Identification of novel heparin-binding domains of vitronectin

Olin D. Liang[a], Sylvia Rosenblatt[b], Gursharan S. Chhatwal[a,*], Klaus T. Preissner[b]

[a]*GBF-National Research Center for Biotechnology/Technical University Braunschweig, Spielmannstrasse 7, D-38106 Braunschweig, Germany*
[b]*Haemostasis Research Unit, Kerckhoff-Klinik, Max-Planck-Institut, Sprudelhof 11, D-61231 Bad Nauheim, Germany*

Received 21 January 1997; revised version received 11 March 1997

Abstract Vitronectin is a multifunctional serum protein which provides a unique regulatory link between cell adhesion, humoral defense mechanism and the hemostatic system, and the heparin-binding properties of vitronectin are thought to have participated in various functional aspects. In addition to the carboxy-terminal glycosaminoglycan-binding motif, we report on two novel heparin-binding domains which were identified using phage display technique. One heparin-binding domain is located between amino acids Asp[82] and Cys[137] at the end of the connector region, while the other is in the second hemopexin-type repeat, between amino acids Lys[175] and Asp[219] of the vitronectin molecule. Our findings may shed new light to the activities of vitronectin and its binding to cells, which could not be explained solely on the basis of the known heparin-binding domain.

1. Introduction

Vitronectin is a multifunctional serum protein produced primarily in the liver and originally defined as S-protein or serum-spreading factor [1]. Vitronectin affects the humoral immune system by binding to and inhibiting the complement C5b-9 membrane attack complex [2]. As a major matrix-associated adhesive glycoprotein, it regulates blood coagulation on the basis of its ability to bind heparin, plasminogen, plasminogen activator inhibitor-1 and thrombin-inhibitor complexes [3,4]. Within vitronectin, distinct interactive sites for these components were identified, whose exposure may depend on the conformation of the vitronectin molecule. Studies on structural requirements for neutralization of heparin-like saccharides by vitronectin suggested that vitronectin might interact through its glycosaminoglycan binding domain(s) with various functional sites of the heparin molecule [5,6]. The phenomenon of heparin neutralization is interpreted primarily in terms of a direct interaction between the highly basic region of the vitronectin molecule comprised by amino acid residues Ala[341]–Arg[379] and various regions of the polysaccharide chain [6,7]. Evidence compatible with the participation of the proposed glycosaminoglycan binding domain of vitronectin is provided by the demonstration of heparin neutralization by a synthetic tridecapeptide (amino acids Lys[348]–Tyr[360]) [4,6]. However, the interaction of this synthetic peptide with heparin is weaker than that of the protein. In addition, while vitronectin can largely neutralize the anti-Factor Xa activity of the synthetic heparin antithrombin-binding pentasaccharide, the tridecapeptide is unable to function in this respect [6]. These findings point to amino acid sequence(s) of vitronectin other than that of Lys[348]–Tyr[360] playing a contributory role in heparin neutralization. In the present study, by using phage display technique, we have identified two novel heparin-binding domains of vitronectin.

2. Materials and methods

2.1. Construction of a vitronectin random epitope phage library

To generate random fragments encoding epitopes of the vitronectin molecule, a 1580 bp DNA fragment containing the full-length vitronectin cDNA [8] was obtained from plasmid pUCVNwt and partially digested with DNAase to produce random fragments of 50–100 bp. To facilitate the cloning and expression of random DNA fragments, we have chosen phagemid vector pComb3B, which contains two non-selfcomplementary *Bst*XI sites, separated by a 350 bp 'replaceable segment', that allow cloning of DNA fragments using *Bst*XI adaptors [9,10]. Once cloned in the correct orientation and translation reading frame, fragments were expressed as fusion proteins composed of an N-terminal *pelB* leader peptide, a fusion peptide F_N, the epitope peptide, a second fusion peptide F_C, a glycine-rich 'flexible' GGGGS peptide linker and the C-terminal half of the bacteriophage M13 gene III-encoded minor coat protein cpIII. Upon superinfection with a helper phage VSCM13 (Stratagene, Heidelberg, Germany), the epitope peptide fused to a part of cpIII were displayed on the surface of newly produced phagemid particles [11].

