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# Hepatocyte-derived fibrinogen-related protein-1 is associated with the fibrin matrix of a plasma clot

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**Abstract**

In order to study the multiple functions of fibrinogen and fibrin, we are investigating which proteins bind to the fibrin matrix of a plasma clot by using a proteomic approach. Extracts from washed plasma clots were analysed by 2-D gel electrophoresis. A relatively abundant spot was identified as hepatocyte-derived fibrinogen-related protein-1 (HFREP-1) by MALDI-TOF analysis, molecular mass (34 kDa), iso-electric point (p*I* 5.5) as well as by Western blot analysis. HFREP-1 in plasma almost completely bound to the fibrin matrix during clot formation. Several purified fibrinogen preparations proved to be contaminated with HFREP-1. It is concluded that HFREP-1 (also named hepassocin), a protein with liver cell growth regulatory properties, occurs in plasma and strongly associates with fibrin and possibly fibrinogen.


*Keywords:* Hepatocyte-derived fibrinogen-related protein-1; Hepassocin; Fibrinogen; Fibrin; Plasma clot; Hepatocellular carcinoma; Regulation of cell growth; Proteomics

Fibrin forms the matrix of a blood clot and is actively involved in various physiological processes by providing binding sites for several hemostatic factors, growth factors, cytokines and cellular receptors [1,2]. In order to improve our knowledge of the multiple functions of fibrin, we are investigating which proteins are bound to the fibrin matrix of a plasma clot by using a proteomic approach. This report focuses on hepatocyte-derived fibrinogen-related protein-1 (HFREP-1) [3], a relatively unknown protein, also named fibrinogen-like protein-1 or hepassocin, which has been found to be present in plasma and to associate non-covalently with the fibrin matrix of a plasma clot.

## Materials and methods

Pooled platelet-poor citrated plasma was prepared from twelve healthy donors from the local blood bank. Clots of 500 μl plasma were prepared by supplementing plasma with calcium chloride (20 mM, f.c.), aprotinin (100 KIU/ml, f.c.) and thrombin (1 NIH U/ml, f.c). After an incubation period of 1 h at room temperature the clots were extensively washed by permeating them overnight at 4 °C with about 10 ml Tris-buffered saline, containing 100 KIU/ml aprotinin. The clots were compacted by centrifugation, washed with water and extracted for 1 h at room temperature with rehydration buffer consisting of 7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 0.5% (v/v) IPG buffer and a trace of bromophenol blue. The extracts were treated with hydroxyethyldisulphide (DeStreak, Amersham Biosciences, Uppsala, Sweden) and analysed by two-dimensional (2-D) polyacrylamide gel electrophoresis following standard procedures using 11 cm IPG strips of pH 3–10 or pH 4–7 (Immobiline DryStrip, Amersham Biosciences) in the first dimension and precast gradient gels (4–12%, Criterion XT Bis–Tris Gels, Bio-Rad Laboratories, Hercules, CA, USA) in the second dimension. Coomassie brilliant blue-stained spots were excised, in-gel digested with trypsin (Trypsin Gold, Promega Corporation, Madison, WI, USA) and analysed by MALDI-TOF–MS using an Ultraflex TOF/TOF apparatus (Bruker–Daltonics, Bremen, Germany) as described earlier [4]. The peptide mass fingerprint spectra were analysed by

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using BioTools (Bruker–Daltonics) and searching the mass spectrometry protein sequence database (MSDB) with MASCOT Peptide Mass Fingerprint software (taxonomy: homo sapiens).

## Results

Fig. 1A shows the protein mixture extracted from a washed plasma clot after 2-D gel electrophoresis using a pH range of 4–7 in the first dimension. The spot indicated by an arrow was excised, digested with trypsin and analysed by mass spectrometry. Mascot analysis identified the spot as HFREP-1 precursor, a protein of 312 amino acids including a predicted signal peptide of 17 [3] or 22 [5] amino acids (significant Mowse scores of 165, 172, and 201 in three separate experiments). The molecular mass and the p*I* of the protein, as derived from its mobility during 2-D gel electrophoresis (Fig. 1), were 32.6 kDa and p*I* 5.5 (mean of 3 experiments), which corresponded well with calculated values of 34.0 kDa and p*I* 5.45, respectively, of HFREP-1 without a signal peptide of 22 amino acids (ProtParam tool on www.expasy.org). The identity of the protein was further studied by Western blot analysis of the 2-D gel using an antibody against HFREP-1. Fig. 1B shows a clear positive spot at the same position as the HFREP-1 spot in Fig. 1A, confirming the identity of the protein. Two minor spots were observed, one with a lower p*I* and one with a higher p*I*, suggesting that at least two additional isoforms of HFREP-1 exist.

