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Heparin binding domain in vitronectin is required for oligomerization and thus enhances integrin mediated cell adhesion and spreading

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A B S T R A C T

Vitronectin is a multi-functional protein found predominantly as a monomer in blood and as an oligomer in the extracellular matrix. We have dissected the minimal regions of vitronectin protein needed for effective integrin dependent cell adhesion and spreading. A fragment of vitronectin containing the RGD integrin binding site showed similar binding affinity as that of full vitronectin protein to purified integrin αvβ3 but had diminished cell adhesion and spreading function in vivo. We demonstrate that the oligomeric state of the protein is responsible for this effect. We provide compelling evidence for the involvement of the heparin binding domain of vitronectin in the oligomerization process and show that such oligomerization reinforces the activity of vitronectin in cell adhesion and spreading.

Structured summary:
MINT-7905703: *Vn* (uniprotkb:P04004) and *Vn* (uniprotkb:P04004) *bind* (MI:0407) by *molecular sieving* (MI:0071)

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1. Introduction

Vitronectin, first identified as serum spreading factor, is a multi-functional glycoprotein found in abundance in the blood and extracellular matrix (ECM). About 30% of the mass of 75 KDa vitronectin protein is a result of glycosylation [1]. There are a plethora of binding partners to vitronectin which play an important role in wound healing, hemostasis, angiogenesis, metastasis, cell adhesion, spreading and migration [2]. The binding sites for different binding partners are contained in various domains in vitronectin. A well studied domain is the somatomedin B domain (SMB), a compact 4 disulphide bond knot at the N-terminus of the protein [3]. This domain binds to the plasminogen activator inhibitor 1 (PAI-1) and urokinase receptor (uPAR) and plays an important role in wound healing [4,5]. Next to the SMB domain is the RGD site which is an integrin binding region involved in cell adhesion and migration [6]. The major integrin receptors that recognize this site in vitronectin are αvβ3, αvβ1 and αvβ5 [7].

An important region and of particular interest in our study is the heparin binding domain (HBD) on the basic C-terminal region. Preissner (1991) hypothesised that the basic region is involved in intra-molecular interaction with the N-terminal acidic region bringing both the ends together, resulting in several binding sites left cryptic in the native inactive vitronectin protein [1]. There is a lack of understanding of the arrangement of different domains as the molecular structure of native protein has not been resolved. A recent report showed that the HBD region interacts directly with the c-loop of β3 subunit in αvβ3 integrin thereby regulating its function [8].

Vitronectin is found in a range of oligomeric forms from monomer to 16-mer [9]. The predominant form of vitronectin in plasma is the monomeric form and the oligomeric form is found in the extracellular matrix. The physiological relevance of such distribution is not clear. There is an increased interest in the multimerization of vitronectin in particular due to the localisation of vitronectin in the amyloid plaques of brain tissue of patients with Alzheimer's disease [10]. The physiological process of multimerization is yet to be understood. Other proteins binding to the native form may result in a conformational change which in turn results in multimerization. It has also been shown that glycosylation of vitronectin plays a role in multimerization. Stepwise deglycosylation of vitronectin appears to increase the multimerization [11]. Here, we have focussed on the domain required for multimerization and how it affects the function of cell adhesion and spreading.

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2. Materials and methods

2.1. Cloning and expression of human vitronectin fragments

Vitronectin insert excluding the region coding N-terminal SMB domain was amplified from full length cDNA clone (OriGene Technologies Inc., USA) by PCR using KOD DNA polymerase (Merck Biosciences Ltd.). This insert was cloned into pRSETa (Invitrogen Ltd., UK) or pGEX6P2 (GE Healthcare UK Ltd., UK) vectors using BamHI (5′) and EcoRI (3′) restriction sites to generate a construct Vn that codes for ^{40}K–^{459}L of mature peptide (Fig. 1a). An insert coding for ^{40}K–^{131}P containing the integrin binding RGD region and downstream unstructured region was cloned in the above vectors to generate a construct sVn. An oligonucleotide corresponding to the HBD (^{342}P–^{373}N) at the C-terminal region of the vitronectin was inserted as a 3′ fusion to sVn construct between EcoRI and XhoI sites resulting in the construct sVnHBD. A construct coding only HBD region was generated by cloning the above HBD coding oligonucleotide into the pGEX6P2 vector.

