Biochemical and Biophysical Research Communications 427 (2012) 421–425

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Binding of carboxypeptidase N to fibrinogen and fibrin

S. Talens [a], J.H.G. Lebbink [b,c], J.J.M.C. Malfliet [a], J.A.A. Demmers [d], S. Uitte de Willige [a], F.W.G. Leebeek [a], D.C. Rijken [a,*]

[a] Department of Hematology, Erasmus Medical Center, Rotterdam, The Netherlands
[b] Department of Cell Biology and Genetics, Erasmus Medical Center, Rotterdam, The Netherlands
[c] Department of Radiation Oncology, Erasmus Medical Center, Rotterdam, The Netherlands
[d] Proteomics Center, Erasmus Medical Center, Rotterdam, The Netherlands

ARTICLE INFO

Article history:
Received 11 September 2012
Available online 21 September 2012

Keywords:
Carboxypeptidase N
Coagulation
Fibrinogen
Fibrinolysis

ABSTRACT

The ultimate step in the blood coagulation cascade is the formation of fibrin. Several proteins are known to bind to fibrin and may thereby change clot properties or clot function. Our previous studies identified carboxypeptidase N (CPN) as a novel plasma clot component. CPN cleaves C-terminal lysine and arginine residues from several proteins. The activity of CPN is increased upon its proteolysis by several proteases. The aim of this study is to investigate the presence of CPN in a plasma clot in more detail. Plasma clots were formed by adding thrombin, $CaCl_2$ and aprotinin to citrated plasma. Unbound proteins were washed away and non-covalently bound proteins were extracted and analyzed with 2D gel electrophoresis and mass spectrometry. The identification of CPN as a fibrin clot-bound protein was verified using Western blotting. Clot-bound CPN consisted of the same molecular forms as CPN in plasma and its content was approximately 30 ng/ml plasma clot. Using surface plasmon resonance we showed that CPN can bind to fibrinogen as well as to fibrin. In conclusion, CPN binds to fibrinogen and is present in a fibrin clot prepared from plasma. Because CPN binds to a fibrin clot, there could be a possible role for CPN as a fibrinolysis inhibitor.

1. Introduction

The final step in blood coagulation is the conversion of fibrinogen into fibrin monomers by the proteolytic cleavage of fibrinogen by thrombin. Polymerization of fibrin monomers and cross-linking by activated factor XIII result in a stable fibrin network [1,2]. Several proteins can bind to fibrinogen and/or fibrin and can thereby influence clot formation, clot structure and function as well as clot degradation [3]. The fibrin clot is degraded by the fibrinolytic system and the main enzyme of the fibrinolytic system is plasmin, which is formed from plasminogen after cleavage by tissue-type plasminogen activator (t-PA). New C-terminal lysine residues are generated by partial degradation of fibrin by plasmin. These lysine residues form new binding sites for plasminogen and t-PA, which results in increased plasmin generation. This positive feedback loop of plasminogen activation is inhibited by activated thrombin activatable fibrinolysis inhibitor (TAFIa). TAFI is a procarboxypeptidase B of the zinc metallocarboxypeptidase family and TAFIa cleaves C-terminal lysine and arginine residues from partially degraded fibrin, thereby removing binding sites for plasminogen and t-PA [4].

Carboxypeptidase N (CPN) belongs to the same family of zinc metallocarboxypeptidases as TAFI and was discovered as an enzyme that cleaves C-terminal arginine residues from bradykinin; it was therefore originally called kininase I [5]. CPN can also inactivate the complement anaphylatoxins C3a, C4a and C5a [6]. CPN exists as a tetramer with a mass of about 280 kDa, consisting of two heterodimers each with a catalytic subunit (CPN1) and a regulatory subunit (CPN2). The catalytic subunit is 55 kDa in its native form, but is *in vivo* proteolytically cleaved at the C-terminus to form a 48 kDa subunit. The two forms are present in blood in a 1:1 ratio [7]. The regulatory subunit of 83 kDa is not active, but stabilizes the protein and inhibits the clearance of the catalytic subunit from the circulation. CPN is sensitive to proteolysis by plasmin, trypsin and plasma or urinary kallikrein. Proteolysis of CPN finally results in a more active protein [8]. Hydrolysis of the 83 kDa regulatory subunit into a 72 and 13 kDa fragment results in the dissociation of the tetramer into two heterodimers. The hydrolysis of the catalytic subunits yields fragments of 27 and 21 kDa with increased CPN activity [9].