In order to study the clot binding properties of HFREP-1 further, the concentration of HFREP-1 in plasma was compared with the HFREP-1 concentration in the supernatant of clotted plasma. The concentrations were compared on a Western blot of an SDS–polyacrylamide gel of varying amounts of immunoprecipitates of plasma and plasma clot supernatants dissolved in equal volumes. Fig. 2 shows that the intensity of the HFREP-1 band of the plasma clot supernatant in lane 2 (40 μl) was significantly lower than the intensity of the HFREP-1 band of plasma in lane 5 (5 μl), indicating that the HFREP-1 concentration in the clot supernatant was significantly lower than 5/40 (12.5%) of the original concentration in plasma. A faint band just above the HFREP-1 band was observed both in plasma and in the clot supernatant (in lane 2). This band might be ascribed to non-specific staining or represent a variant of HFREP-1. It was concluded that HFREP-1 in plasma is almost completely bound to the fibrin matrix upon the clotting of the plasma.

Different commercially available fibrinogen preparations were tested on the presence of HFREP-1 as a contaminant. To this end fibrinogen was clotted in wells of a microtiter plate, dried and incubated with antibodies against HFREP-1. Fig. 3 shows no response with a bovine fibrinogen preparation, possibly because the antibodies might not cross-react with bovine HFREP-1. By contrast, two different human fibrinogen preparations showed a significant dose-response, indicating that these preparations were contaminated with HFREP-1. This suggests that

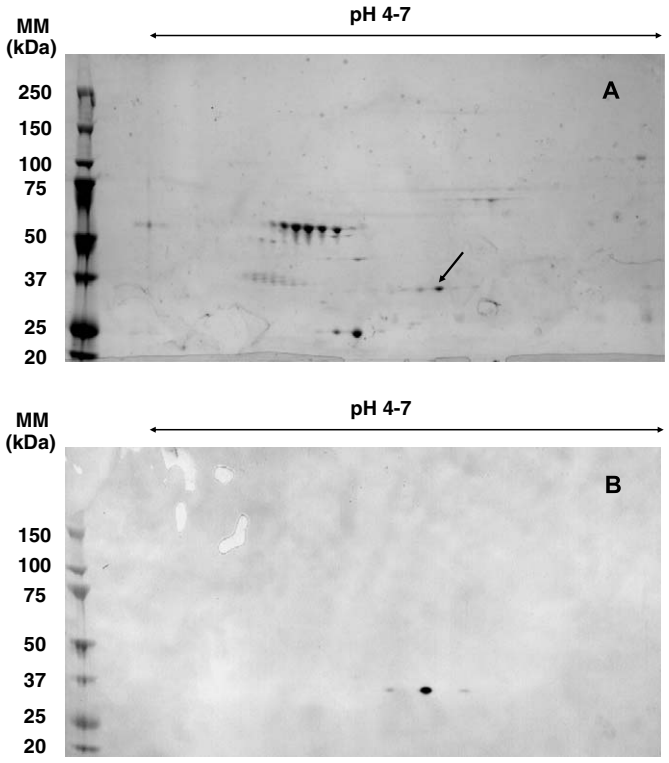


Fig. 1. Two-dimensional gel electrophoresis of proteins extracted from three extensively washed plasma clots of 500 μl. The molecular mass (MM) of a series of protein standards (Precision Plus Protein Standards, dual color, Bio-Rad) is indicated. The gels were either stained by colloidal Coomassie brilliant blue (A) or blotted and incubated with affinity-purified goat antibody against human HFREP-1/hepassocin (R&D Systems, Abingdon, UK), rabbit anti-goat immunoglobulins/HRP (DakoCytomation, Glostrup, Denmark) and BM blue stain (Roche, Basel, Switzerland), successively (B). The spot indicated by an arrow was excised and identified by mass spectrometry as HFREP-1.

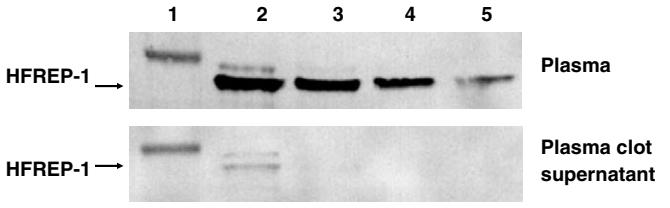


Fig. 2. SDS–polyacrylamide gel electrophoresis (4–12%, Criterion XT Bis–Tris Gels, Bio-Rad) of HFREP-1 in plasma (upper panel) and in the supernatant of clotted plasma (lower panel). The gel was blotted and stained with affinity-purified goat antibody against human HFREP-1/hepassocin (R&D Systems), rabbit anti-goat immunoglobulins/HRP (DakoCytomation) and BM blue (Roche).The plasma clot was prepared by supplementing plasma with calcium chloride (20 mM, f.c.), aprotinin (100 KIU/ml, f.c.) and thrombin (1 NIH U/ml, f.c) and incubating the mixture for 1 h at room temperature. The clot supernatant was obtained by centrifugation. HFREP-1 in plasma and clot supernatant was obtained by immunoprecipitation using the affinity purified goat anti-HFREP-1/hepassocin antibody and Protein G–Sepharose. Lane 1, 37 kDa protein standard; lane 2, 40 μl HFREP-1; lane 3, 20 μl HFREP-1; lane 4, 10 μl HFREP-1; lane 5, 5 μl HFREP-1. 100 μl HFREP-1 was isolated from 500 μl plasma or clot supernatant.