The expression vectors were transformed into BL21(DE3) strain of *Escherichia coli* for recombinant vitronectin production. Bacterial culture was induced with 0.1 mM IPTG and incubated for 5 h at 30 °C. NiNTA (Qiagen Ltd., UK) was used to purify poly-histidine tagged proteins and Glutathione-Sepharose (GE Healthcare UK Ltd.) was used to purify GST tagged proteins. Purification was performed according to the manufacturer's instructions.

2.2. Determination of binding affinity of vitronectin fragments to native $\alpha v\beta 3$ integrin

An ELISA assay was used to determine the binding affinity of vitronectin fragments to the native αvβ3 integrin receptor. Integrin receptor was purified from human placenta tissue using a protocol described previously with modifications [12]. Anti-αvβ3 integrin antibody produced from HB11029 clone was used for purification of the integrin. ELISA assay was performed according to the previously described protocol with modifications. Doubling dilutions of 2 μM recombinant vitronectin proteins were applied in triplicate. 1:2500 diluted anti-GST antibody (Sigma-Aldrich Company Ltd., UK) solution was used as a primary antibody and 1:2500 diluted anti-mouse-HRP conjugated antibody (Sigma-Aldrich Company Ltd.) was used as a secondary antibody. The data were fitted for one site binding using SigmaPlot11 software to obtain the apparent K_d values.

2.3. Cell adhesion assays

Wells of a Maxi-sorp 96 well plate were coated with the desired protein constructs in triplicates. Fifty microliters of 2 μM recombinant vitronectin protein solution was used for each well. We confirmed that equal amounts of each protein is coated on the wells using an ELISA assay for the tag (Supplementary Fig. 1). The cell adhesion assay was performed as described previously using human endometrial stromal fibroblasts [13].

2.4. Immunostaining

Cell adhesion was performed as described done on round glass cover slips placed in a four well plate. Immunostaining of the cells was performed as described previously with minor changes [13]. 1:200 diluted anti αvβ3 integrin antibody (MAB1976, Millipore UK Ltd., UK) was used to detect integrin and 1:75 diluted anti-mouse-FITC antibody (Jackson ImmunoResearch Laboratories Inc., USA) was used as secondary antibody. 1:75 diluted Texas-red Phalloidin (Invitrogen Ltd.) was used for actin staining and DAPI was used to stain nuclei.

3. Results and discussion

3.1. SDS–PAGE analysis of purified recombinant vitronectin fragments

Vn, sVn, sVnHBD and heparin binding domain HBD were produced in modest levels of up to 2–5 mg/L of *E. coli* culture. All the proteins were purified from the cytoplasm without the need of any denaturation steps. The purified proteins analysed using SDS–PAGE (Fig. 1b) showed that the Vn protein had undergone partial degradation but the sVn and sVnHBD were homogeneous. The recombinant proteins run slightly higher than the expected size on SDS–PAGE probably due to acidic nature of the protein (calculated p*I* for GST tagged Vn = 5.37, sVn = 4.74, sVnHBD = 5.54). In all our constructs the N-terminal SMB domain was deleted since it was reported that deletion of SMB domain did not affect cell adhesion and spreading [14]. More over we know from our own mass spectrometry studies (not shown here) and from earlier studies that the SMB domain is incorrectly folded in *E. coli* with wrong disulphide topology as compared to the plasma vitronectin [3,15].