Abbreviations: t-PA, tissue-type plasminogen activator; TAFI, thrombin activatable fibrinolysis inhibitor; CPN, carboxypeptidase N; BSA, bovine serum albumin.

* Corresponding author. Address: Erasmus University Medical Center Rotterdam, Department of Hematology, Room Ee 1393, Dr. Molewaterplein 50, 3015 GE Rotterdam, The Netherlands. Fax: +31 10 70 44 745.
E-mail address: d.rijken@erasmusmc.nl (D.C. Rijken).

0006-291X/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2012.09.081

In a previous study we identified CPN as a plasma clot component [10] with possible antifibrinolytic properties [11]. However, detailed information about the presence of CPN in the plasma clot could not be derived from that study. Therefore the aim of this study was to investigate the binding of CPN to a fibrin clot in more detail. We determined the amount of protein present in the plasma clot, that the protein is present in the forms that are also present in plasma and that CPN is able to bind directly to fibrin and fibrinogen.

2. Materials and methods

2.1. Materials

Human thrombin and albumin from bovine serum were obtained from Sigma–Aldrich (St. Louis, MO, USA). Aprotinin (Trasylol) was from Bayer (Leverkusen, Germany). Human fibrinogen (plasminogen, von Willebrand factor and fibronectin depleted) and human CPN purified from plasma were obtained from Enzyme Research Laboratories (South Bend, IN, USA). Bis–Tris (10% and 12%) Criterion XT precast gels and XT MOPS buffer were from Bio-Rad (Hercules, CA, USA). Sypro Ruby was obtained from Invitrogen (Paisley, UK) and 0.45 μm nitrocellulose transfer membrane from Whatman (Dassel, Germany). DeStreak, IPG buffer pH 3–10, immobiline strips, Ettan Spot Picker, IPGphore and Typhoon Trio apparatus were from GE Healthcare (Little Chalfont, Buckinghamshire, UK). The Biacore T100, CM5 sensor chips and amine coupling kit were obtained from GE Healthcare (Uppsala, Sweden). The rabbit polyclonal IgG to human CPN1 was obtained from Abbiotec (San Diego, CA, USA) and the mouse monoclonal IgG to human CPN2 was from Abfrontier (Seoul, Korea). The Odyssey apparatus and IRDye® 800CW secondary donkey-anti-mouse and donkey-anti-rabbit antibodies were obtained from Li-Cor Bioscience (Lincoln, NE, USA).

2.2. Plasma clot preparation

In vitro clots of 500 μl citrated platelet-poor plasma (from a pool of 10 healthy volunteers, Sanquin, Leiden, the Netherlands) were prepared by adding calcium chloride (final concentration 20 mM), thrombin (final concentration 1 NIH U/ml) and aprotinin (final concentration 100 KIU/ml) [12]. After 2 h of incubation at room temperature, the clots were extensively washed by perfusing them with 10 ml Tris-buffered saline (50 mM Tris–HCl, 100 mM

