

To the Graduate Council:

I am submitting herewith a thesis written by Yacynth Ruwansara entitled "Investigating the Oligomerization of Vitronectin." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Biochemistry, Cellular and Molecular Biology.

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Investigating the Oligomerization of Vitronectin

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Master's Thesis

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Abstract.

Vitronectin is a multi-functional glycoprotein that is present in the plasma and extra-cellular matrix of eukaryotes. It is capable of binding a wide variety of structurally different ligands, including plasminogen activators, plasminogen activator-inhibitors, proteases, cell surface receptors and components of the extra-cellular matrix. Vitronectin exists in two conformations – as a monomer in circulation, and as a multimer in the extra-cellular matrix. The pathway by which vitronectin undergoes the transition from monomer to multimer is not well characterized, but this laboratory has put forward evidence to suggest that the binding of vitronectin with plasminogen activator inhibitor type-1 (PAI-1), facilitates higher order complex formation.

Because multimeric vitronectin remains long after PAI-1 has dissociated from the complex, the question of which region(s) within vitronectin are interacting to maintain the multimeric conformation has been asked. To address this question, candidate domain regions in vitronectin were analyzed for potential aggregative properties. Based on this analysis, the Central domain of the protein was hypothesized to be the region responsible for self-association. In support of this hypothesis, the predicted structure of the Central domain is a 4-bladed β -propeller fold, a motif that is capable of mediating protein-protein interactions. However, not all β -propeller proteins have self-associative behavior. Examples include hemopexin, gelatinase-A and collagenase-3, all of which share distant sequence homology with vitronectin.

In this study, a negative-design approach was used in which the Central domain of vitronectin was replaced with the homologous β -propeller regions from hemopexin and gelatinase-A. The substitution of this domain with β -propeller regions that do not

demonstrate self-association was expected to alter the aggregative properties of the chimeras. An additional recombinant form of vitronectin was also designed which did not possess the C-terminal domain to conclusively rule out this domain as the candidate region. In addition to this approach, limited proteolysis was used to try and isolate the Central domain region of vitronectin so that it could be better characterized both structurally and functionally.

All three recombinant proteins were synthesized and expressed in insect Sf9 cells. However, purification of these proteins was met with difficulty due to the low expression levels observed. Optimization attempts to improve expression were unsuccessful, and so structural and functional analysis studies were limited. Nevertheless, all three proteins were able to bind to wild-type PAI-1 in solution, as well as demonstrate Western blot reactivity with two monoclonal antibodies. Initial results from the limited proteolysis study also showed potential peptide fragments for the Central domain of vitronectin.

Based on the results from this study, the region responsible for the self-association of vitronectin should be further examined and resolved to critical residues within the Central domain. These results may offer insight into the process of oligomerization in other proteins, many of which are implicated in fatal diseases, e.g. Alzheimer's and Creutzfeldt-Jakob disease.

Introduction.

Vitronectin (VN), is a 72 kD glycoprotein that exists both in plasma and the extracellular matrix (ECM). It circulates in the bloodstream at micromolar levels as a monomer, where it regulates proteolytic cascades that mediate blood coagulation and fibrinolysis. Monomeric vitronectin exists in two forms – as a single chain, and as a two-chain, disulfide-linked form. This two chain form is produced by proteolysis by an unknown protease on the carboxyl-terminal side of R379, dividing the protein into heavy chain (62 kD) and light chain (10 kD) fragments. The presence of a threonine residue at position 381 instead of a methionine seems to increase the likelihood of proteolytic cleavage [1]. In the ECM, the protein adopts a multimeric form, where it modulates cell adhesion and migration.

In order to accomplish these different functions, vitronectin has adopted the ability to bind to many different biomolecules. The soluble monomeric form for example, is able to bind glycosaminoglycans such as heparin [2, 3] , serine protease inhibitors such as plasminogen activator inhibitor type I (PAI-1) [4], and proteases such as thrombin [5]. The tissue-associated multimeric form on the other hand, is capable of binding to certain integrin receptors [6, 7], cell surface receptors such as the urokinase plasminogen activator receptor (uPAR) [8, 9] and other components of the ECM (e.g. collagen) [10].

The structure of vitronectin.

A full, three-dimensional structure of vitronectin still remains elusive – however, a substantial amount of research has been done in order to understand both the structural

and functional properties of the protein. Through various studies utilizing approaches such as sequence comparison, peptide mapping, limited proteolysis, as well as work with recombinant fragments [11-13], a general organization of the protein has been formed. It is believed to be organized into 3 discrete domains,

1. The N-terminal domain : this domain encompasses amino acids 1-51. The region between amino acids 1-44 is also referred to as the somatomedin B (SMB) domain (so named because of its identity in sequence to the somatomedin B protein, a growth-hormone-dependant factor that stimulates DNA synthesis in human glia cells [14]). The SMB domain houses the primary binding site for PAI-1 [15-17], along with the binding site for uPAR [8, 9]. Following the SMB sequence are additional amino acids, including an RGD sequence at positions 45-47, which allows binding to the integrins [6, 7].

2. The Central domain : amino acids 130-320 encompass the Central domain of vitronectin, sometimes referred to as the ‘hemopexin1’ domain, due to its several repeat motifs that resemble the protein hemopexin [13].

3. The C-terminal domain : sometimes referred to as the ‘hemopexin2’ domain (for the same reason as the Central domain), amino acids 320-459 contain a heparin binding sequence, and may also bind complement factors as well as plasminogen [18].

While the three-dimensional structure of the entire protein has not been discerned, much work has been done in attempting to solve the individual structures of the three domains listed above. The solution structure of the N-terminal domain of vitronectin from human plasma has been solved by this laboratory, using 2-dimensional NMR [19]. Other groups have also reported its structure using recombinant methods [20-22]. There has been some controversy over the disulfide bond arrangements predicted for this

domain between this laboratory and other groups [20, 22, 23]. This laboratory maintains the idea that the plasma-purified structure for this domain should serve as the standard for other recombinant matches, but does acknowledge the difficulties in mapping disulfide bond arrangements. In agreement with all groups, however is the fold of the N-terminal domain, which is a tightly packed structure with the only notable secondary structural element being a single-turn α -helix that houses the binding sites for PAI-1 and uPAR [19].

The independent structures of the Central and C-terminal domains have also been predicted using a threading algorithm encompassing fold recognition and sequence-structure alignment [24]. The structure of the Central domain is predicted to be a 4-bladed β -propeller fold, similar to the C-terminal domains of hemopexin and gelatinase-A [24]. The C-terminal domain is predicted also to assume that of a 4-bladed β -propeller structure, but only over half the fold, and interrupted by the highly-charged heparin binding sequence [24, 25]. The same study also proposed a model for the docking of the Central and C-terminal domains to one another. The computer program used to generate the docked model was guided by the experimental identification of the inter-domain disulfide linkage between the Central and C-terminal domains (present in the two-chain form). **Figure 1** shows the linear sequence of vitronectin, along with the corresponding structures of the N-terminal (solved), Central and C-terminal (predicted) domains.

In addition to the aforementioned studies, small-angle x-ray scattering (SAXS) experiments were carried out on monomeric, unbound vitronectin to try and discern an overall shape and fold of the protein [26], as well as to try and formulate a plausible arrangement of the three individual domains within the entire protein. **Figure 2** shows the

proposed arrangement of all three domains within vitronectin from those experiments.

Together, this information provides a useful working model by which a study of the relationship between the structural and functional properties of vitronectin can be better examined.

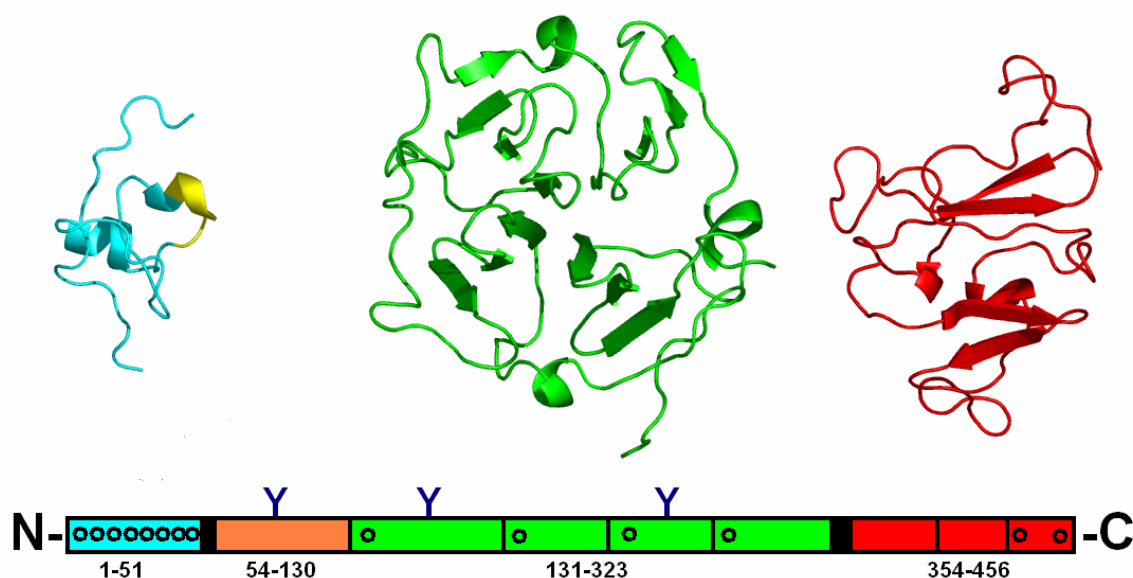


Figure 1. The linear sequence of vitronectin.

