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The cyclopentapeptide plactin enhances cellular binding and autoactivation of the serine protease plasma hyaluronan-binding protein

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ABSTRACT

Plactin, a family of cyclopentapeptides of fungal origin, enhances fibrinolytic activity by promoting of single-chain urokinase-type plasminogen activator (scu-PA) activation on the cell surface. For this activity, factor(s) in the blood plasma is absolutely required. In the previous studies, we identified prothrombin as a plasma cofactor involved in this mechanism, while the presence of another independent cofactor was suggested. The objective of this study was to identify the second cofactor and investigate the mechanism involved. Using plactin-affinity and ion-exchange chromatographies, we purified plasma hyaluronan-binding protein (PHBP) ~4,000-fold from human plasma as an independent plactin cofactor. PHBP, at ~10 nM, was effective in plactin-dependent promotion of scu-PA activation by U937 cells. PHBP is a serine protease that is produced as a single-chain proenzyme (pro-PHBP) and autoproteolytically converted to an active two-chain form. Pro-PHBP was comparable to PHBP in activity to promote plactin-dependent scu-PA activation by U937 cells. Plactin enhanced both cellular binding and autoproteolytic activation of pro-PHBP. The two activities were obtained with a plactin concentration at ~30 μM, which resulted in a significant increase in intrinsic fluorescence and self association of pro-PHBP. Thus, it is suggested that such changes account for both enhanced cellular binding and autoactivation of pro-PHBP, resulting in an enhancement of scu-PA activation.

Introduction

Plactin is a family of cyclic pentapeptides isolated from a fungus belonging to Agonomycetales (mycelial imperfect fungi that do not produce spores) in the screening for a small molecule that enhances cellular fibrinolytic activity [1]. As well as its activity in the cultured cell system, plactin enhances fibrinolytic activity in the lungs of mice after intravenous injection [2]. The plactin action *in vitro* involves an increase in cellular urokinase-type plasminogen activator (u-PA) activity [2]. In this mechanism, the presence of plasma is absolutely required [1,2]. In the previous studies, we identified prothrombin as a plactin cofactor in plasma from chromatography on plactin-coupled affinity matrix [3]. The plactin action on prothrombin is to modulate its proteolytic activation. Plactin inhibits prothrombin activation by membrane-associated processes, especially physiological coagulation catalyzed by prothrombinase complex (factor Xa assembled with factor Va on phospholipid membranes in the presence of Ca^{2+}) [3]. Therefore, plactin inhibits physiological coagulation. On the contrary, plactin enhances prothrombin activation by membrane-free Xa, affording α-thrombin, which can cleave single-chain u-PA (scu-PA) to form an inactive two-chain species, which, in turn, is processed to active two-chain u-PA (tcu-PA) by dipeptidyl peptidase I-like activity of U937 cells. Thus, an implication of the dual modulation of prothrombin activation in anticoagulant and profibrinolytic activities of plactin is proposed [3].

In addition to prothrombin, the presence of another independent cofactor in plasma is suggested by the plactin-affinity chromatography [3]. The objective of this study was to identify the second cofactor and investigate the mechanism involved. After the plactin-affinity chromatography, the second cofactor was purified through repeated ion-exchange chromatography. The cofactor was identified as plasma hyaluronan-binding protein (PHBP) from its amino acid sequence. PHBP, also known as factor VII activating protease or plasma hyaluronan binding serine protease, is a serine protease that can proteolytically activate scu-PA and the coagulation factor VII [4–6]. In plasma, PHBP is present as a single-chain 70-kDa zymogen (pro-PHBP) at a concentration of 12 μg mL^{-1} (170 nM) [7]. Pro-PHBP is proteolytically converted to an active two-chain form, which consists of the 50-kDa A-chain (Phe^{1}-Arg^{290}) and the 30-kDa B-chain (Ile^{291}-Phe^{537}) that are held together through a disulfide bond (numbering based on the human sequence). Pro-PHBP activation is catalyzed by

Abbreviations: u-PA, urokinase-type plasminogen activator; scu-PA, single-chain u-PA; tcu-PA, two-chain u-PA; PHBP, plasma hyaluronan-binding protein; pro-PHBP, proenzyme form of PHBP; FUT-175, 6-amidino-2-naphthyl-4-guanidinobenzoate dimethanesulfonate; SDS-PAGE, SDS-polyacrylamide gel electrophoresis.

