Elucidation of the Heparin Binding Domain of Goat Vitronectin by Deletion Mutagenesis

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ABSTRACT

Vitronectin is a multifunctional glycoprotein present in the extracellular matrix. The RGD motif and the heparin-binding domains of the human protein play a major role in cell adhesion and bacterial binding. Goat vitronectin is unique in having two RGD motifs compared to human vitronectin. The heparin-binding domain of goat vitronectin has not yet been identified, and the amino acids from 340 to 380 of human vitronectin are known to be involved in heparin binding primarily. The proposed HBD of goat protein contains consensus sequences of many known heparin-binding proteins. PCR-based deletion mutagenesis of this region indicated that the basic residues are essential for heparin binding and protein multimerisation.

Keywords: Goat vitronectin, Heparin binding domain, HBD, Deletion mutagenesis

INTRODUCTION

Vitronectin (Vn) is a matrix glycoprotein synthesized predominantly by the liver. It exists in two different conformations: as a monomer in blood and as an oligomer in the extracellular matrix and platelet releasates. It is involved in diverse physiological roles such as cell adhesion, wound healing, angiogenesis and is also implicated in the metastasis of tumours, internalisation of pathogens, bacterial serum resistance, etc. (Chillakuri *et al.,* 2010; Singh *et al.,* 2010; Pellegrini and Pietrocola, 2024). Human vitronectin has a domain arrangement with three different domains, such as the somatomedin B domain, hemopexin-like domains and the heparin binding domain (Schvartz *et al.,* 1999). Studies on goat vitronectin revealed similar structural features to those of human vitronectin and is unique in having an additional RGD motif (Mahawar and Joshi, 2008). The heparin-binding domain (HBD) of human Vn has been studied on its possible roles in multimer formation, cell adhesion and bacterial binding, but the data on goat Vn are lacking. The present study aimed to elucidate the heparin-binding domain of goat Vn by mutagenesis and its possible role in multimerisation of the protein.

MATERIALS AND METHODS

Generation of Recombinant Mutant Goat Vitronectin by Deletion Mutagenesis: Heparin-binding domains are characterized by the presence of clusters of positively charged amino acids with spatially arranged hydrophobic residues. Sequence analysis of goat Vn revealed the presence of many such heparin-binding consensus regions between 341-380 residues. This putative region was subjected to deletion mutagenesis, and three mutants were generated. The methodology for the creation of a full-length HBD deletion mutant, viz. HBDFLD (Δ 341-380) using an inverse PCR-based method was described earlier (Pathak *et al.*2019). Partial HBD deletions, viz. HBD R1D(Δ341-355) and HBD R2D(Δ356-380) representing this region were generated similarly using the method as described for HBD FLD (Δ 341-380) with minor modifications. The details of primers used are given in Table I. The mutants were sequenced and found to have only the desired alterations. The mutants were expressed in a prokaryotic expression system - pPROEXHTa. The proteins were expressed as inclusion bodies, which were then solubilized with 8M urea and purified by Nickel-agarose affinity chromatography exploiting the hexahistidine tag. The purified protein was dialyzed against

decreasing concentrations of urea and salt viz. 4M, 2M, 1M, 1M urea and 1M NaCl, 0.5M NaCl and normal saline, to remove urea. The dialysed proteins were soluble, suggesting correct refolding.

Heparin Affinity Chromatography: The dialysed HBD mutant proteins were subjected to affinity chromatography on a heparin-agarose column to check their affinities for heparin. The column was washed extensively with 2M NaCl and equilibrated with 20 mM sodium phosphate (pH 7.4) containing sodium azide. About 6-8 ml (~1-2 mg of protein) of the dialysed aliquot was passed through the column. The absorbance of the column load and flow through was checked at 280 nm. Fractions of 1.5 ml of the flow-through were collected. After extensive washing with equilibration buffer, the bound proteins were eluted sequentially with 0.15M and 0.5M NaCl in equilibration buffer and fractions of 1.5ml were collected. The presence of proteins in the fractions was checked by SDS- PAGE following standard protocols.