3.2. Heparin binding domain is required for oligomerization of vitronectin

The recombinant histidine tag fusion proteins were subjected to gel-filtration to estimate the approximate molecular size of the

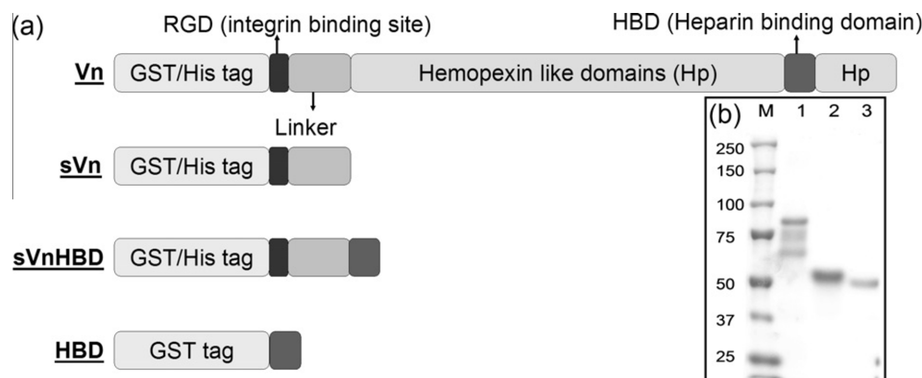

Fig. 1. (a) Recombinant vitronectin constructs: Vn, sVn, and sVnHBD constructs were generated by cloning respective vitronectin inserts into pRSETa for poly-histidine tag fusion or pGEX6P2 vector for GST fusion. (b) Purified vitronectin proteins were subjected to 12% reducing SDS-PAGE to assess the level of purity. Lane 1 is GST-Vn, lane 2 is GST-sVn and lane 3 is GST-sVnHBD. M represents the marker lane showing the marker weight in KDa.

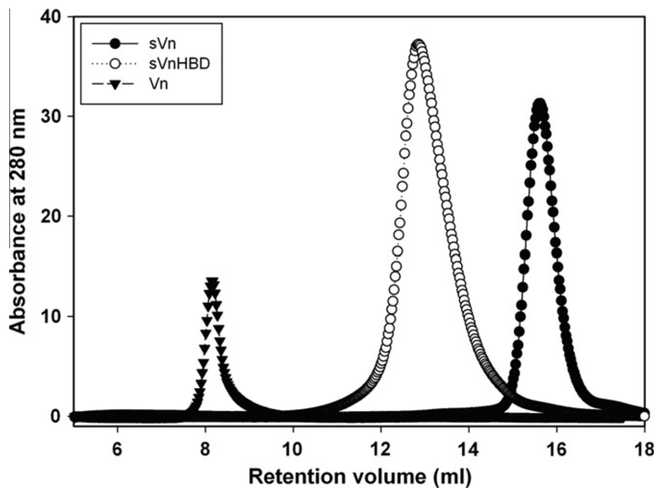

Fig. 2. Gelfiltration analysis of vitronectin oligomeric state: Vn protein eluted out in the void volume of Superdex 200 column indicating that it is a large oligomer. sVn protein eluted at a retention volume of 16.2 ml corresponding to a monomer size. Addition of HBD domain to sVn promotes the oligomerization and the resulting protein eluted at 12.8 ml which is a 200 KDa complex of ⩾10 units.

proteins. Vn fragment analysed on Superdex 200 column showed that it is a large oligomer which eluted out in the void volume (Fig. 2). sVn fragment eluted at a retention volume of 16.2 ml indicating that it is a monomer. This has also been confirmed using multiple angle light scattering experiment. The sVnHBD protein eluted at a retention volume of 12.8 ml which corresponds to a molecular weight of around 200 KDa. This molecular weight corresponds to an oligomeric number ⩾10 (18 KDa being the monomer size of sVnHBD). The chromatogram for sVnHBD was quite broad as seen in the Fig. 2 showing that the protein exists in several minor intermediate oligomeric forms. Though it is difficult to assign a particular multimer number to the protein, it is clear that it undergoes multimerization. Vn and sVnHBD containing the HBD domain formed large oligomers and sVn lacking HBD did not. The HBD domain had earlier been proposed to be a probable site for multimerization [9], although, another report suggested that there is no involvement of HBD in multimerization in their refolding studies [16]. Our study clearly shows that sVn protein multimerizes to a large complex when HBD domain is fused to it and confirms its role in oligomerization.