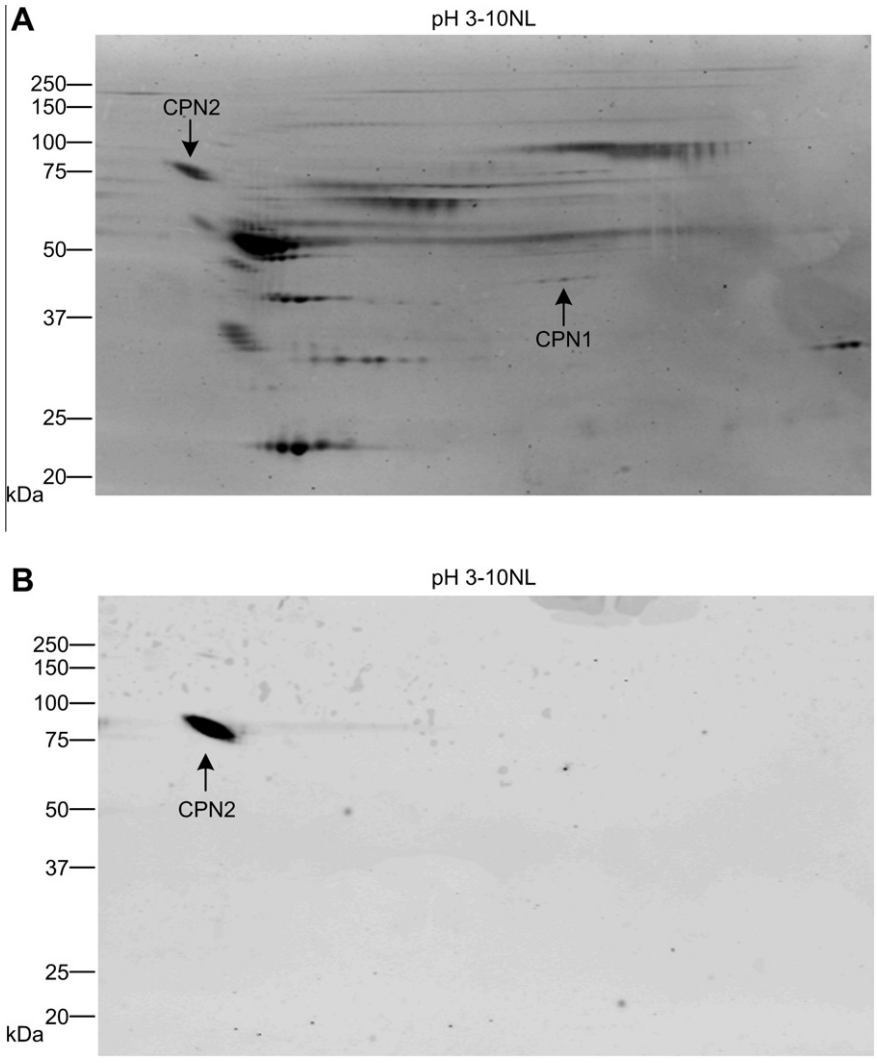

Fig. 1. An overview of plasma clot components. Plasma clots were made by adding $CaCl_2$, thrombin and aprotinin to platelet-poor citrated normal plasma, unbound proteins were washed away and bound proteins were extracted. (A) Proteins in the clot extract were separated with 2D gel electrophoresis and visualized by staining with Sypro Ruby. CPN1 and CPN2 were identified using mass spectrometry and are indicated by arrows. (B) Western blot analysis of a 2D gel using specific CPN antibodies. CPN2 is indicated by an arrow. CPN1 was not detected on this Western blot using a specific antibody to CPN1.

NaCl, pH 7.4) containing aprotinin (100 KIU/ml) at 4 °C. The clots were compacted by centrifugation, washed with deionized water and non-covalently clot-bound proteins were extracted for 2D gel electrophoresis with 150 μl rehydration buffer (7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 0.5% (v/v) IPG 3–10 buffer) for 1 h at room temperature. For optimal 2D gel electrophoresis, 1% (v/v) DeStreak was added to the extract. The non-covalently clot-bound proteins were extracted for SDS–PAGE with sample buffer (11% (v/v) glycerol, 3% (w/v) SDS, 50 mM Tris–HCl, pH 8.5) for 1 h at room temperature. To reduce samples, 5% (v/v) β-mercaptoethanol was added to the sample buffer when indicated.

2.3. 2D gel electrophoresis and mass spectrometry

Plasma clot extract was analyzed with 2D gel electrophoresis. The proteins in the 150 μl extract were separated in the first dimension using a 11 cm immobiline drystrip with a 3–10NL pH range by isoelectric focusing on the IPGphor with the following running protocol: 30 V for 12 h (rehydration), 1000 V for 4 h (gradient), 8000 V for 5 h (step-n-hold), with a 50 μA limit per gel. After isoelectric focusing, the gel strip was equilibrated in buffer (6 M urea, 50 mM Tris–HCl pH 8.8, 20% (v/v) glycerol, 2% (w/v) SDS) with 1% (w/v) DTT for 15 min followed by a second equilibration step with equilibration buffer containing 1% (w/v) iodoacetamide for 15 min. For the second dimension the gel strip was placed on a 12% Bis–Tris gel and run for 1 h at 200 V constant, using XT MOPS buffer as running buffer. The proteins in the gel were visualized by Sypro Ruby staining according to the manufacturer's instructions and scanned on a Typhoon Trio at an excitation wavelength of 532 nm and an emission wavelength of 610 nm. Protein spots were excised from gel and analyzed using nanoflow LC–MS/MS as described before [10].