RESULTS AND DISCUSSION

Deletion mutagenesis of the proposed region was observed, as evidenced by sequencing and the recombinant mutant proteins were generated. The dialysed proteins were passed over a heparin-agarose column to check their binding with heparin. The HBD FLD (Δ 341-380) fraction did not bind to the column, as evident by its presence in the unbound fraction. The 0.5M column wash did not show any protein (Fig. 1). The HBD R1D (Δ 341-355) mutant bound to the column and was eluted at 0.5M NaCl concentration. The eluted fraction had both monomeric and multimeric forms (Fig. 2). The HBD R2D (Δ 356-380) mutant showed less affinity to heparin as compared to HBD R1D (Δ 341-355). Most of the HBD R2D (Δ 356-380) protein was present in the unbound fraction. The 0.5M NaCl wash contained only multimers (Fig. 3).

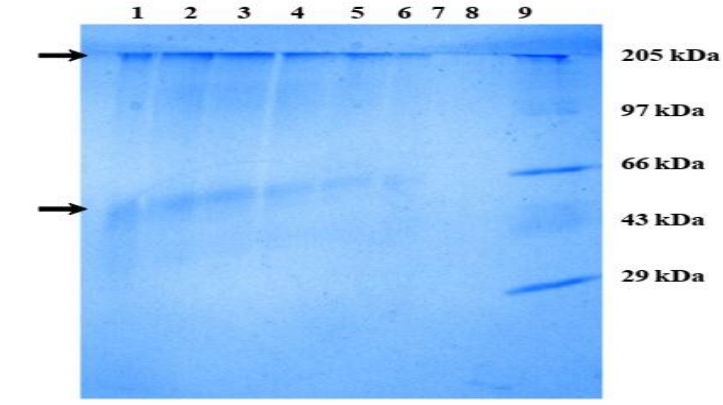

Fig. 1: Heparin affinity chromatography of HBD FLD (Δ341-380)
Lane 1: Column load; **Lanes 2 – 6:** Column flow through; **Lane 7:** 0.5M NaCl eluted fraction (2); **Lane 8:** 0.5M NaCl eluted fraction (3); **Lane 9:** Molecular Weight Markers.
Arrows indicate monomer and multimer

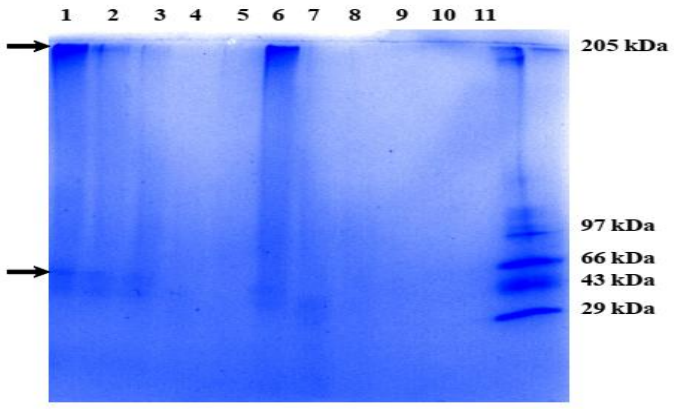

Fig. 2: Heparin affinity chromatography of HBDR1D (Δ341-355)
Lane 1: Column load; **Lanes 2-3:** Column flow through; **Lane 4:** 0.15M NaCl eluted fraction (1); **Lane 5:** 0.15M NaCl eluted fraction (2); **Lane 6:** 0.5M NaCl eluted fraction (1); **Lane 7:** 0.5M NaCl eluted fraction (2); **Lane 8:** 0.5M NaCl eluted fraction (3); **Lane 9:** 1 M NaCl eluted fraction (1); **Lane 10:** 1 M NaCl eluted fraction (2); **Lane 11:** Molecular Weight Markers.
Arrows indicate monomer and multimer