2.2. Panning of heparin-binding phagemid particles

One hundred microliters of heparin–agarose (pore size 0.01 μm) (Sigma, Deisenhofen, Germany) were washed 3 times with phosphate buffered saline (PBS) and resuspended in 100 μl of PBS containing 3% bovine serum albumin (BSA). The suspension was allowed to stand at room temperature for 1 h with gentle agitation. Fifty microliters of the vitronectin fragment library containing 4.5×10^{10} phagemid particles were mixed with 50 μl of BSA-blocked heparin–agarose in an Eppendorf tube, and the mixture was incubated at room temperature for 2 h with gentle agitation. The heparin–agarose beads and bound phagemid particles were then spun down using a conventional bench centrifuge at 3000 rpm for 3 min. The beads were washed 10 times using PBS containing 0.1% Tween 20 (PBST), and the final suspension was transferred into a new Eppendorf tube where the beads were sedimented by centrifugation and supernatant was discarded. Heparin-binding phagemid particles were eluted stepwise using 200 μl of respective buffer, first 0.3 M sodium acetate (pH 5.2), then 0.1 M glycine-HCl (pH 2.2). The eluates were immediately neutralized using 12 μl of 2 M Tris (pH 10). The eluted phagemid particles were then used to infect 100 μl of *E. coli* XL-1 Blue (Stratagene), which were subsequently spread onto Luria-Bertani agar plates containing 50 μg/ml carbenicilin and 10 μg/ml tetracyclin for overnight incubation at 37°C.

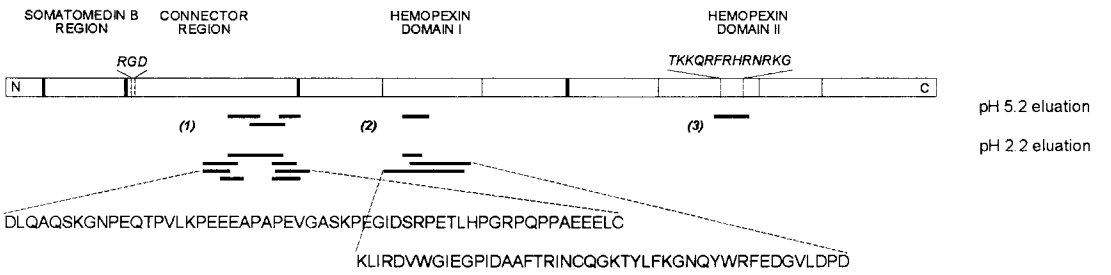
In preliminary experiments, 50 μl of the vitronectin phage library (containing 4.5×10^{10} phagemid particles) were added onto microtiter wells coated with 3% BSA. After 10 times washing with PBST, no phagemid particle was obtained using the elution steps described above. The possibility of vitronectin phage library binding to the blocking agent BSA was therefore excluded.

2.3. Sequencing of DNA inserts

Individual clones, harboring recombinant phagemids from the previous step, were picked and cultivated with gentle shaking at 37°C overnight in 5 ml of Luria-Bertani broth containing antibiotics as above. Phagemids containing a vitronectin cDNA fragment were purified according to the QIAprep Spin Plasmid Kit Protocol (QIAGEN, Hilden, Germany). The sequences of the inserts were deter-

*Corresponding author. Fax: (49) 531-391-5858.
E-mail: gsc@gbf-braunschweig.de

PII S0014-5793(97)00330-X



(1)