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PHBP itself (autoactivation), and no other physiologically relevant protease that activates pro-PHBP has been found yet [8]. The autoactivation is promoted both by negatively charged substances (such as heparin and RNA) and polyamines [8–12]. Some of these molecules are postulated to serve as potential pathophysiologic modulators of pro-PHBP activation [10,12,13].

We describe in this paper the purification of PHBP as a second plactin cofactor and show that plactin enhances cell-binding and autoactivation of pro-PHBP, leading to activation of scu-PA. Since PHBP predominantly exists as the zymogen pro-PHBP in plasma, this mechanism may partly explain how plactin enhances fibrinolytic activity *in vivo* [2].

Materials and methods

Materials

Plactin D [*cyclo*(-D-Val-L-Leu-D-Leu-L-Phe-D-Arg-)], plactin14 [*cyclo*(-D-Val-L-Lys-D-Leu-L-Phe-D-Arg-)], and a plactin-affinity matrix (plactin-14 coupled with CNBr-activated Sepharose 4B) were prepared as described previously [2,3]. For assays, plactin D dissolved in dimethy sulfoxide was used at the solvent concentration of 1% (vol vol^{-1}). Pro-PHBP was isolated from human plasma as described by Etscheid *et al.* [8] to an apparent homogeneity. The purified pro-PHBP preparation was stored in 6 M urea, in which pro-PHBP autoactivation/degradation was completely inhibited. The preparation was diluted with appropriate buffers just before assays. Active form of PHBP was prepared from pro-PHBP [12]. Human scu-PA was kindly provided by Mitsubishi Tanabe Pharma (Osaka, Japan). Glycosaminoglycans were gifts from Dr. Yoshihiro Nomura, Tokyo Noko University. 6-Amidino-2-naphthyl-4-guanidinobenzoate dimethanesulfonate (FUT-175), a serine protease inhibitor that effectively inhibited PHBP, was donated by Torii Pharmaceutical (Tokyo, Japan). Other materials were from the following sources: unfractionated heparin from Sigma (St. Louis, MO); glutaryl-Gly-Arg-4-methylcoumarin-7-amide from Peptide Institute (Osaka, Japan); Spectrozyme TH, Spectrofluor TH, and Spectrozyme UK from American Diagnostica (Greenwich, CT); $Na^{125}I$ from Perkin Elmer (Boston, MA). Human monocytoid line U937 cells were cultured as described previously [3].

Buffers used were: buffer A, 20 mM sodium phosphate, pH 7.4, and 150 mM NaCl; buffer B, 50 mM Tris-HCl, pH 7.4, and 100 mM NaCl; buffer C, 50 mM sodium phosphate, pH 7.4; buffer D, 50 mM Tris-HCl, pH 7.4; buffer E, 50 mM Tris-HCl, pH 8.3, 75 mM NaCl, 5 mM $CaCl_2$, and 0.05% (wt vol^{-1}) Tween 20; buffer F, 250 mM Tris-HCl, pH 6.8, 8% (wt vol^{-1}) SDS, 20% (vol vol^{-1}) 2-mercaptoethanol, 40% (wt vol^{-1}) glycerol, and 0.008% (wt vol^{-1}) bromophenol blue.

Assay for U937 cell-mediated scu-PA activation

U937 cells (5.0×10^6 mL^{-1}) were incubated in buffer A with human plasma or the cofactor preparation at 37 °C for 20 min in the absence or presence of plactin D. After washing with buffer B, cells were incubated at 22 °C for 1 h with buffer B containing 5 nM scu-PA and 0.1 mM glutaryl-Gly-Arg-4-methylcoumarin-7-amide, a fluorogenic peptide substrate for u-PA. After addition of acetic acid (10%, vol vol^{-1}) to stop the reaction, fluorescence of the supernatant was measured (excitation, 380 nm; emission, 480 nm).