3.3. Binding affinity of recombinant vitronectin fragments to αvβ3 integrin

Saturation binding assays were performed to measure the binding affinity of Vn, sVn and sVnHBD proteins to the native αvβ3 integrin. The apparent K_d values for Vn, sVn, sVnHBD were 12.3 ± 2.5 nM, 10.9 ± 3.4 nM and 8.2 ± 4.4 nM. These values were not statistically different showing that integrin binding affinity was not affected by removal of much of the region outside the RGD site.

3.4. HBD mediated oligomerization of vitronectin enhances cell adhesion

The levels of cell adhesion to plasma vitronectin and recombinant Vn were indistinguishable. sVn protein had a considerable loss of function (Fig. 3a). Only 40% cell adhesion was observed to sVn compared to Vn (Fig. 3b). A similar construct but with an intact

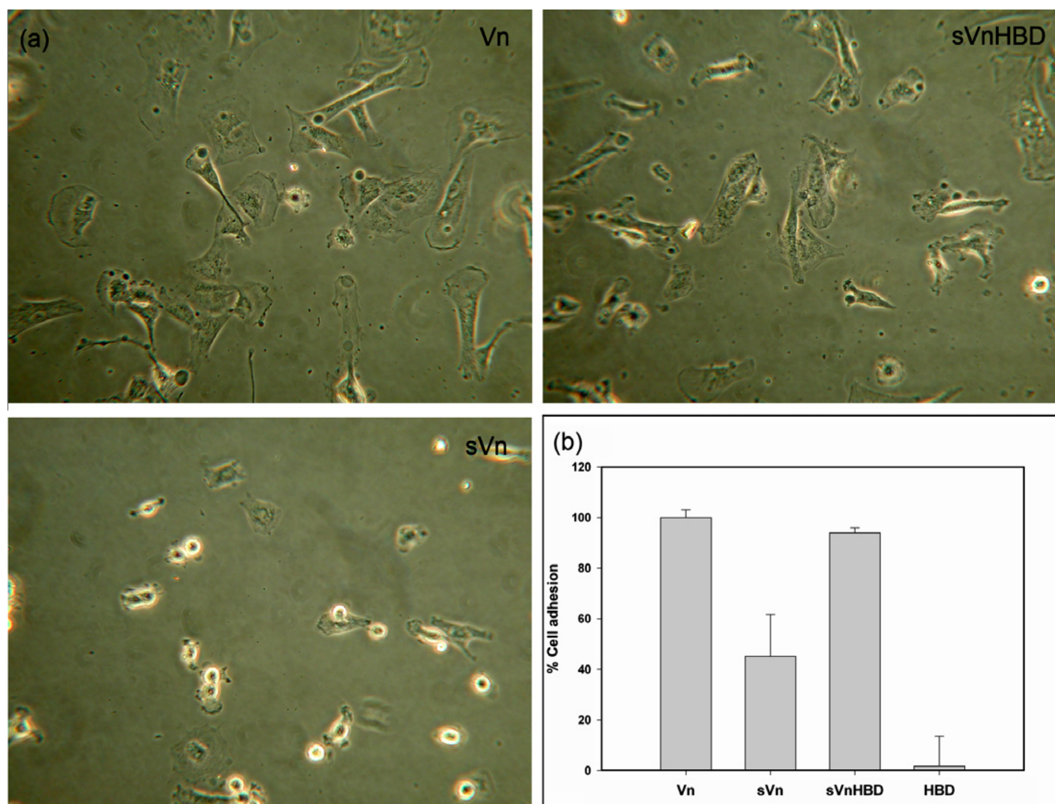

Fig. 3. (a) Light microscope images of cell adhesion: human endometrial stromal fribroblast cell adhesion on Vn and sVnHBD fragments was similar where as cell adhesion was hindered on sVn fragment. (b) Cell adhesion on vitronectin fragments: 2 μM solution of each protein was used for coating the plates. Value of each bar is a mean of nine wells and the error bar represents the standard deviation.