PVLKPEEEAPAPEVGAS ETLHPGRPQPPA
PEVGASKPEGIDSRPETLH

PVLKPEEEAPAPEVGASKPEGIDSRPETL
DLQAQSKGNPEQTPVLKPE DSRPETLHPGRPQP
DLQAQSKGNPEQTPV RPETLHPGRPQPPAEEELC
PEQTPVLKPEEEA DSRPETLHPGRPQPPAE

(2)

EGPIDAAFTRINCQ

EGPIDAAFTRI
DAAFTRINCQGKTYLFKGNQYWRFEDGVLDPD
KLIRDVWGIEGPIDAAFTRINCQGKTYLFKGNQYWRFEDGVL

(3)

PSLTKKQRFRHRNRKGYRS

Fig. 1. Alignment of vitronectin cDNA fragment-encoding peptides, which were displayed as cpIII fusion protein on the surface of M13 phages and bearing heparin-binding activity. Amino acid sequences Asp^{82}–Cys^{137}, Lys^{175}–Asp^{219}, and of all 15 phage-displayed vitronectin peptides are shown.

mined according to the ABI PRISM™ DYE Terminator Cycle Sequencing Ready Reaction Kit Protocol (PERKIN-ELMER), using an ABI automatic sequencer. The following two synthesized oligonucleotides were used as primers: 5′-GCCCAGGTGAAACTGCTCG-3′ and 5′-CAAACGAATGGAGAGCCACC-3′. A computer program 'Genework' was used to analyze the obtained sequences. Estimation of pI values of the displayed peptides encoded by a vitronectin cDNA fragment was made using the same computer program.

2.4. Microtiter plate binding assay

Three representative heparin-binding phagemid clones H24-2.2 (encoding vitronetin peptide Asp^{82}–Glu^{100}), H25-2.2 (encoding vitronectin peptide Asp^{117}–Pro^{130}) and H58-2.2 (encoding vitroenctin peptide Lys^{175}–Asp^{216}) were selected, and their corresponding phagemid particles were propagated and tested for their ability to bind to immobilized heparin. Briefly, 96-well microtiter plates were coated with heparin–albumin (Sigma) at a concentration of 20 μg/well in 100 μl of PBS at 4°C overnight. Additional binding sites were blocked using 3% BSA in PBST for 1 h at 37°C. A series of concentrations of phagemid particles in 100 μl were added and the plates were incubated for 1 h at 37°C. After six washes of the microtiter plates with PBST, bound phagemid particles were detected using horseradish peroxidase conjugated to sheep anti-M13 polyclonal antibodies (1:5000, Pharmacia Biotech). The plates were developed for 20 min with *o*-phenylenediamine dihydrochloride in 0.1 M citric acid-phosphate buffer (pH 5.0) and approximately 0.01% hydrogen peroxide as recommended by the manufacturer. The reaction was stopped using 50 μl of 1 M H_2SO_4, and the plates were then read at 490 nm with a Bio-Rad ELISA reader. Several repetition of duplicate determinations were made and averaged in all cases.

3. Results

3.1. Construction of a vitronectin fragment phage display library

A vitronectin random peptide library was constructed containing approximately 10^5 independent clones and having a titer of 9×10^{11} phagemid particle/ml. Fifty randomly picked clones were sequenced, revealing inserts in the designated range with equal participation of both orientations and a non-biased distribution of fragments throughout the vitronectin cDNA. Approximately one in 18 inserts expressed a vitronectin peptide (data not shown).

3.2. Affinity selection of heparin-binding phagemid particles

After stringent washing with PBST, bound phagemid par-

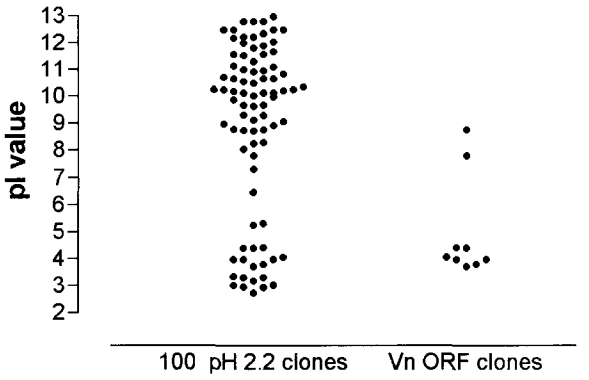


Fig. 2. Distribution of p*I* values of 100 heparin-binding peptides, selected from the vitronectin random peptide phage library by pH 2.2 elution. The computer-assisted estimation of p*I* values was based on the sequence of the vitronectin cDNA inserts. Vn ORF: vitronectin open reading frame.