The linear sequence of vitronectin, showing the residue ranges for the N-terminal (cyan, 1-51), Central (green, 131-323), and C-terminal (red, 354-456). Residues 54-130 (tan) comprise a linker region between the N-terminal and Central domains that is thought to be highly unstructured. Cysteine residues within the domains are shown as open-circles (o), while glycosylation sites are represented as dark blue Ys. Shown directly above the linear sequence are the structures of the N-terminal (PDB file 2JQ8 [19]), and the predicted structures of both the Central and C-terminal domains. The yellow area shown on the N-terminal structure spans residues 26-30, which houses the primary binding site for PAI-1. The seven hemopexin homology domains are shown as well (green and red boxes) [12, 19, 26].

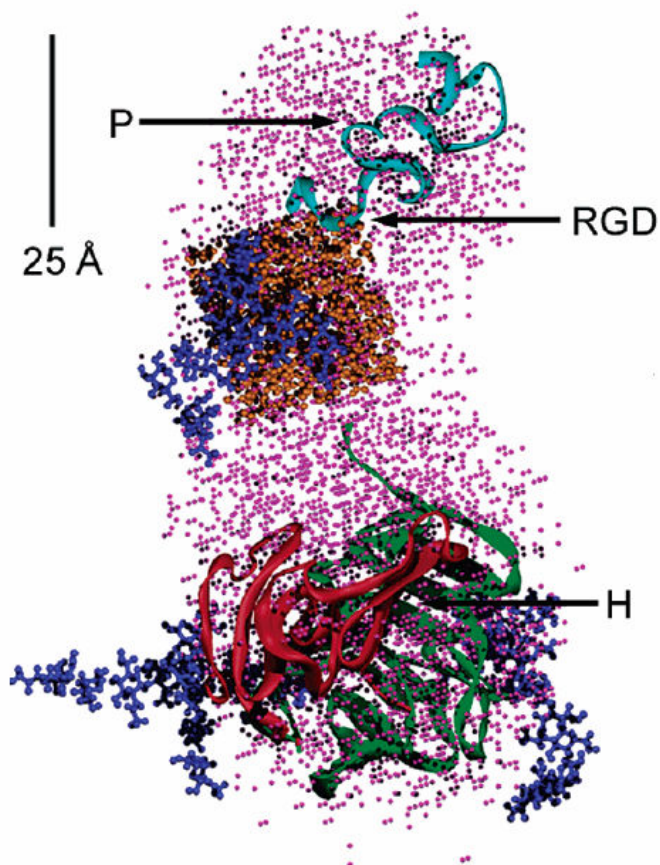


Figure 2. The solution structure of monomeric vitronectin from SAXS.

[26] The solution structure of monomeric, unbound vitronectin as determined from SAXS. Shown are the N-terminal (cyan), Central (green), and C-terminal (red) domains, along with the linker region between the N-terminal and Central domains (amber). The 3 glycosylation sites are shown in blue, while the purple dots display the consensus envelope model for the protein. The binding regions for PAI-1 (**P**), the integrins (**RGD**) and Heparin (**H**) are also shown.

The relationship between PAI-1 and vitronectin.

Of the many ligands which vitronectin binds to, its interaction with plasminogen activator inhibitor type 1 (PAI-1), is of most importance to this laboratory. PAI-1 is a member of the serpin (serine protease inhibitor) superfamily of proteins [27]. The serpin mechanism of inactivation involves cleavage of the reactive center peptide bond (denoted P1-P1') by the protease, resulting in the formation of a stable acyl-intermediate [28, 29]. The P1-P1' bond is found on the surface-exposed reactive center loop (RCL) of the protein [30]. The cleaved ends of the RCL then separate, and the P1-end, covalently bound to the protease inserts into the central β -sheet of the serpin [31, 32]. Thus, the protease is inactivated by the formation of a stable 1:1 complex with the serpin.

PAI-1 is the primary inhibitor of both urokinase plasminogen activator (uPA), and tissue-type plasminogen activator (tPA) [16]. It is unique among other serpins as it prone to convert from an active to latent/non-inhibitory form over time via the spontaneous insertion of its reactive center loop (RCL), into the central β -sheet of the protein [33]. This insertion is thermodynamically driven, as the latent form of PAI-1 is more stable than the active form [34]. **Figure 3** shows both the active and latent structures of PAI-1, highlighting the repositioning of the RCL.

Free PAI-1 is unstable, easily converting to its latent form by insertion of the reactive center loop (RCL) into its core β -sheet (as shown in Figure 3). However, the binding of vitronectin greatly increases the half-life of active PAI-1 [3]. Vitronectin binds across the base of the central β -sheet of PAI-1, thereby inhibiting the movement necessary to allow the RCL to insert in between the other β -sheets [35], thus maintaining it in an active conformation. The interaction between vitronectin and PAI-1 is of

considerable physiological significance. As mentioned, PAI-1 is the primary inhibitor of tPA and uPA. These proteins are the main activators of plasminogen, converting it from its inactive zymogen form to its active protease form of plasmin. Plasmin is responsible for degrading fibrin clots. Therefore, PAI-1 and vitronectin play an important regulatory role in fibrinolysis.

Vitronectin has already been identified as the main PAI-1-binding protein in the ECM of cultured endothelial cells [36]. This laboratory has also shown that PAI-1 is localized to the fibrin matrix of blood clots exclusively via vitronectin [37]. Vitronectin and PAI-1 are frequently detected at sites of tissue injury and tumor invasion, with vitronectin adopting its multimeric form [38], to allow it to participate in co-regulating cell adhesion and migration with PAI-1. The exact mechanism of the formation of multimeric, ECM-associated vitronectin and its association with PAI-1 at these sites however, is unknown. PAI-1 binding also interferes with the ability of vitronectin to associate with certain cell surface receptors (such as the urokinase plasminogen activator receptor (uPAR), and the integrins) [39, 40]. Therefore, it is clear that vitronectin and PAI-1 exert effects on one another at both the structural and functional levels, and that these effects no doubt have a consequence in physiological processes.

The self-association of vitronectin into higher order oligomers.

The pathway by which vitronectin undergoes self-association to form higher order multimers that constitute the ECM-associated form has not been well characterized. Since nearly all circulating PAI-1 is found complexed with vitronectin [38], and since PAI-1 is thought to produce conformational changes in vitronectin which might induce this transition [17, 41], the self-association of vitronectin into higher order multimers via PAI-

1 binding was investigated. In support of this, sedimentation velocity experiments using varying concentrations of vitronectin and PAI-1, in the analytical ultracentrifuge [16, 17] have shown that oligomerization most likely proceeds in a step-wise, concentration-dependent manner, with PAI-1 binding initially to a high-affinity site (residues 26-30 in the SMB domain) on vitronectin. Then, with increasing concentrations of PAI-1 a secondary site is bound to form a 2:1 (PAI-1:VN) complex. This complex is thought to be the key intermediate by which higher order complexes (i.e. multimers of 4:2, 8:4, etc.) are formed.

The binding of two PAI-1 molecules to vitronectin overturned the previous belief of a 1:1 association between the two proteins. However, this 2:1 stoichiometry has been supported in other studies as well [15, 18, 42-44]. Most recently, the binding interface between PAI-1 and vitronectin has been shown to include dual binding sites on each protein for one another, with the secondary binding site for PAI-1 lying outside the SMB domain of vitronectin [45, 46]. An overall scheme for the self-association of vitronectin via PAI-1 is shown in **Figure 4**.

Whether the 2:1 complex triggers a structural change that facilitates the formation of the 4:2 complex is unknown. Also unknown is at what limits the oligomerization of vitronectin? Measurements taken in the analytical centrifuge indicated the presence of intermediates greater than the 10S species shown **Figure 4** (e.g. 16.4S, 20.7S and higher). Therefore, while it is unlikely that oligomerization proceeds unchecked, it is not known at this point in time how the process ends.