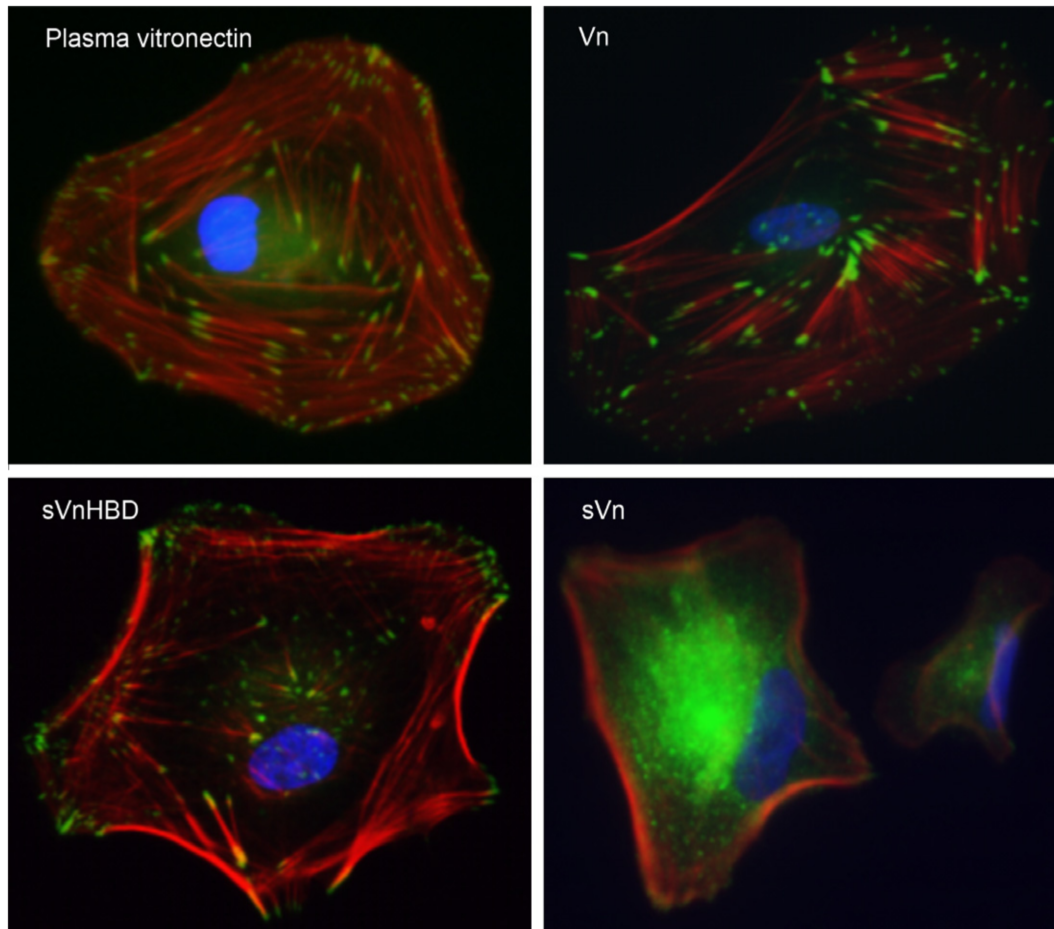

Fig. 4. Immunostained images of human endometrial stromal fibroblasts: The cells were stained for actin fibres (red) using Texas Red-Phalloidin, nucleus using DAPI. Integrin $\alpha v\beta 3$ was stained using anti-human integrin αv subunit antibody MAB1976Z and antimouse antibody conjugated to FITC as secondary antibody. The cells spreading on plasma vitronectin, Vn and sVnHBD show clear focal adhesion points and well organised actin fibres. Most of the cells spreading on sVn protein show poorly formed actin cytoskeleton and diffused integrin staining.