ticles were eluted from the heparin–agarose stepwise by lowering the pH. After pH 5.2 elution, approximately 10^4 phagemid particles, reflected by carbenicilin resistant colonies, were obtained. Eighty recombinant phagemids were selected randomly and inserts were sequenced. By pH 2.2 elution, a total of 200 phagemids were obtained, and 160 of them were selected randomly and inserts were sequenced. In control experiments, where helper phages containing the phagemid vector pCom3b without a insert were used to pan against heparin–agarose beads, no carbenicilin resistant colony was obtained.

3.3. *Sequences of the inserts and their alignment against the vitronectin gene*

All of the 240 sequenced recombinant phagemids were found to have an insert. As clearly shown in Fig. 1, 10 inserts (3 from pH 5.2, and 7 from pH 2.2 elution), with correct orientation and open reading frame, clustered and many of them to some extent overlapped each other between amino acid Asp^{82} and Cys^{137} of the vitronectin molecule. Similarly, all four inserts (1 from pH 5.2, and 3 from pH 2.2) having correct orientation and open reading frame, clustered and overlapped each other between amino acid Lys^{175} and Asp^{219} of the vitronectin molecule. Furthermore, one insert from pH 5.2 elution aligned itself to the known heparin-binding domain of vitronectin, at the carboxy-terminus (Pro^{344}–Ser^{362}). Interestingly, the known heparin-binding region Ala^{341}–Arg^{379} seemed less represented in the experiments. However, the estimated p*I* values indicated that most of the heparin-binding peptides, encoded by a vitronectin cDNA fragment and displayed on the phage surface, were very basic in nature (Fig. 2).

3.4. *Binding of heparin-binding phagemid particles to immobilized heparin*

As shown in Fig. 3, phagemid particles from clones H24-2.2, H25-2.2 and H58-2.2 were able to bind to immobilized heparin in a dose-dependent manner. The phagemid particles from these three clones did not bind to microtiter wells coated with 3% BSA (data not shown).

4. Discussion

The phage display technique, introduced in 1985 by Smith [12], utilizes filamentous M13 phages to express foreign peptides or proteins in fusion with one of the coat proteins. During the past decade this technique has been developed into a powerful method to study protein–ligand interactions [13]. Epitope libraries have been constructed and used to identify specific amino acids involved in protein–protein interactions, such as the RGD-motif recognized by integrins [14], and epitopes in proteins recognized by antibodies [15]. Other large peptides that have been successfully displayed in the native conformation are the enzyme, alkaline phosphatase, and an immunogenic fragment of the HIV Gag protein [see reviews [13] and [16]], as well as plasminogen activator inhibitor-1 [17]. The so-called panning procedure in fact is an affinity purification of fusion protein, and the advantage of phage display cloning lies in the physical linkage of each protein to the genetic material that encodes it.