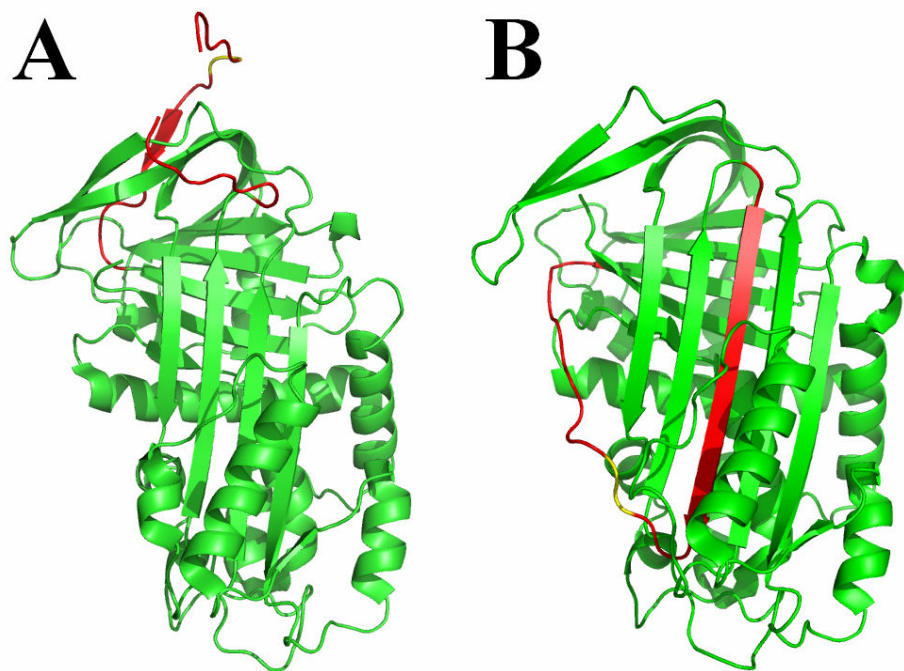


Figure 3. The active and latent structures of PAI-1.

The active (**A**) and latent (**B**) structures of PAI-1 (PDB files 1DVM and 1DVN respectively). The reactive center loop (RCL, residues 331-357) is highlighted in red, with the R346 and M347 (the P1-P1' bond which is cleaved during protease inactivation) residues shown in yellow [47].

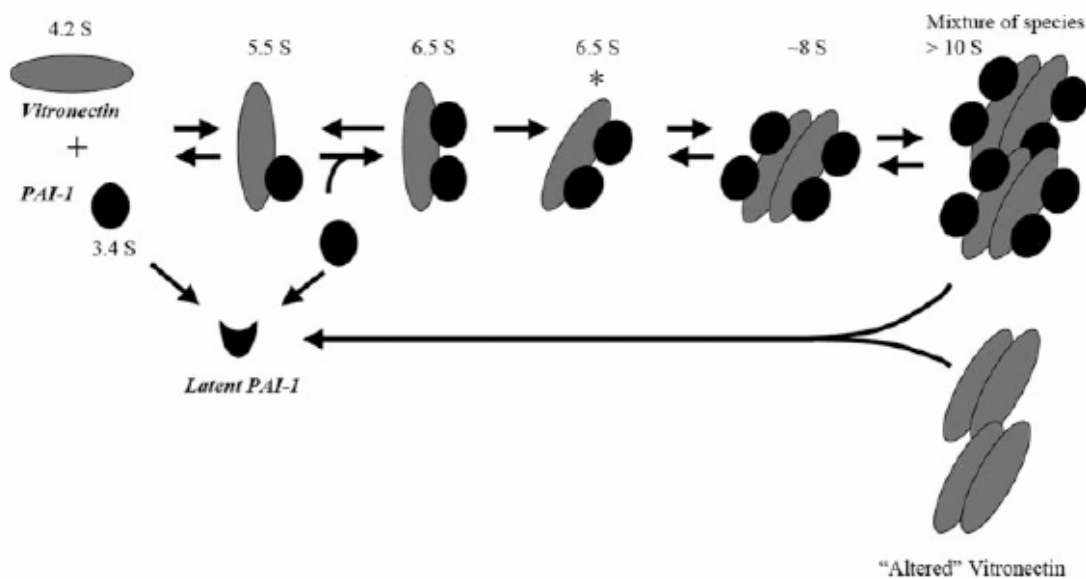


Figure 4. The self-association of vitronectin via PAI-1 binding.

The proposed association of PAI-1 and vitronectin, and the formation of oligomeric complexes. The asterisk shown above the 6.5S species indicates that a conformational change may occur to promote the formation of the ~8S (4:2, PAI-1:VN) species. (PAI-1 eventually dissociates from the higher order complexes, leaving behind the 'altered' vitronectin) [16].

The same study showed that higher order complexes of vitronectin persist long after PAI-1 has lost its ability to bind vitronectin, assuming its latent conformation and dissociating. Thus, there must be a region (or regions) within vitronectin that are able to associate with one another. This is the central focus of this study.

The Hypothesis.

The central hypothesis for this work is that the region that participates in the oligomerization of vitronectin is the Central domain. As was mentioned earlier, the Central domain is thought to be folded as a 4-bladed β -propeller. β -propellers are known to participate in protein-protein/intermolecular interactions [48-50]. Examples include WD40 structures involved in signal transduction [51-53], the head regions of integrins that recognize ECM ligands [50, 54, 55], and the β -propeller domains of matrix metalloproteinases [56, 57].

In addition to this, previous research done on the N-terminal and C-terminal domains of vitronectin also point towards the Central domain as being the region responsible for the self-association of vitronectin. The isolated N-terminal domain does not form higher order complexes when mixed with PAI-1 [19], thus ruling it out as a candidate for oligomerization. Early folding studies on vitronectin [58, 59], showed that denaturation followed by renaturation in the presence of heparin still resulted in oligomeric complex formation. Because the heparin binding site is localized to the C-terminal domain [18], the conclusion from this study was that if vitronectin did undergo

self-association via the C-terminal domain, the binding of heparin would have interfered with this process.

In yet another study, experiments done on a recombinant form of vitronectin, in which the terminal 80 amino acids had been deleted showed that this ‘truncated’ form of vitronectin possessed the same functionality and binding capabilities of full-length vitronectin, indicating that at least the terminal 80 amino acids were not necessary for oligomerization [60]. Although these findings do not conclusively rule out the C-terminal domain as being the region required, they do make it a less likely candidate for oligomerization when compared to the Central domain.

Rationale and Aims for this Project.

For this study, a ‘negative design’ strategy has been utilized in determining whether the Central domain is indeed the critical region in the oligomerization of vitronectin. As shown in **Figure 1**, the Central domain of vitronectin contains several hemopexin-like motifs. The ‘pexin’ motif is common to several homologous proteins resembling hemopexin, including interstitial collagenase (collagenase-3), gelatinase-A, and vitronectin [61]. The crystal structures of the C-terminal portion of rabbit serum hemopexin, along with the crystal structures of the C-terminal domains of collagenase-3 and gelatinase-A are shown in **Figure 5**.

All three of these proteins share the 4-bladed β -propeller fold that is predicted for the Central domain of vitronectin. However, in contradiction to the evidence suggesting that β -propellers can mediate intermolecular interactions, all three of these proteins do not exhibit self-associative behavior. Richardson and coworkers have attempted to

decipher the strategies employed by β -sheet proteins to prevent aggregation [62], with particular attention being paid to the amino acid residues that line the edge strands. Structural elements such as β -bulges have been postulated to help inhibit aggregation, as well as the presence of charged residues along the edge strands themselves. An initial study has been performed by Kevin Walters, who pursued an honors thesis based on analyzing and comparing the edge residues between vitronectin, hemopexin, gelatinase-A and collagenase, with respect to the results found in the Richardson study. His analysis of the edge residue patterns in vitronectin concluded that the edge residues localized in the upper left and lower left quadrants of the Central domain of vitronectin were most likely to assist in β -sheet extension. The edge residue sequences in these quadrants do not contain any structural motifs that would inhibit the self-association of the protein, such as β -bulges and charged residues that might be buried as a result of self-association. The edge residues in the upper and lower right quadrants however, were highlighted by both the inhibitory motifs mentioned. These findings corroborated well the three-dimensional arrangement of vitronectin deduced from SAXS measurements [26], which showed the C-terminal domain partially shielding the upper and lower right quadrants of the Central domain.

However, the rationale behind this analysis could not be extended to explain why vitronectin was able to undergo self-association, but hemopexin, gelatinase-A and collagenase-3 were not. This was because no striking differences in the edge residue sequence alignment between vitronectin and those of the other three proteins were found with respect to factors that influence β -sheet extension.