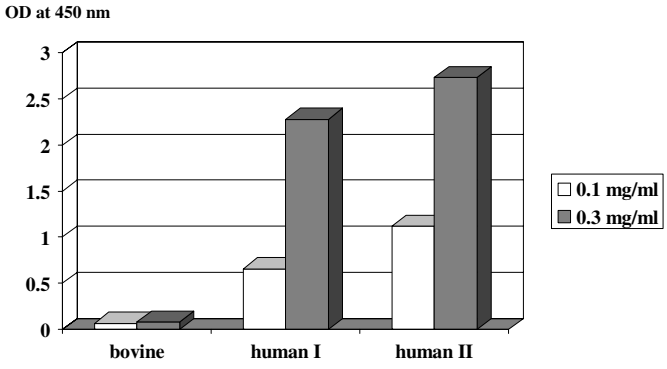


Fig. 3. Detection of HFREP-1 in different fibrinogen preparations. Dilutions of 0.1 or 0.3 mg/ml of plasminogen-free bovine fibrinogen (Povite, Organon Teknika, Boxtel, The Netherlands), human fibrinogen (Chromogenix, Molndal, Sweden) (human I) and human plasminogen-, von Willebrand- and fibronectin-free fibrinogen (Enzyme Research Laboratories, South Bend, IN, USA) (human II) were clotted with thrombin (0.1 NIH U/ml, f.c.) in the presence of calcium chloride (2 mM) in wells of a microtiter plate. The clots were dried overnight at 45 °C and incubated successively with affinity-purified goat antibody against human HFREP-1/hepassocin (R&D Systems), rabbit anti-goat immunoglobulins/HRP (DakoCytomation) and tetramethylbenzidine (TMB) substrate. The optical density (OD) was measured at 450 nm.

fibrinogen and HFREP-1 might interact during purification procedures.

## Discussion

HFREP-1 belongs to a fibrinogen superfamily that includes for instance fibrinogen, fibroleukin (fibrinogen-like protein-2), angiopoietin, angiopoetin-related protein, tenascin, and ficolin [6]. The members share a domain at their carboxy-termini, which is homologous to the carboxy-terminal two-thirds (about 270 amino acids) of the beta- and gamma chains of fibrinogen [7]. HFREP-1 has a domain with 41.1% and 41.3% homology to these parts of the beta- and gamma chains of fibrinogen, respectively [3].

The literature about HFREP-1 is limited [3,5,8–10]. The recombinant protein is a homodimer (68 kDa) with a disulphide bridge between the two subunits of each 34 kDa [5]. HFREP-1 lacks the thrombin-sensitive site of the beta chain of fibrinogen as well as the platelet-binding site and the cross-linking site in the carboxy-terminus of the gamma chain of fibrinogen, and is therefore considered to be a protein with biological functions outside coagulation [3]. HFREP-1 was originally detected by subtractive and differential cDNA cloning as a liver-specific protein that was markedly over-expressed in a hepatocellular carcinoma [3]. A more recent study, however, showed that HFREP-1 is frequently down-regulated in hepatocellular carcinoma and might possess growth suppressor activity [10]. The protein was also discovered as hepassocin in rat [8] and human [5] liver. Hepassocin showed mitogenic activity on isolated hepatocytes and was strongly up-regulated during liver regeneration. Up-regulation of the protein during liver regeneration after partial hepatectomy was confirmed in the mouse system [9], but the exact action of HFREP-1 during cell growth remains to be established.

We demonstrated for the first time that HFREP-1 occurs in plasma. The finding that HFREP-1 is associated with a plasma clot may be ascribed to the direct binding of HFREP-1 to the fibrin matrix. This could be explained if the polymerisation pocket in the beta or gamma chain in the D domain of fibrin monomer, which interacts with the complementary polymerisation site in the beta or alpha chain, respectively, in the E domain of a second fibrin monomer [11], were to be sufficiently conserved in HFREP-1. In that case, HFREP-1 might not only associate with fibrin, but also interfere with the polymerisation of fibrin. Therefore, the association of HFREP-1 with fibrin, as observed in this study, demands an experimental evaluation of the haemostatic properties of HFREP-1.

The presence of HFREP-1 in highly purified fibrinogen preparations suggests, however, that HFREP-1 may not only bind to fibrin, but also to fibrinogen. The association of HFREP-1 with fibrin and fibrinogen might localise and possibly regulate the growth regulatory activities of HFREP-1. Other growth factors that bind to fibrinogen and fibrin are basic fibroblast growth factor (bFGF) [12] and vascular endothelial growth factor (VEGF) [13]. These growth factors have multiple functions, including the stimulation of angiogenesis. Fibrin binding of bFGF and VEGF may play a coordinating role in angiogenesis [12,13]. It has been suggested that HFREP-1 acts specifically on liver cells [5], but the association of HFREP-1 with fibrin prompts the further study of potential target cells, including vascular endothelial cells.

Finally, our study shows that a proteomic approach to studying the composition of a plasma clot represents a powerful tool for identifying proteins that are bound to the fibrin matrix and may improve our insight into the multiple functions of fibrin.

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