As a cell adhesion and spreading factor, vitronectin has been identified in the extracellular matrix and isolated from plasma and serum [see review [1]]. Its interactions with heparin and cellular receptors were documented by several studies emphasizing the importance of conformationally different isoforms [18]. The well-documented heparin-binding site is comprised of a cluster of basic residues between Arg^{341} and Arg^{379} [8]. More than 40% of the amino acids in this sequence are

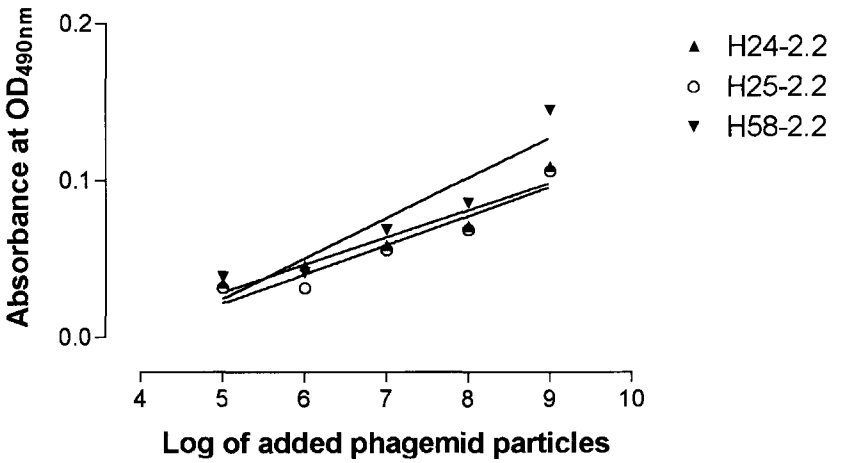


Fig. 3. Binding of heparin-binding phagemid particles to immobilized heparin. Background values of each phagemid particle concentration, estimated using helper phages containing phagemid vector pComb3B without insert, were subtracted.

basic, and only a few are hydrophobic, suggesting that this region has a loop structure, rather than pronounced α-helix or a β-sheet. Moreover, the core region contains two heparin-binding consensus sequences that allow tight interactions with complementary acidic sites of sulfated glycosaminoglycans [7]. It is worth noting that the known heparin-binding region Ala^{341}–Arg^{379} seemed less represented in our experiments. This could be explained by the fact that most heparin-binding peptides, displayed on the phage surface and selected by panning, were very basic in nature as judged by their pI values (Fig. 2). Subsequently, these basic peptides could compete with the peptides corresponding to the known heparin-binding domain. In contrast, no other sequences with clusters of basic amino acids are present in vitronectin. Thus, both regions Asp^{82}–Cys^{137} and Lys^{175}–Asp^{219}, where two novel heparin-binding domains were proposed based on this communication, provide a different type of interaction with glycosaminoglycans that is reminiscent to the interaction of heparin with antithrombin [19] or the NH_2-terminus of human plasma fibronectin [20]. In these proteins the organization of proposed loops together with hydrophilic and flexible segments results in the provision of several arginine- or lysine-containing sites for potential interaction with the sulfate groups of heparin. Similarly, folding of the protein backbone in vitronectin mostly within 8-sheet structures and loops, as proposed by secondary structure prediction [5], may bring basic residues in close proximity into functional heparin binding site(s) that were previously not recognized.

Indeed, several observations point to the fact that heparin-binding properties of native vitronectin are different from the well-known binding of glycosaminoglycans to the C-terminal basic cluster, which is normally buried in this conformational state but becomes accessible following denaturation/unfolding and/or multimer formation of vitronectin [5,6,21]: (a) at low ionic strength, native vitronectin is retained on immobilized heparin together with a ~40 kDa NH_2-terminal fragment lacking the basic cluster [22]; (b) differences in the binding to and uptake by cells of both forms of vitronectin, which are competed for by heparin, have been recognized [23,24]. It is tempting to believe that phage surface-displayed vitronectin peptides could resemble the presentation of amino acids in their native conformation and that the subsequent overall surface charge in these two regions might account for the heparin-binding activity.

Finally, the heparin-binding domain(s) of vitronectin are involved in the interaction of the adhesive protein with certain pathogenic bacteria [25]. The two novel heparin-binding domains described in the present communication might act separately from or synergistically with the known C-terminal heparin-binding site and thereby dictate conformation-dependent heparin neutralization activities of vitronectin as well as cell surface receptor interactions also relevant in bacterial pathogenesis.