Therefore, to begin this investigation, recombinant proteins of vitronectin were constructed in which the Central domain of vitronectin was replaced with the homologous regions from the proteins hemopexin and gelatinase-A. The hemopexin-like motifs within the central domain of vitronectin make the former a suitable 'donor' for this type of exchange. With respect to the latter, the best match for a structural template for the Central domain of vitronectin in the sequence-structure alignment study [24] was gelatinase-A. Collagenase-3 was also considered, based on the structural and functional properties of the hemopexin and gelatinase-A chimeras.

Because hemopexin and gelatinase-A do not aggregate, it was expected that the vitronectin chimeras generated from these experiments should have an altered propensity to form multimeric complexes when mixed with PAI-1. This was the basis of the 'negative design' approach.

In addition to the two chimeras mentioned above, a third recombinant was pursued in which the C-terminal domain of vitronectin was eliminated. This construct was used to evaluate the importance of the C-terminal domain in higher order complex formation.

Yet another approach was to use limited proteolysis to isolate the Central domain of vitronectin, so that structural information of the domain region could be ascertained, as well as any aggregative properties.

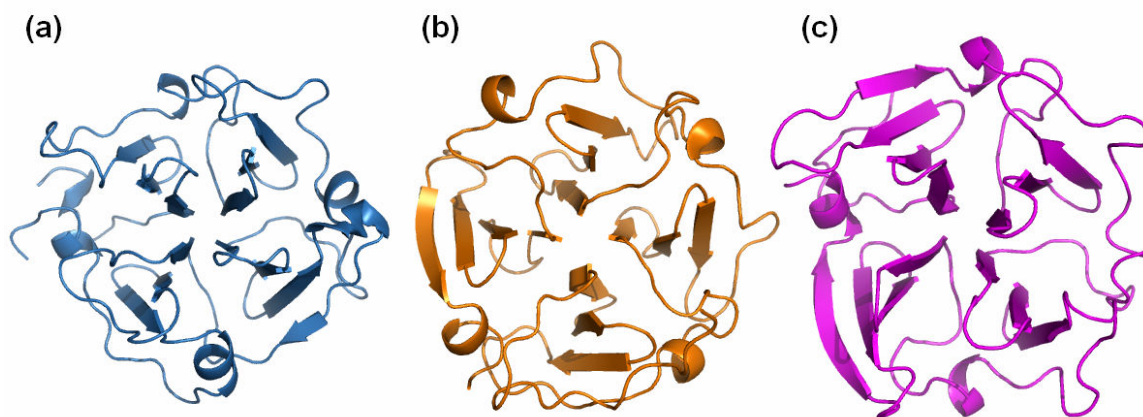


Figure 5. The crystal structures of hemopexin, gelatinase-A and collagenase-3.

The crystal structures of (a) the C-terminal domain of rabbit hemopexin (PDB file 1HXN [63]), (b) the C-terminal domain of gelatinase-A (PDB file 1RTG [64]), and (c) the C-terminal domain of collagenase-3 (PDB file 1PEX [65]).

The primary goals of this study were as follows –

1. To generate chimeric proteins of vitronectin, in which the Central domain of vitronectin was replaced with the homologous domains from hemopexin and gelatinase-A.
2. To generate a recombinant form of vitronectin, which lacked the C-terminal domain.
3. To express and purify all three of the constructs mentioned above.
4. To use limited proteolysis in an attempt to isolate the Central domain of vitronectin.
5. To test the structural and functional properties of the chimeras, recombinant and isolated Central domain in comparison to native, full-length vitronectin, with emphasis being placed on the ability to form higher order complexes when mixed with PAI-1.

Based on the results generated from these studies, the next step will be to try and narrow the region(s) of focus from the entire Central domain for example, to isolated residues along the edge strands.

Materials and Methods.

I. Cloning.

DH5 α cells containing pFASTBAC DNA (plus the full-length vitronectin cDNA) were grown up on LB-agar plates (containing 50ug/ml ampicillin) from an existing stock belonging to this laboratory (CP625). The plasmid DNA was then extracted from the cells using an SV Miniprep kit (Promega). The isolated DNA sample was then sequenced with P_{PH} polyhedrin primers (Invitrogen) to verify that there were no unwanted mutations present in the vitronectin cDNA. **Figure 6** shows the pFASTBAC1 vector used.

(a) Primer design.

(i) *VNHX*.

DH5 α cells containing pDNR-LIB DNA (plus full-length hemopexin cDNA) were ordered from ATCC. The cells were then grown up on LB-agar plates (containing 34ug/ml chloramphenicol). Plasmid DNA was extracted in the same manner as that of the pFASTBAC DNA. The isolated DNA was then sequenced with M13 forward and reverse primers to verify that there were no unwanted mutations present.

Mutagenesis using a Site-Directed Mutagenesis Kit (Stratagene) was then done to engineer the XhoI and NdeI restriction enzyme cut sites bordering the amino acid regions used in the domain exchange in both vitronectin and hemopexin DNA sequences.

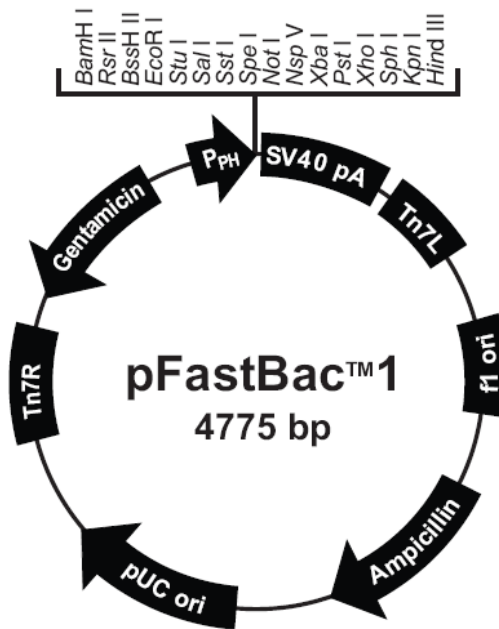


Figure 6. The pFASTBAC expression vector (Invitrogen).

(Taken from the Bac-to-Bac Expression Manual) The multiple cloning site is shown. The (**P_{PH}**) polyhedrin promoter, allows for high expression of protein in insect cells. The ‘expression cassette’ containing the vitronectin DNA is flanked by the left (**Tn7L**) and right (**Tn7R**) arms of the Tn7 transposon, to allow for site-specific transposition into DH10Bac E.coli cells. **Ampicillin** and **Gentamycin** resistance markers are also shown.

The primers that were used were as follows – for vitronectin/pFASTBAC, the forward primer sequence for the XhoI site was,

5' CCAGCAGAGGAGGAGCTCGAGAGTGGGAAGCCCTTCGACGCC 3'

and the reverse primer sequence was,

5' GGCGTCGAAGGGCTTCCCACTCGTGAGCTCCTCCTCTGCTGG 3'

The forward primer sequence for the NdeI site was,

5' GGCTGCGTCCATATGCCCTGGCACACCGTGCC 3'

and the reverse primer sequence was,

5' GGCACGGTGTGCCAGGGCATCTGGACGCAGCC 3'.

For hemopexin/pDNR-LIB, the forward primer sequence used for the XhoI site was,

5' GGGAACAGTATATGCTCGAGAGCCCACATCTAGTCTTGTCTGC 3'

and the reverse primer sequence was,

5' GCAGACAAGACTAGATGTGGGCTTGTGCGCATATACTGTTCCC 3'

.The forward primer sequence used for the NdeI site was,

5' GGCTGCATTCAGTTTCTCCACACATATGTAGCAGTACAAATTGGGACC 3'

and the reverse primer sequence was,

5' GGTCCCAATTTGTACTGCTACACACTTGTGGAGAACTGAATGCAGCC 3'.

(ii) *VNGA*.

Unique restriction enzyme sites for XhoI and EcoRI were engineered into both pFASTBAC and pOTB7 DNA samples. pOTB7, containing full-length gelatinase-A cDNA (ATCC) colonies were grown up on LB-agar plates containing 34ug/ml chlroamphenicol. Plasmid DNA extraction was done using an SV miniprep kit

(Promega). DNA samples were sequenced to verify the lack of unwanted mutations. The primer sequences were as follows – for pFASTBAC, the forward primer sequence for the XhoI site was,

5' CCCAGCAGAGGAGGAGCTCTCGAGTGGGAAGCCCTTCGACGC 3'

and the reverse primer sequence was,

5' GCGTCGAAGGGCTTCCCACTCGAGAGCTCCTCCTCTGCTGGG 3'.

The forward primer sequence for the EcoRI site was,

5' CGGGACTGGCACGGTGTGCCGAATTCAGTGGACGCAGCCATGGC 3'

and the reverse primer sequence was,

5' GGAGCCAGGGCCAGCTCAGGAATTCAGCCAGTCGGATTTGATGC 3'.

