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

Chandramouli R Chillakuri, Céline Jones, Helen J Mardon *

Nuffield Department of Obstetrics and Gynaecology, The John Radcliffe Hospital, University of Oxford, Oxford OX3 9DU, United Kingdom

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ABSTRACT

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)

1. Introduction

Vitronectin, first identified as serum spreading factor, is a multifunctional 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.

* Corresponding author. Address: Nuffield Department of Obstetrics and Gynaecology, University of Oxford, Level 3, Women's Centre, The John Radcliffe Hospital, Headington, Oxford OX3 9DU, United Kingdom. Fax: +44 (0)1865 221001.
E-mail address: Helen.Mardon@obs-gyn.ox.ac.uk (H.J. Mardon).

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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 αvβ3 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

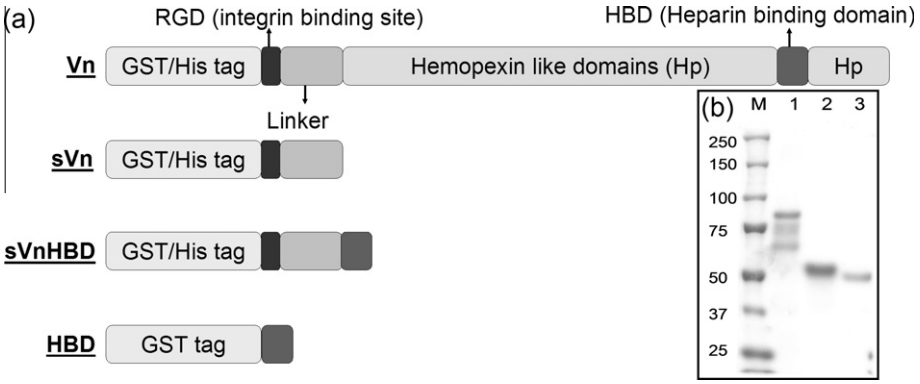

Fig. 1. (a) Recombinant vitronectin constructs: Vn, sVn, and sVnHBD constructs were generated by cloning respective vitronectin inserts into pRSETa for poly-histidine tag fusion or pGEX6P2 vector for GST fusion. (b) Purified vitronectin proteins were subjected to 12% reducing SDS-PAGE to assess the level 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.