2.4. Western blot analysis

To confirm the identification of CPN as a plasma clot component, a clot extract was made and analyzed by 2D gel electrophoresis as described above. The proteins were transferred from a 12% Bis–Tris precast gel to a nitrocellulose membrane by blotting at 100 V constant for 1 h. The membrane was incubated with block buffer (PBS, 1% BSA, pH 7.4). CPN1 was analyzed by incubating the blot after the blocking step with the rabbit polyclonal CPN1 antibody (1 μg/ml) and CPN2 was analyzed with the mouse monoclonal CPN2 antibody (1:2000), all diluted in 5% (w/v) milk and 0.1% (v/v) Tween 20 in PBS, pH7.4 and incubated overnight at 4 °C. The membrane was then incubated for 1 h at room temperature with the secondary antibodies IRDye® 800CW donkey-anti-mouse and IRDye® 800CW donkey-anti-rabbit diluted 10,000 times in 5% (w/v) milk and 0.1% (v/v) Tween 20 in PBS, pH 7.4. To visualize the proteins, the membrane was scanned on an Odyssey scanner.

To investigate further which CPN subunits or fragments were bound to a washed plasma clot and the amount of protein present, a plasma clot extract was made as described above. CPN1 was analyzed by SDS–PAGE using a 12.5% Tris–HCl gel at 0.02 Å constant. CPN2 was analyzed using a 10% Criterion XT Bis–Tris gel with MOPS running buffer at 150 V constant. The proteins were transferred from the gels to a nitrocellulose membrane by blotting at 100 V constant for 1 h and CPN1 and CPN2 were analyzed as described above.

2.5. Surface plasmon resonance

Binding of CPN to fibrinogen and fibrin was analyzed using a Biacore T100. Fibrinogen, additionally purified using Superdex 200, or bovine serum albumin (BSA) in 10 mM sodium acetate pH 4.0, was immobilized on a CM5 sensor chip using the amine coupling kit according to manufacturer's instructions. Approximately 10,000 resonance units (RU) of fibrinogen and BSA were covalently attached to the chip. Immobilized fibrinogen on one flow cell was converted to fibrin monomer by treatment with thrombin (1 U/ml) in running buffer (20 mM HEPES buffer, pH 7.4 with 150 mM NaCl, 2.5 mM $CaCl_2$ and 0.1% (v/v) Tween 20) for 20 min at 2 μl/min. Thrombin was removed by injecting 2 M NaCl for 60 s at 30 μl/min. Binding experiments were performed in running buffer at a flow rate of 30 μl/min and 25 °C. CPN (0–450 nM) in running buffer was injected for 11 min and the dissociation was monitored for 10 min. BSA (100 nM) in running buffer was tested as a control. To regenerate the surface, 0.5 M potassium thiocyanate (KSCN) was injected twice for 60 s. The data was visualized using Biacore T100 evaluation software (version 2.0.3). Before data analysis, all data were corrected for the response obtained from the flow cell with immobilized BSA (which did not exceed 21% of the uncorrected response of CPN) and for buffer injection. Due to high response levels and non-steady-state behavior, data were not analyzed quantitatively.