For pOTB7, the forward primer sequence for the XhoI site was,

5' CTGTCACTCCTGAGATCTGCAACTCGAGCATTGTATTTGATGGCATCGC 3'

and the reverse primer sequence was,

5' GCGATGCCATCAAATACAATGCTCGAGTTGCAGATCTCAGGAGTGACAG
3'.

The forward primer sequence for the EcoRI site was,

5' GCATCAAATCCGACTGGCTGAATTCCTGAGCTGGCCCTGGCTCC 3'

and the reverse primer sequence was,

5' GGAGCCAGGGCCAGCTCAGGAATTCAGCCAGTCGGATTTGATGC 3'.

(iii) *VNΔC*.

The forward primer sequence used to engineer the 'STOP' codon was,

5' GGTGTGCCAGGGCAATAGGACGCAGCCATGGCTGG 3'

and the reverse primer sequence was,

5' CCAGCCATGGCTGCGTCCTATTGCCCTGGCACACC 3'.

All primers used in (i), (ii), (iii) were ordered from Invitrogen. All primers were also designed according to the parameters specified in the Site-directed Mutagenesis kit instruction manual.

(b) Site-directed Mutagenesis.

Mutagenesis of each plasmid DNA sample was done with one set of primers at a time. PCR was used, with temperature and cycle parameters for the polymerase chain reaction given in the kit instruction manual. The reaction mixture was also prepared according to the instruction manual, with the reaction buffer, dNTPs and DNA polymerase components all provided with the kit. Following PCR completion, 1uL of Dpn1 was added to the PCR reaction mixture to digest the parental, non-mutated DNA. The mixture was incubated at 37°C for 1 hour to allow the digestion to proceed. Following digestion, 2uL of the PCR reaction was then transformed into 45uL of XL-10 Gold Ultracompetent cells (Stratagene), using a heat-shock method. First both the DNA and cells were incubated on ice for 30 minutes. This was followed by a 37°C heat shock for 45 seconds, followed by incubation on ice for 2 minutes. 500uL of **S.O.C.** (Super Optimal Broth with Catabolite repression, [66]) medium was then added, and the cells were allowed to grow for 1 hour at 37°C with shaking at 225-250 rpm. 100uL of cells were then plated onto LB-agar plates containing the antibiotic specific to the vector used in the mutagenesis (e.g. pFASTBAC – ampicillin, pOTB7 and pDNR-LIB – chloramphenicol). The plates were incubated at 37°C overnight and inspected for colonies the following day.

Colonies found on plates were selected and individually grown up in 10ml of Terrific Broth (Research Products International) containing an appropriate amount of antibiotic at 37°C overnight with shaking. Plasmid DNA was isolated using SV miniprep kits. The DNA samples were then submitted for sequencing to verify both successful mutagenesis, and no unwanted mutations.

(c) Performing the ‘Domain Exchange’.

Figure 7 shows a diagram illustrating the regions of both vitronectin and hemopexin that were to be substituted to accomplish the domain exchange.

The mutated DNA vectors that contained both unique restriction enzyme sites were then digested with the appropriate pair of enzymes for at least 1 hour at 37°C. The amount of each enzyme added varied depending on the concentration of the DNA sample and the activity of the enzyme. Digested samples were then electrophoresed on 0.8% agarose gels containing Ethidium Bromide (0.01 %) at 100V for 1 hour. The gels were then observed under UV-light to identify the bands corresponding to the DNA fragments generated by digestion within the newly designed restriction enzyme sites. These bands represented the pieces of DNA from each vector that would be ligated together to complete the domain exchange.

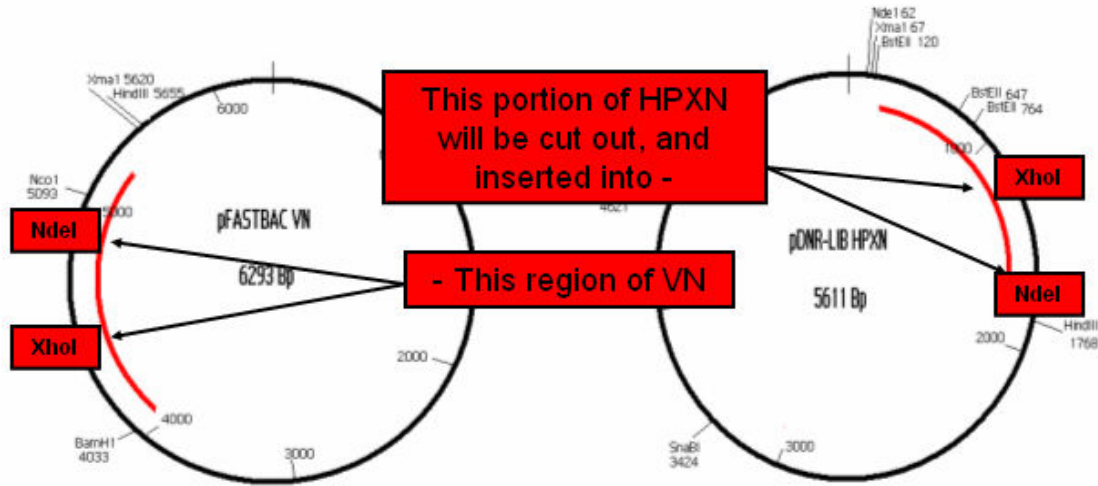


Figure 7. Diagram illustrating the domain exchange between vitronectin and hemopexin.

Plasmid maps of both the **pFASTBAC** and **pDNR-LIB** vectors, showing the locations of the cDNA sequences (red lines) in both vitronectin cDNA and hemopexin cDNA, and the known restriction enzyme sites (labeled). The unique restriction sites (**Xho1** and **Nde1**, red boxes) are also shown.

The appropriate bands were then excised from the gel manually, and the band fragments were purified by centrifugation, using Ultra-free DA tubes (Amicon). The gel extraction samples were then ligated using T4 DNA ligase, in a ratio of 1:5 (vector:insert). For example, 1uL of the DNA from the band highlighted in [B] (Figure 8) was mixed with 5uL of DNA from the band highlighted in [A]. The reaction was done overnight, at 15°C. T4 DNA ligase and reaction buffer (New England Biolabs), were combined according to the accompanying instruction manual.

Two microliters of the reaction mixture was then transformed into XL-10 Gold Ultracompetent cells by the same protocol used for the mutagenesis experiments. Cells were plated on antibiotic plates containing 50ug/mL ampicillin and incubated overnight at 37°C. Colonies that grew were then picked manually and grown individually in 10ml cultures containing the appropriate amount of antibiotic, and the plasmid DNA isolated using an SV miniprep kit. DNA samples were then sequenced to verify the completed construction of both the VNHX and VNGA constructs, as well as the absence of any unwanted mutations.

II. Virus generation.

A schematic diagram outlining the steps taken in utilizing the Bac-to-Bac Baculovirus expression system is shown in **Figure 8**.

