence. This result indicates that the phosphorylation of fibrinogen by CK-1 is reverted by this phosphatase.

Years ago, it was reported that the extent of phosphorylation of fibrinogen might affect its clotting time (18,19), but whether the phosphorylation ocurred in the liver or in the circulating blood was not known yet. The α-chains of fibrinogen from human blood are heterogeneous and only approximately one third of them are of the phosphorylated α(AP) type. It has been pointed out that heterogeneity could develop secondary to biosynthesis in the liver, being the α(AP) chain the primary product of the biosynthesis and the other types of α-chains corresponding to derivatives produced by exopeptidases and phosphatases of the blood (5). Recent studies on the phosphorylation of blood plasma proteins, including fibrinogen, have indicated that the protein kinases that might be operative "in vivo" in the phosphorylation of these proteins are not present in blood plasma (20). The data reported herein suggest that phosphorylation of fibrinogen by CK-1 could take place in liver prior to its secretion to circulating blood, although the contribution of other protein kinases to this process can not be disregarded.

ACKNOWLEDGMENTS

This work was supported by grant 0339/81 from the "Comisión Asesora de Investigación Científica y Técnica" of M.E.C. Spain.

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