SMB domain was studied earlier by another group [17]. This study reported that this construct retained 70% activity of the native vitronectin. But they achieved this activity at fairly high concentrations of protein (10 μM). We found in a separate study that the fibronectin cell binding fragment (FnIII9′10) forms bilayers at higher concentrations [18]. This might also be the case for sVn and the high concentrations of protein coated onto the well may result in bilayers mimicking oligomeric state of the vitronectin protein. To avoid such a possibility we used a relatively low concentration of vitronectin fragments with the aim of retaining similar physical properties both in solution and attached to the well. Surprisingly, 95% cell adhesion was observed to sVnHBD fragment compared to Vn (Fig. 3b) showing that the HBD domain enhances sVn function. There was no significant difference in cell adhesion compared to the HBD domain alone.

3.5. Cell morphology of spreading cells

The immunostained images showed that there was no distinguishable difference in the morphology of spreading among the cells spread on plasma vitronectin, Vn and sVnHBD proteins. These cells had a well organised actin cytoskeleton (Fig. 4). Staining for the integrin $\alpha v\beta 3$ showed prominent focal adhesions co-localized with actin at the cell membrane. Most of the cells attached to sVn protein had a poorly formed actin cytoskeleton (Supplementary Fig 2). There were very few focal adhesions. This result is consistent with an earlier report which showed that the C-terminal heparin binding site is necessary for cytoskeletal organisation and migration of endothelial cells though this study did not say why [19].

Taken together, our data to show that the RGD region itself is sufficient for integrin binding but not for its full function in cell adhesion and spreading. Our data confirm that the HBD is involved in the multimerization process of vitronectin and that the sVnHBD construct represents the minimal domain requirement for integrin dependent cell adhesion and spreading function of vitronectin. Sano et al. (2007) showed that the larger the multimer of vitronectin the more efficiently it binds to collagen [11]. It has also been shown that stiffness of ECM is required for activation of integrin $\alpha 5\beta 1$ [20]. In addition, the oligomeric form has been demonstrated to increase the adhesion strength of the ECM protein [21]. We propose that oligomeric vitronectin forms strong bonds with collagen creating a stiff ECM, which allows formation of stronger adhesion to integrin and a well organized actin cytoskeleton.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2010.06.023.

References

[1] Preissner, K.T. (1991) Structure and biological role of vitronectin. Annu. Rev. Cell Biol. 7, 275–310.

[2] Schvartz, I., Seger, D. and Shaltiel, S. (1999) Vitronectin. Int. J. Biochem. Cell Biol. 31 (5), 539–544.

[3] Zhou, A., Huntington, J.A., Pannu, N.S., Carrell, R.W. and Read, R.J. (2003) How vitronectin binds PAI-1 to modulate fibrinolysis and cell migration. Nat. Struct. Biol. 10 (7), 541–544.

[4] Declerck, P.J., De Mol, M., Alessi, M.C., Baudner, S., Paques, E.P., Preissner, K.T., Muller-Berghaus, G. and Collen, D. (1988) Purification and characterisation of a plasminogen activator inhibitor I binding protein from human plasma. Identification as a multimeric form of S protein (vitronectin). J. Biol. Chem. 263, 15454–15461.

[5] Wei, Y., Waltz, D.A., Rao, N., Drummond, R.J., Rosenberg, S. and Chapman, H.A. (1994) Identification of the urokinase receptor as an adhesion receptor for vitronectin. J. Biol. Chem. 269, 32380–32388.

[6] Jin, H. and Varner, J. (2004) Integrins: roles in cancer development and as treatment targets. Br. J. Cancer 90, 561–565.

[7] Felding-Habermann, B. and Cheresh, D.A. (1993) Vitronectin and its receptors. Curr. Opin. Cell Biol. 5, 864–868.

[8] Maile, L.A., Aday, A.W., Busby, W.H., Sanghani, R., Veluvolu, U. and Clemmons, D.R. (2008) Modulation of integrin antagonist signalling by ligand binding of the heparin-binding domain of vitronectin to the $\alpha v\beta 3$ integrin. J. Cell Biochem. 105, 437–446.