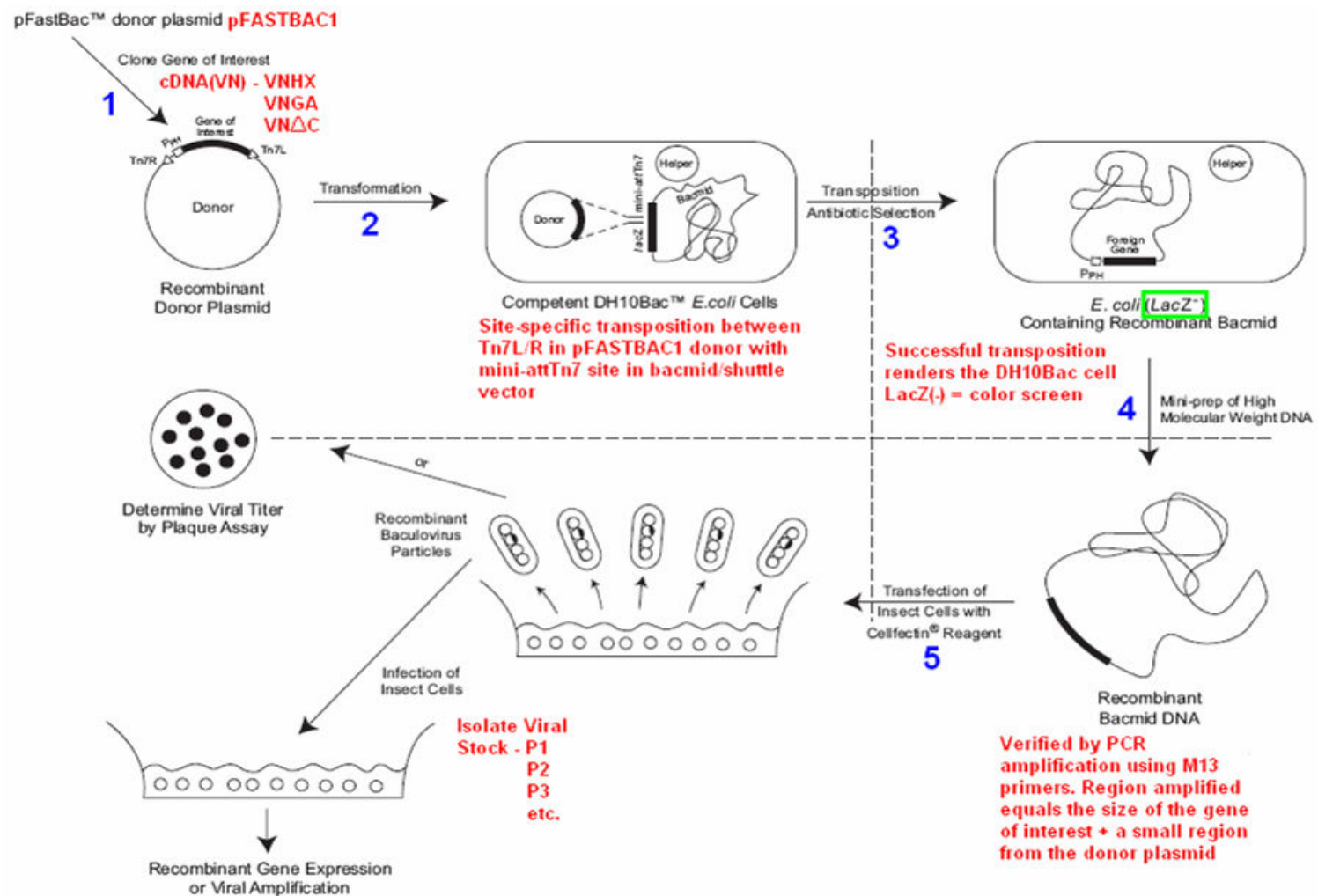


Figure 8. The Bac-to-Bac baculovirus expression system. (Taken from the Bac-to-Bac Expression Manual)

(a) Transforming into DH10Bac cells.

The DH10Bac cells contain a portion of the baculovirus genome, or 'bacmid' which contains a segment that allows for site-specific transposition between the bacmid and the gene of interest in the pFASTBAC vector.

One nanogram of each pFASTBAC construct was added to 100uL of DH10Bac cells and allowed to incubate on ice for 30 minutes. The cells then underwent heat-shock for 45 seconds at 42°C without shaking, and were immediately transferred back to ice for 2 minutes. 900uL of S.O.C. medium at room temperature was then added, and the mixture was incubated for 4 hours at 37°C with shaking at 225 rpm.

Following this, 10-fold serial dilutions of each mixture were prepared with S.O.C. medium (10^{-1} , 10^{-2} , 10^{-3}). 100uL of each dilution were then plated onto LB-agar plates containing 50ug/mL kanamycin, 7ug/mL gentamycin, 10ug/mL tetracycline, 100ug/mL Blueo-gal, and 40ug/mL isopropyl β -D-1-thiogalactopyranoside (IPTG). The plates were then allowed to incubate for 48 hours at 37°C.

After the 48 hour incubation period, any white colonies that were found were then picked and re-streaked onto fresh LB-agar plates containing the antibiotics, Blueo-gal and IPTG in the same concentrations as mentioned above. The plates were allowed to incubate overnight at 37°C.

(b) Recombinant Bacmid DNA prep.

White colonies that grew on the re-streaked plates were then picked and grown up in 20ml of TB containing 50ug/mL kanamycin, 7ug/mL gentamycin, and 10ug/mL tetracycline. The culture was grown overnight at 37°C with shaking. The recombinant bacmid DNA prep was then performed, following the instructions given in the Bac-to-

Bac manual. (Note – the Bac-to-Bac manual offered the option of using a S.N.A.P. midiprep kit to isolate the DNA, but this was not used. Instead a procedure that had been previously used to isolate large plasmids was referred to). The DNA was stored at -20°C.

(c) Verification of site-specific transposition by PCR.

As illustrated in Figure 10, steps 1-5 were performed for each of the constructs designed. Prior to transfection into the insect Sf9 cells however, PCR amplification was done using M13 primers as a second level of verification to ensure successful transposition before continuing onto step 5.

Following the instructions in the Bac-to-Bac manual, the PCR reaction was done using all bacmid DNA samples with M13 primers (Invitrogen).

(d) Transfection of Insect Cells.

[Note – all steps here were performed in a Fisher Hamilton Class II Type A (A/B3) Biological Safety Cabinet, Model # 54L936]

(i) *Culturing Sf9 cells.*

Sf9 (*Spodoptera frugiperda*) cell cultures were initiated from a frozen stock following the directions given in the accompanying instruction manual (Invitrogen, Insect Cell Lines Instruction Manual, Version E, 180427). In short, cells were weaned off 10% FBS, and kept at a concentration of 0.5×10^6 – 4.0×10^6 cells/mL in serum-free insect cell culture media (HyClone SFX-Insect, Fisher Scientific) in 10ug/mL gentamycin. Cultures were incubated at room temperature under gentle shaking at 150 rpm.

(ii) Transfection of Sf9 cells using recombinant bacmid DNA.

In a T75 rectangular flask, 1.2×10^7 cells/ml were added with sufficient antibiotic-free insect cell growth medium to coat the cells along the bottom of the flask. The cells were allowed to attach for an hour at 27°C. While the cells were incubating, the following samples were prepared –

1. 15ug of bacmid DNA was mixed with 1.5mL of antibiotic-free insect cell growth medium.
2. 90uL of Cellfectin reagent (Invitrogen) was diluted in 1.5mL of antibiotic-free insect cell growth medium.
3. The solutions in 1 and 2 were then mixed together and allowed to incubate for 45 minutes at room temperature.

After the cell attachment period, the existing media was removed, and the cells were washed once with 10mL of antibiotic-free growth medium. The medium was then also removed, and 12mL of antibiotic-free growth medium was added to the cells, along with the solution prepared in 3 above. The cells were then allowed to incubate for 5 hours at 27°C in the T75 flask.

After the incubation period, the media inside the flask was removed, and 15mL of fresh insect cell growth medium containing 10 mg/mL gentamycin was added to the cells. The cells were then incubated at 27°C for at least 72 hours, to allow for viral infection.

(d) Harvesting P1 Viral Stock.

Viral infection was allowed to proceed for a maximum of 7 days (Bac-to-Bac manual). To harvest the initial P1 viral stock, the medium in the T75 flask was collected and centrifuged at 500xg for 5 minutes to remove cellular debris. The supernatant, which

contained virus was then collected, and stored at +4°C protected from light. Subsequent amplification of the P1 viral stock was then done by infecting 50mL cell cultures of Sf9 cells at $1-2 \times 10^6$ cells/mL with 2mL of viral stock. Infection was allowed to proceed for 72 – 96 hours at 27°C with shaking, and virus collection was done in the same manner as described above to generate P2, P3 and P4 viral stocks for each construct.

III. Expression/Detection/Purification of Recombinant Protein.

(a) Expression.

50 ml cultures of insect Sf9 cells at 1.0×10^6 cells/ml were infected with 5 ml of P4 viral stocks of each construct (VNHX, VNGA, VNΔC). After a period of 4-7 days, at which point the cell count dropped to 5.0×10^5 cells/ml, the cultures were centrifuged at 500 rpm for 10 minutes to remove cellular debris, and the supernatants were concentrated down to 1ml each using Amicon Ultra Centrifugal Filter Devices (Millipore). 5uL protease inhibitor cocktail (Sigma) was then added at a ratio of 1:200 (protease:supernatant).

Cell pellet samples were lysed using homemade lysis buffer containing 10mM NaH_2PO_4 , 10% glycerol (w/v), 0.6% CHAPS (w/v), and protease inhibitors (filter-sterilized, 1:200 ratio). Pellet samples were resuspended in two milliliters of buffer, vortexed/sheared for 2 minutes and then spun down at 14,000 rpm. The supernatant was then analyzed using SDS-PAGE and Western Blotting under reducing conditions, and the remaining pellet sample re-lysed using the same lysis buffer in the presence of 2M urea. The same steps were then repeated as described above, and the final supernatant and final pellet were analyzed by SDS-PAGE and Western blot analysis.

(b) Detection.