In some experiments, U937 cell-mediated scu-PA activation was also determined as the proteolytic cleavage of ^{125}I-scu-PA. In this assay, ^{125}I-scu-PA (25 nM) was included in place of unlabeled scu-PA. After incubation for the indicated duration, aliquots of supernatant were resolved on reduced SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

Purification of plactin cofactor from plasma

The purification was achieved by affinity chromatography on plactin-14-coupled matrix and anion-exchange chromatography, tracing plactin-dependent activity to promote U937 cell-mediated scu-PA activation. Plactin-14 itself was an inactive analog of plactin D, while it became active after modification of its primary amino group (ε-amino group of the lysine residue), and the compound was immobilized through amino-coupling with Sepharose beads [3]. Human citrated plasma (100 mL) was applied to a plactin-14-Sepharose column (10×50 mm) equilibrated with buffer C. After washing, the column was developed with buffer C containing 0.5 M NaCl (20 mL), followed by 6 M guanidine-HCl (20 mL). Total of ten batches of the 6 M guanidine-HCl fraction were dialyzed against buffer A and treated with ammonium sulfate (75% saturation). Precipitates formed were dialyzed against buffer A before chromatography on Protein PAK G-DEAE (20×100 mm, Waters). The column was developed with a linear gradient of NaCl (from 0 to 1 M) in buffer D. Fractions containing cofactor activity were dialyzed against buffer C and re-chromatographed on Protein PAK G-DEAE. The final preparation was obtained from the third ion-exchange chromatography.

Affinity chromatography on hyaluronan-Sepharose and AC4-Sepharose

Operation was carried out at 4 °C using buffers containing 1 μg mL^{-1} FUT-175 to inhibit degradation PHBP/pro-PHBP. Human plasma (50 mL) was applied to hyaluronan-Sepharose or Sepharose beads coupled with the AC4 monoclonal anti-PHBP IgG (5 mL) [14]. From the hyaluronan-Sepharose, PHBP/pro-PHBP was eluted with 50 mL of 10 mM sodium phosphate, pH 7.0, containing 0.5 M NaCl and 0.1% (wt vol^{-1}) Nonidet-P40. The AC4-Sepharose was developed with 0.2 M Gly-HCl, pH 2.5, after washing with 50 mL of the same buffer and 10 mL of water. The Gly-HCl eluates were neutralized with 1 M Tris base. Before determination of plactin cofactor activity, each fraction was dialyzed against buffer A to remove FUT-175.

Assay for PHBP/pro-PHBP binding to U937 cells

U937 cells (5.0×10^6 mL^{-1}) were incubated with ^{125}I-PHBP or ^{125}I-pro-PHBP (concentrations given in each experiment) at 4 or 37 °C in 2 mg mL^{-1} bovine serum albumin in buffer A (buffer A/BSA) containing 1 μg mL^{-1} FUT-175. After incubation for 60 min or the duration where specified, an aliquot (40 μL) of the mixture was placed onto buffer A/BSA containing 20% (wt vol^{-1}) sucrose (300 μL) in a 0.5-mL polypropylene centrifuge tube. After centrifugation at 1,600 ×*g* for 5 min, the tip of the tube with cell pellet was amputated to count radioactivity [15]. Data were normalized by subtracting blank values obtained from incubations without cells.

Assay for pro-PHBP autoactivation

Pro-PHBP (20 nM) was incubated at 37 °C in buffer E in the absence or presence of plactin D. After incubation for the indicated durations, the mixture was diluted 10-fold with buffer E containing 0.1 mM Spectrozyme TH, a chromogenic substrate for PHBP. The change in absorbance at 405 nm was kinetically monitored at 37 °C. Alternatively, autoactivation was assayed by directly incubating pro-PHBP (10 nM) with 0.1 mM Spectrozyme TH in buffer E in the presence of the indicated concentrations of plactin D (*see* Fig. 4A, *inset*). Kinetic data were tentatively analyzed by plotting changes in absorbance at 405 nm versus t^2 to estimate autoactivation rate.

The status of activation cleavage was confirmed by incubating ^{125}I-pro-PHBP (20 nM) in the absence or presence of plactin D (30 μM) in buffer E at 37 °C. Reaction was stopped by mixing with 0.33 volume of buffer F at 100 °C. Aliquots were resolved on reduced SDS-PAGE. Alternatively, ^{125}I-pro-PHBP (150 nM) was incubated in 65% (vol vol^{-1})