[9] Stockmann, A., Hess, S., Decklerck, P., Timpl, R. and Preissner, K.T. (1993) Multimeric vitronectin: identification and characterization of conformation-dependent self-association of the adhesive protein. J. Biol. Chem. 268 (30), 22874–22882.

[10] Shin, T.M., Isas, J.M., Hsieh, C.-L., Kayed, R., Glabe, C.G., Langen, R. and Chen, J. (2008) Formation of soluble amyloid oligomers and amyloid fibrils by the multifunctional protein vitronectin. Mol. Neurodegen. 3, 16.

[11] Sano, K., Asanuma-Date, K., Arisaka, F., Hattori, S. and Ogawa, H. (2007) Changes in glycosylation of vitronectin modulate multimerization and collagen binding during liver regeneration. Glycobiology 17 (7), 784–794.

[12] Altroff, H., van der Walle, C.F., Asselin, J., Fairless, R., Campbell, I.D. and Mardon, H.J. (2001) The eighth FIII domain of human fibronectin promotes integrin $\alpha 5\beta 1$ binding via stabilization of the ninth FIII domain. J. Biol. Chem. 276 (42), 38885–38892.

[13] Klemmt, P.A.B., Carver, J.G., Koninckx, P., McVeigh, E.J. and Mardon, H.J. (2007) Endometrial cells from women with endometriosis have increased adhesion and proliferative capacity in response to extracellular matrix components: towards a mechanistic model for endometriosis progression. Hum. Reprod. 22 (12), 3139–3147.

[14] Schar, C.R., Blouse, G.E., Minor, K.H. and Peterson, C.B. (2008) A deletion mutant of vitronectin lacking the somatomedin B domain exhibits residual plasminogen activator inhibitor-1-binding activity. J. Biol. Chem. 283 (16), 10297–10309.

[15] Kjaergaard, M., Gårdsvoll, H., Hirschberg, D., Nielbo, S., Mayasundari, A., Peterson, C.B., Jansson, A., Jørgensen, T.J.D., Poulsen, F.M. and Ploug, M. (2007) Solution structure of recombinant somatomedin B domain from vitronectin produced in *Pichia pastoris*. Protein Sci. 16, 1934–1945.

[16] Zhuang, P., Li, H., Williams, J.G., Wagner, N.V., Seiffert, D. and Peterson, C.B. (1996) Characterisation of the denaturation and renaturation of human plasma vitronectin: investigation into the mechanism of formation of multimers. J. Biol. Chem. 271 (24), 14333–14343.

[17] Jang, J.H., Koak, J.Y., Kim, S.C., Hwang, J.H., Lee, J.B., Jang, I.T., Chung, C.P. and Heo, S.J. (2003) Expression and characterization of recombinant NH_2-terminal cell binding fragment of vitronectin in *E. coli*. Biotech. Lett. 25, 1973–1975.

[18] Kreiner, M., Chillakuri, C.R., Pereira, P., Fairhead, M., Li, Z., Mardon, H.J., Holt, S.A. and van der Walle, C.F. (2009) Orientation and surface coverage of adsorbed fibronectin cell binding domains and bound integrin a5b1 receptors. Soft Matter 5, 3954–3962.

[19] Thiagarajan, P., Le, A., Snuggs, M.B. and Vanwinkle, B. (1996) The role of carboxy-terminal glycosaminoglycan-binding domain of vitronectin in cytoskeletal organization and migration of endothelial cells. Cell Commun. Adhes. 4 (4), 317–325.

[20] Friedland, J.C., Lee, M.H. and Boettiger, D. (2009) Mechanically activated integrin swith controls $\alpha 5\beta 1$ function. Science 323, 642–644.

[21] Roca-Cusachs, P., Gauthier, N.C., del Rio, A. and Sheetz, M.P. (2009) Clustering of $\alpha 5\beta 1$ integrins determines adhesion strength whereas $\alpha v\beta 3$ and talin enable mechanotransduction. PNAS 106 (38), 16245–16250.