All samples were subjected to SDS-PAGE using the buffer system of Laemmli [67]. Proteins were then transferred onto nitrocellulose using a modified version of the buffer system of Towbin et. al [68]. Both procedures were done under reducing and non-reducing conditions. With regard to the latter, nonspecific binding was blocked with 10% non-fat skim milk in PBS buffer (137mM NaCl, 2.7mM KCl, 4.3mM Na₂HPO₄, 1.47mM KH₂PO₄, pH 7.4). The nitrocellulose membrane was then incubated (at room temperature, with gentle shaking) with rabbit polyclonal anti-vitronectin antibody (1:4000 dilution) for 1 hour, and then washed three times with Western Wash buffer (PBS + 0.1% (w/v) Tween). Peroxidase-conjugated goat anti-rabbit (Vector laboratories) was then added (1:6000 dilution) and the mixture was incubated in the same manner as before. After 1 hour, the membrane was washed in the same manner as previously described. The membrane was then developed by adding PBS and 4-chloro-1-naphthol (in methanol) in a ratio of 35:6 (ml:ml). Hydrogen peroxide (Fisher) was then added at a 1:2000 dilution to facilitate color development. The reaction was then stopped by washing the membrane three times for 15 seconds with de-ionized water. Both SDS-PAGE gels and Western blots were imaged using a Bio-Rad ChemiDoc XRS Photo-documentation system, using the Quantity-One program (version 4.6.5).

(c) Purification (VNΔC).

Four 1L Erlenmeyer flasks were filled with 500mL each of insect Sf9 cell cultures at a density of 1.25x10⁶ cells/ml. 50mL of a P5 VNΔC viral stock was then equally distributed over the four flasks, and viral infection was allowed to proceed for 5 days at room temperature with shaking (100-125 rpm). On Day 5, the cultures were harvested

and centrifuged at 500xg for 30 minutes. The supernatant was then separated from the cell pellet and brought to pH 7.4. The supernatant was then allowed to sit at 4°C for at least 1.5 hours to allow any precipitate to form. After this time, the supernatant was centrifuged for 30 minutes at 500xg and pH adjusted to 7.4. The final mixture was then filtered using a 500mL filter system equipped with a 0.22 micron filter (Corning) and then loaded onto a 15mL Blue Sepharose column (Blue Sepharose 6 Fast Flow, GE) that had been equilibrated with a solution of 50mM sodium phosphate, 0M NaCl, 0.1mM EDTA (pH 7.4). After loading, the column was washed to baseline conditions using the same buffer. Bound proteins were then eluted using a gradient of 0M-1M NaCl. 8ml fractions were collected and checked for absorbance at 280nm. Every third fraction was then subjected to SDS-PAGE electrophoresis and Western blot analysis under reducing conditions as previously described, with a rabbit anti-vitronectin polyclonal antibody (Bunny12) used as the primary antibody. Fractions were then pooled and mixed with Ammonium Sulfate in a ratio of 472 g/mL.

The preparation of the immuno-affinity column was attempted by using the PIERCE Protein G IgG Plus Orientation Kit (#44990). Monoclonal antibody 1244 was fixed onto the column using the instructions provided in the kit. After assembly, VNΔC (under Ammonium Sulfate), was spun down and resuspended in 1X Tris buffer (1M Tris-HCl plus 1mM EDTA, 0M NaCl, pH 7.4), and dialyzed overnight against the same buffer. The sample was then loaded onto the column, washed with 1X Tris buffer, and then eluted using PIERCE's Gentle Ag/Ab Elution Buffer (pH 6.6, #21067). Samples (load, wash, elute) were collected, concentrated and dialyzed (only elute sample)

overnight against 1X Tris buffer prior to being subjected to SDS-PAGE and Western Blot analysis under reducing conditions.

IV. Structural/Functional Binding Studies.

(a) Monoclonal antibody experiments.

All monoclonal antibodies (153, 1244, 2C3, 1E9, 2410, 8H1, 4A1) were kept in our lab, donated/purchased from various sources. For the assay, a total of sixteen 8% SDS-PAGE gels were poured. Seven gels were allocated for each monoclonal antibody to be used, and the last gel was used a control reaction for Bunny 12. All samples were tested under both reducing and non-reducing conditions. All construct/control samples were then added to the wells. After transferring the proteins to a nitrocellulose membrane in the same manner as described for III(b), the membrane was blocked with 10% non-fat skim milk in PBS for 1 hour, at room temperature with gentle shaking. The membrane was then washed three times with Western wash buffer, and then each monoclonal antibody was added at a 1:1500 dilution to the appropriate membrane in milk/PBS. The membrane/antibody solution was then incubated for one hour, after which the membrane was again washed as previously described. Peroxidase-conjugated anti-mouse antibody (Vector laboratories) was then added at a 1:5000 dilution, and the solution incubated a final time for one hour. The membrane was then washed and developed in the same way as described in III(b), and the results imaged using the same Bio-Rad Chemidoc-XRS Photo-documentation system..

(b) Surface Plasmon Resonance experiments.

[Note – all experiments were conducted using a Biacore3000 instrument (Biacore, Uppsala, Sweden), coupled with the Biacore3000 control software program (version 4.1).]

A CM5 Sensor chip (BR-1000-14) was coated with rabbit anti-mouse antibody using a primary amine coupling method. Two of the four flow cells on the chip were used in these experiments. One was used as a control, over which no antibody (1E9/1244) was injected over. The other one was used as the experimental surface over which binding between the analytes was to be measured. Using a flow rate of 10uL/min, 10uL of a 5ug/mL stock solution of 1E9 was injected over one flow cell only. A binding response change of between 70-90 units was observed (for mAb 1244, 10uL of an unknown stock solution was injected over one flow cell, and a binding response change of between 190-230 units was observed). After establishing a new baseline, 20uL injections of each construct were then done over both flow cells, and the binding responses recorded as a difference between flow cells to remove any nonspecific binding. After again establishing a new baseline, 20uL of 1uM wild-type PAI-1 was then injected over both flow cells, and the binding response change recorded. Wild-type PAI-1 was then injected again in the same manner until no binding response change could be detected. Using the formula that 1 response unit change is equivalent to 50 pg/mm² bound material [69], the response unit changes observed over each injection were then translated into concentrations. After each construct was tested, the surface over both flow cells was regenerated by flushing the chip with 10mM glycine, pH 1.7. This was made by mixing 4.6 ml of 10mM glycine, pH 1.5 with 5.4 ml of 10mM glycine, pH 2.0. HBS-EP (0.01 M HEPES, pH 7.4, 0.15M

NaCl, 3mM EDTA, 0.005% Surfactant P20), filtered, degassed was used as running buffer. All of these solutions were purchased from Biacore.

V. Limited Proteolysis Study.

(a) Digestion of Vitronectin with Plasmin.

Plasma vitronectin was purified using a modification of the original procedure by Preissner, et. al [3]. From the purification, 1 milligram of vitronectin was obtained. The vitronectin sample was dialyzed twice over one 6 hour period and then overnight in 20mM Tris/135mM NaCl buffer at 4°C. The buffer was adjusted to pH 7.4 at 37°C to, which would be the temperature that the proteolysis would be performed at. After dialysis, iodoacetamide (Sigma) was added to the solution to a final concentration of 10mM. The mixture was allowed to incubate at 37°C for 45 minutes to allow the iodoacetamide to alkylate any of the free sulfhydryls present.

Human Plasmin (Hematologic Technologies) was then added to the mixture in a 1:20 (enzyme:substrate) ratio. Prior to the addition, a 100uL sample was removed and added to a 1.5ml Eppendorf tube containing 11.1uL of 10% TFA (Pierce). The tube was marked "Time 0". After the addition of plasmin, subsequent 100uL aliquots were removed from the reaction mixture at 1 hour, 3 hour and 24 hour time points.

9uL samples from each tube were then subject to SDS-PAGE electrophoresis under both reducing and non-reducing conditions. To obtain good separation of fragments, the samples were run on a 4-12% Tris-Glycine gel (Invitrogen). The gel was then Coomassie-stained, and developed.

Following the development of the gel, 100uL of each sample were injected onto RP-HPLC column (Bydac). The column was developed using a 5-70% acetonitrile

gradient in water at a flow rate of 1mL/min. Because no identification of the peaks could be made simply on the basis of retention time, samples from the fractions collected for each injected sample were then analyzed by mass spectrometry.

(b) MALDI-TOF analysis of digest samples.

An α -Cyano-4-Hydroxycinnamic acid matrix was dispensed onto a MALDI plate (MSP 96 ground steel target, Bruker Daltonics) using the thin layer method outlined in Zhao, et. al 2004 [70]. Samples from fractions corresponding to the retention times in **Figure 25** were then added to the matrix by the dried droplet method. Mass spectra were obtained using a Bruker Daltonics Microflex MALDI-TOF spectrometer, in the linear mode. 500-1000 shots per spectrum were generated, with an acceleration voltage of 20KV.

VI. Bioinformatics Study.

Using the Seqbuilder program of Lasergene (DNASar), the amino acid sequence of human vitronectin was blasted against the database of known protein sequences at NCBI (Server – <http://www.dnastar.com/blast/ncbi-blast.html>). As expected, the top matching results were of vitronectin sequences belonging to different organisms. These were then downloaded and compared by a ClustalV alignment with one another and human vitronectin, using the MegAlign program of Lasergene.