Acknowledgements: The invaluable help of Dr. H. Pannekoek (Academic Medical Center, Amsterdam, The Netherlands) in the preparing of the phage-display library is gratefully acknowledged. The Fond der Chemischen Industrie is acknowledged for generous support, and part of the work was supported by a grant from the Deutsche Forschungsgemeinschaft (Pr 327/1-3), Bonn, Germany. O.D.L. is supported by a post-doctoral fellowship from the GBF. The authors wish to thank the DNA Sequencing Unit at the GBF.

References

[1] K.T. Preissner, Annu Rev Cell Biol 7 (1991) 275–310.
[2] K.T. Preissner, E.R. Podack, H.J. Müller-Eberhard, Eur J Immunol 19 (1989) 69–75.
[3] Preissner KT, Rosenblatt S, Kost C, Wegerhoff J, Mosher, DF (1993) Biology of Vitronectins and their Receptors, Elsevier, Amsterdam.
[4] C. Kost, W. Stüber, H.J. Ehrlich, H. Pannekoek, K.T. Preissner, J Biol Chem 267 (1992) 12098–12105.
[5] K.T. Preissner, G. Müller-Berghaus, J Biol Chem 262 (1987) 12247–12253.
[6] D.A. Lane, A.M. Flynn, G. Pejler, U. Lindahl, J. Choay, K. Preissner, J Biol Chem 262 (1987) 16343–16348.
[7] A.D. Cardin, H.J.R. Weintraub, Arteriosclerosis 9 (1989) 21–31.
[8] D. Jenne, K.K. Stanley, EMBO J 4 (1985) 3153–3157.
[9] C.F.I. Barbas, A.S. Kang, R.A. Lerner, S.J. Benkovic, Proc Natl Acad Sci USA 88 (1991) 7978–7982.
[10] A.J. Van Zonneveld, B.M.M. van den Berg, M. van Meijer, H. Pannekoek, Gene 167 (1995) 49–52.
[11] A.S. Kang, C.F. Barbas, K.D. Janda, S.J. Benkovic, R.A. Lerner, Proc Natl Acad Sci USA 88 (1991) 4363–4366.
[12] G.P. Smith, Science 228 (1985) 1315–1317.
[13] G. Cesareni, FEBS Lett 307 (1992) 66–70.
[14] E. Koivunen, B. Wang, E. Ruoslahti, J Cell Biol 124 (1994) 373–380.
[15] J. Winter, Drug Dev Res 33 (1994) 71–89.
[16] R.H. Hoess, Curr Opin Struct Biol 3 (1993) 572–579.
[17] H. Pannekoek, M. van Meijer, H. Gaardsvoll, A.J. van Zonneveld, Cytotechnology 18 (1995) 107–112.
[18] S. Hess, S.M. Kanse, C. Kost, K.T. Preissner, Thromb Haemostasis 74 (1995) 258–265.
[19] K.T. Preissner, Haemostasis 18 (1988) 271–306.
[20] N.S. Jaikaria, L. Rosenfeld, M.Y. Khan, I. Danishefsky, S.A. Newman, Biochemistry 30 (1991) 1538–1544.
[21] A. Stockmann, S. Hess, P. Declerck, R. Timpl, K.T. Preissner, J Biol Chem 268 (1993) 22874–22882.
[22] S.V. Bittorf, E.C. Williams, D.F. Mosher, J Biol Chem 268 (1993) 24838–24846.
[23] T.S. Panetti, P.J. McKeown-Longo, J Biol Chem 268 (1993) 11988–11993.
[24] de Boer, HC, Preissner, KT, Bouma, BN, and de Groot, PG (1992) 267, 2264-2268.
[25] G.S. Chhatwal, K.T. Preissner, G. Müller-Berghaus, H. Blobel, Infect Immun 55 (1987) 1878–1883.