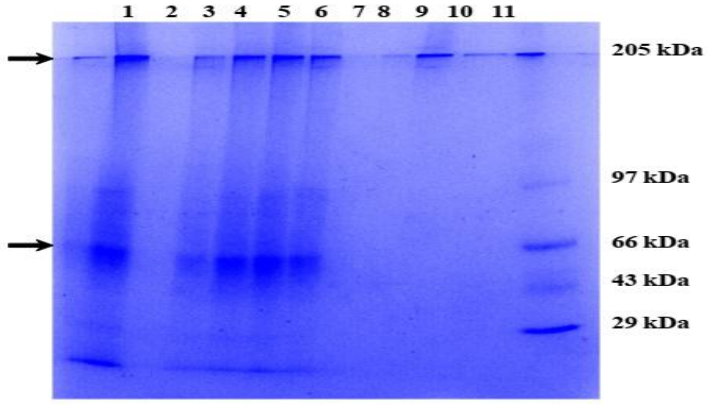

Fig. 3. Heparin affinity chromatography of HBDR2D (Δ356-380)
Lane 1: Column load; **Lanes 2-7:** Column flow through; **Lane 8:** 0.15M NaCl eluted fraction (1); **Lane 9:** 0.5M NaCl eluted fraction (2); **Lane 10:** 1 M NaCl eluted fraction (2); **Lane 11:** Molecular Weight Markers
Arrows indicate monomer and multimer

Table I: Details of Mutagenic Primers Used

1) **HBD R1D Forward:** TCCCCCCGGGCCCTCCGAAGCCGTGGCCGAGGC
2**) HBD R1D Reverse:** ACGGCTTCGGAGGGCCCGGGGGGAGCTGGGAGC
3) **HBD R2D Forward:** CGTTACCGCTCGTCCTGGTTCTCCAGCGAGGAG
4) **HBD R2D Reverse:** GGAGAACCAGGACGAGCGGTAACGTTTGCGATG

In the present study, HBD FLD (Δ 341-380) did not bind to heparin–sepharose. Wild Vn has amino acids 341-380 intact, bound to a matrix containing heparin (Mahawar and Joshi, 2008). The residues 341-380 also possess heparin binding consensus sequences (RRFR; BBXB) as reported earlier (Cardin and Weintraub, 1989; Mann *et al.,*1994). A recombinant polypeptide comprising the C-terminal 129 amino acids of human Vn exhibited heparin-binding affinity that is comparable to that of full-length protein, and theputative secondary sites were reported to be non-functional (Gibson *et al.*1999). The partial deletion of HBD in goat Vn did not alter heparin binding property significantly. In case of HBD R1D (Δ 341-355), both monomeric and multimeric forms bound to heparin–sepharose, whereas in HBD R2D (Δ 356-380), only multimers interacted with heparin–sepharose as the monomer was present in the unbound fraction. HBD R1D (Δ 341 - 355) and HBD R2D (Δ 356 - 380) both contain eight basic amino acids. However, in HBD R1D (Δ 341-355), these basic residues are clustered together (RRHRKRYR), whereas in HBD R2D (Δ356- 380), they are segregated. It therefore appears that the presence of basic residues spread all over the partial deletion HBD R2D (Δ 356-380) may impart heparin-binding property to the monomeric form of the protein moiety.

The present study indicated the role of the HBD of goat Vn in protein multimerization as partial deletion of this region did not affect protein oligomerization. The presence of partial HBD induced protein multimerization as HBD R1D(Δ 341-355) and HBD R2D(Δ356-380) existed in the oligomeric state. These results complement an earlier study where goat Vn fragment (residues 323-444) with a complete heparin-binding site showed multimerisation, and also a truncated HBD fragment (residues 363-444) underwent oligomerisation (Mahawar and Joshi, 2008*).* This was also observed in another study by Chillakuri *et al.* (2010), where the addition or presence ofa recombinant HBD fragment of vitronectin (aa residues 342-373) triggered protein oligomerization.

CONCLUSION

The amino acid residues 341-380 are essential for heparin binding by goat Vn and the role of basic amino acids of HBD in protein oligomerization is amply demonstrated. The

binding of multimers present in the truncated Vn(s) having partial HBD may be due to an increased number of basic residues as a result of protein multimerization/oligomerisation. Therefore, amino acid residues between 341 - 380 may be considered as the primary HBD of goat Vn. Further studies on its role in cell and bacterial adhesion are needed to understand its role in host-pathogen interactions. The presence of many basic residues in this region is important for heparin binding activity. The residues 341-355 might be involved in triggering the multimerisation of the goat vitronectin.

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