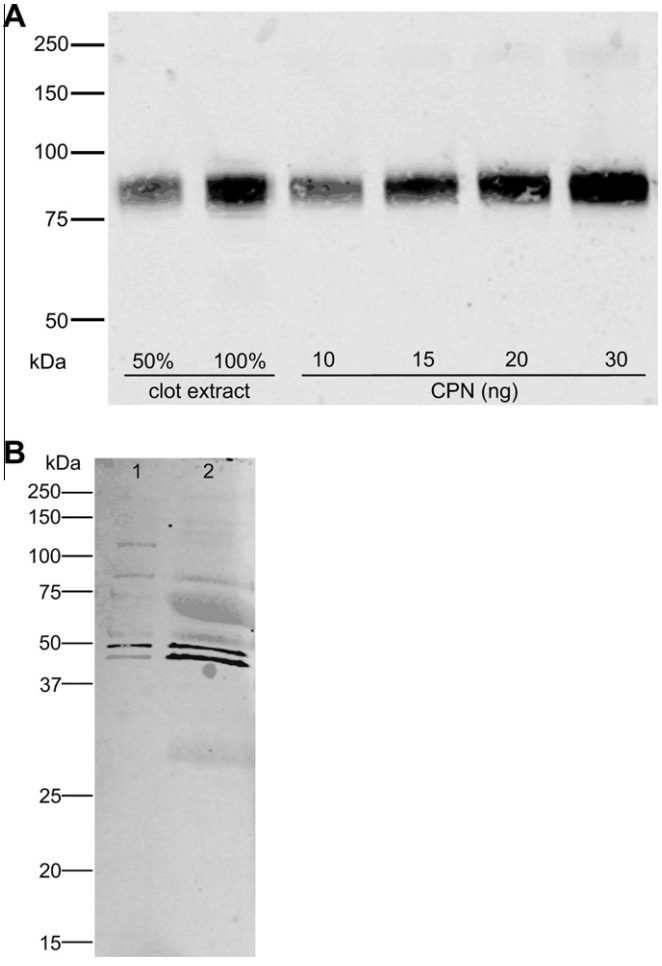

Fig. 2. SDS–PAGE and Western blot analysis of CPN. Plasma clots were made by adding $CaCl_2$, thrombin and aprotinin to platelet-poor citrated normal plasma, unbound proteins were washed away and bound proteins were extracted. (A) The clot extract (50% and 100%) and different amounts of purified CPN (10–30 ng) were analyzed with SDS–PAGE and Western blot analysis using a specific CPN2 antibody. A calibration curve was made from the different purified CPN concentrations and the amount of CPN in the plasma clot extract was calculated using the calibration curve. (B) The plasma clot extract (1) and 100 ng purified CPN (2) were analyzed by using SDS–PAGE and Western blot with a specific CPN1 antibody.

3. Results and discussion

In line with previous results [10,13], 2D gel electrophoresis and mass spectrometry analysis revealed that CPN1 and CPN2 were present in a washed fibrin clot made from plasma (Fig. 1A). The apparent molecular masses on the 2D gel were comparable to what was published for CPN1 (55 kDa) and CPN2 (83 kDa). The theoretical isoelectric point (pI) of CPN1 is around 6.9 and of CPN2 around 5.5 (www.expasy.org). The apparent pI on the 2D gel of CPN2 differed from the theoretical pI, probably due to glycosylation of the protein. We confirmed by Western blot analysis that the protein identified with mass spectrometry was CPN2 (Fig. 1B). The protein spot identified as CPN1 was flanked by at least two other proteins spots, which most probably were post-translationally modified CPN1 subunits. Unfortunately we could not detect CPN1 by Western blot analysis, possibly due to the low amount of protein on the 2D gel in combination with a low affinity antibody.

Both CPN1 and CPN2 are sensitive to proteolytic cleavage by e.g. plasmin which results in a more active enzyme when the catalytic subunit of 48 kDa is cleaved into fragments of 27 and 21 kDa [9]. We investigated which forms of CPN are present in a fibrin clot using mass spectrometry data [10] and Western blot analysis. The peptides obtained from mass spectrometry analysis that were matched to the CPN2 sequence included the peptides Ser^{479}-Arg^{500} and Trp^{501}-Arg^{509} in the C-terminal region of the protein located after the plasmin cleave site at the arginine residue on position 478 [9], suggesting that the intact CPN2 subunit is present in the plasma clot. In addition, SDS–PAGE, in combination with Western blot analysis using a specific CPN2 antibody, showed only one broad band of the highly glycosylated CPN2 and did not show the cleaved subunit of around 73 kDa (Fig. 2A). Using SDS–PAGE followed by Western blot analysis with a specific CPN1 antibody, the presence of CPN1 in a plasma clot was confirmed (Fig. 2B). In addition, both the intact CPN1 subunit of 55 kDa and the cleaved 48 kDa subunit were present as plasma clot components. The cleaved fragments of 27 and 21 kDa were not detected, suggesting that the catalytic subunit present in a fibrin clot is not a further cleaved, more active enzyme. The cleaved CPN1 subunit of 48 kDa is present in plasma in a 1:1 ratio with the intact CPN1 subunit of 55 kDa and this cleavage occurs most likely upon its excretion into the circulation directly after synthesis [7]. The formation of the 48 kDa subunit has no effect on the activity of the enzyme. Taken together, we concluded that the forms of CPN that are normally present in plasma were also found within the fibrin clot. We did not find any evidence of the presence of substantially cleaved CPN forms within fibrin clots.

The amount of CPN present in the plasma clot extract was estimated with Western blot analysis using the specific CPN2 antibody. Different amounts of purified CPN and two different concentrations of the plasma clot extract were run on SDS–PAGE and Western blot (Fig. 2A). The estimated amount of CPN per ml plasma clot was approximately 30 ng.

The binding of CPN to fibrinogen and fibrin was investigated using surface plasmon resonance. CPN solutions (0–450 nM) were run over immobilized fibrinogen and fibrin. Increasing concentrations of CPN showed increasing amounts of protein bound to the fibrinogen surface (Fig. 3). Similar results were found for a fibrin surface (data not shown). BSA (100 nM) did not bind to immobilized fibrinogen or fibrin (data not shown). These results imply that the binding of CPN to the fibrin clot is specific and does not require another plasma component. It was not possible to perform kinetic analysis because data could not be fitted according to standard binding models due to the high amounts of protein that were bound to the fibrinogen and fibrin immobilized on the chip. Although equilibrium binding was not reached with the CPN concentrations used, we estimated an apparent Kd for complexes with fibrinogen and fibrin around 100 nM.

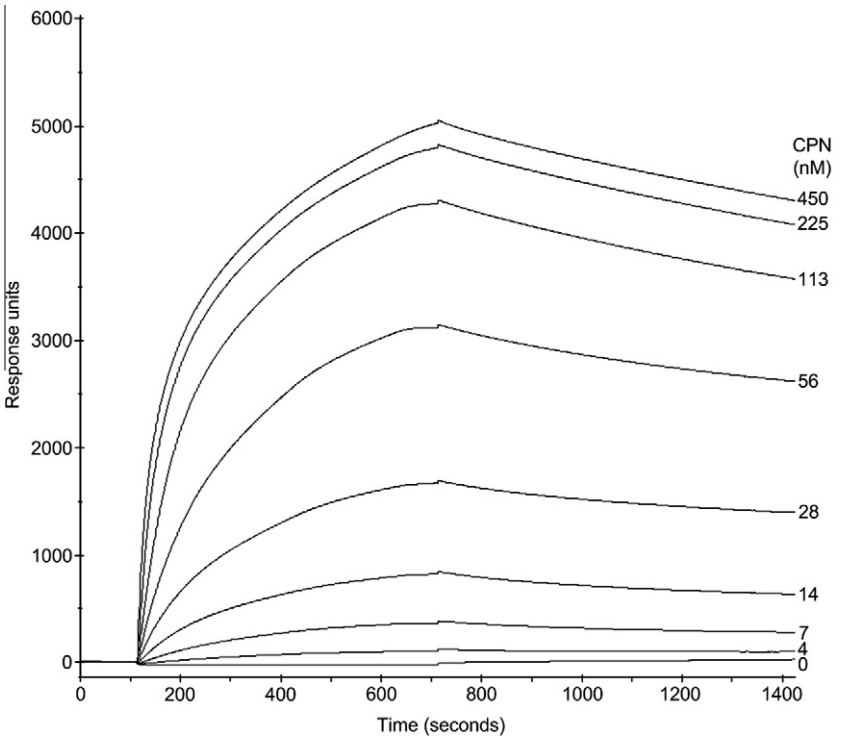

Fig. 3. Binding of CPN to fibrinogen. Surface plasmon resonance sensorgrams of CPN binding to fibrinogen obtained using Biacore T100. Different amounts of CPN (0–450 nM) were injected over a CM5 chip containing immobilized fibrinogen. Equilibrium analysis was performed using Biacore T100 evaluation software to estimate the apparent K_d (100 nM).

The physiological function of the binding of CPN to fibrinogen and a fibrin clot is not yet clear. CPN is known to inactivate bradykinin and the complement anaphylatoxins C3a, C4a and C5a [5,6]. Two case reports suggest that a CPN deficiency causes angioedema. In these cases no predisposition for bleeding was reported [14,15]. CPN cleaves C-terminal lysines and arginines from similar target proteins as activated TAFI [16,17]. However, while TAFIa is a clear fibrinolysis inhibitor the role of CPN in the inhibition of fibrinolysis has not been fully established. Walker et al. [11] investigated the antifibrinolytic properties of CPN extensively. They concluded that CPN may be a long-term antifibrinolytic enzyme, in particular when it is proteolytically cleaved. Activated TAFI seems to be the main inhibitor of fibrinolysis but has a half-life of only 10 min [18]. Therefore CPN present in a fibrin clot may prolong the inhibition of fibrinolysis after TAFIa has been inactivated.

In conclusion, the data presented here suggests that the forms of CPN that are normally present in plasma bind to fibrinogen and fibrin and are present in a fibrin clot and may therefore play a role in the inhibition of fibrinolysis.

Acknowledgment

This research was supported by the Nederlandse Organisatie voor Wetenschappelijk Onderzoek (VIDI 700.58.428 to J.L. and NWO-groot).

References

[1] J.W. Weisel, Fibrinogen and fibrin, Adv. Protein Chem. 70 (2005) 247–299.
[2] S.T. Lord, Fibrinogen and fibrin: scaffold proteins in hemostasis, Curr. Opin. Hematol. 14 (2007) 236–241.
[3] A.S. Wolberg, Plasma and cellular contributions to fibrin network formation structure and stability, Haemophilia 16 (Suppl. 3) (2010) 7–12.
[4] D.C. Rijken, H.R. Lijnen, New insights into the molecular mechanisms of the fibrinolytic system, J. Thromb. Haemost. 7 (2009) 4–13.
[5] E.G. Erdos, E.M. Sloane, An enzyme in human blood plasma that inactivates bradykinin and kallidins, Biochem. Pharmacol. 11 (1962) 585–592.
[6] V.A. Bokisch, H.J. Muller-Eberhard, Anaphylatoxin inactivator of human plasma: its isolation and characterization as a carboxypeptidase, J. Clin. Invest. 49 (1970) 2427–2436.
[7] R.A. Skidgel, E.G. Erdos, Structure and function of human plasma carboxypeptidase N the anaphylatoxin inactivator, Int. Immunopharmacol. 7 (2007) 1888–1899.
[8] Y. Levin, R.A. Skidgel, E.G. Erdos, Isolation and characterization of the subunits of human plasma carboxypeptidase N (kininase i), Proc. Natl. Acad. Sci. USA 79 (1982) 4618–4622.
[9] M.O. Quagraine, F. Tan, H. Tamei, E.G. Erdos, R.A. Skidgel, Plasmin alters the activity and quaternary structure of human plasma carboxypeptidase N, Biochem. J. 388 (2005) 81–91.
[10] S. Talens, F.W. Leebeek, J.A. Demmers, D.C. Rijken, Identification of fibrin clot-bound plasma proteins, PLoS One 7 (2012) e41966.
[11] J.B. Walker, T.M. Binette, M. Mackova, G.R. Lambkin, L. Mitchell, L. Bajzar, Proteolytic cleavage of carboxypeptidase N markedly increases its antifibrinolytic activity, J. Thromb. Haemost. 6 (2008) 848–855.
[12] D.C. Rijken, S.P. Dirkx, T.M. Luider, F.W. Leebeek, Hepatocyte-derived fibrinogen-related protein-1 is associated with the fibrin matrix of a plasma clot, Biochem. Biophys. Res. Commun. 350 (2006) 191–194.
[13] J.M. Howes, V.R. Richardson, K.A. Smith, V. Schroeder, R. Somani, A. Shore, K. Hess, R. Ajjan, R.J. Pease, J.N. Keen, K.F. Standeven, A.M. Carter, Complement C3 is a novel plasma clot component with anti-fibrinolytic properties, Diab. Vasc. Dis. Res. 9 (2012) 216–225.
[14] J.L. Willemse, D. Chen, D.F. Hendriks, Major carboxypeptidase N deficiency, Clin. Chim. Acta 389 (2008) 181–182.
[15] K.P. Mathews, J.G. Curd, T.E. Hugli, Decreased synthesis of serum carboxypeptidase N (SCPN) in familial SCPN deficiency, J. Clin. Immunol. 6 (1986) 87–91.
[16] B.N. Bouma, P.F. Marx, L.O. Mosnier, J.C. Meijers, Thrombin-activatable fibrinolysis inhibitor (TAFI, plasma procarboxypeptidase B, procarboxypeptidase R, procarboxypeptidase U), Thromb. Res. 101 (2001) 329–354.
[17] E.F. Plow, K. Allampallam, A. Redlitz, The plasma carboxypeptidases and the regulation of the plasminogen system, Trends Cardiovasc. Med. 7 (1997) 71–75.
[18] J.B. Walker, L. Bajzar, The intrinsic threshold of the fibrinolytic system is modulated by basic carboxypeptidases but the magnitude of the antifibrinolytic effect of activated thrombin-activable fibrinolysis inhibitor is masked by its instability, J. Biol. Chem. 279 (2004) 27896–27904.