For the blade/ β -strand homology study, the amino acid sequences of VNHX and VNGA were entered into the MegAlign program, alongside native human vitronectin. Each chimera's sequence was then aligned to native vitronectin using a Lipman-Pearson alignment (default parameters : Ktuple – 2, Gap Penalty – 4, Gap Length Penalty – 12). The β -strands for each protein were identified using the available crystal/predicted

structures. For VNGA, the β -strands were identified using PDB file 1RTG; for VNHX, PDB file 1HXN was used, and the equivalent amino acid sequences in human hemopexin were used, etc. All PDB files, along with the predicted Central domain structure of native vitronectin were visualized using PyMol software (© 2006 DeLano Scientific LLC).

Results and Discussion.

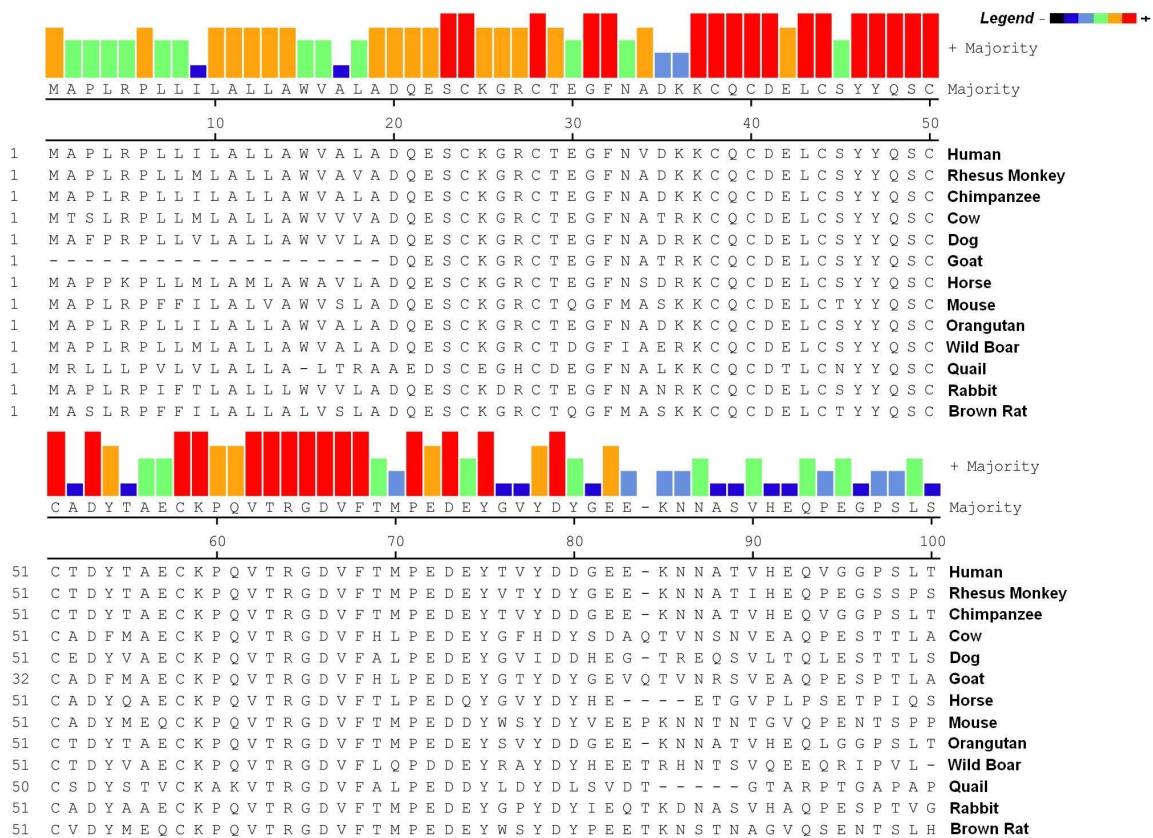
Sequence analysis of Vitronectin among certain Eukaryotes.

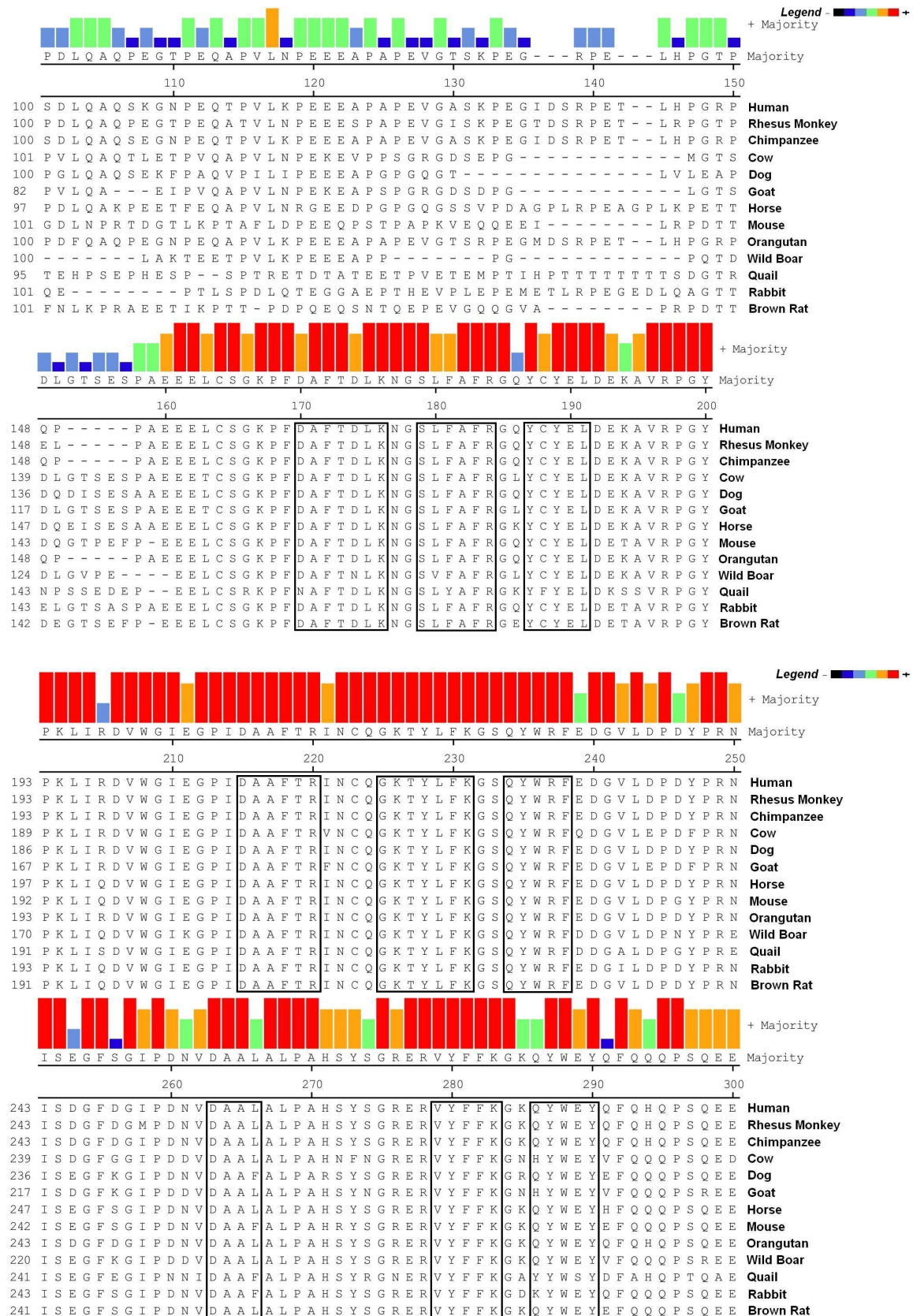
As was stated in the ‘Rationale and Aims’ section, the Central domain of vitronectin is believed to play a critical role in the self-association of the protein. The Central domain is predicted to assume a 4-bladed β -propeller fold structure [24], based on its homology with the β -propeller regions in hemopexin and gelatinase-A [24, 71]. To see whether this homology was reflected in the vitronectin sequences in other organisms, a sequence-alignment study was done. The results from this analysis of the amino acid sequences of vitronectin among different eukaryotes further strengthened the potential function of the Central domain. The sequence alignment reveals two pieces of information (**Figure 9**). Firstly, two main regions within the sequences are conserved. One encompasses the Central domain of the protein (residues 160-360). The other is the N-terminal domain (residues 20-68). The ‘RGD’ sequence that is responsible for binding integrins [6, 7] is completely conserved, and the PAI-1 binding sequence [19] is almost totally conserved. The conservation of the Central domain of vitronectin between organisms lends strong support to it having a functional role in the protein.

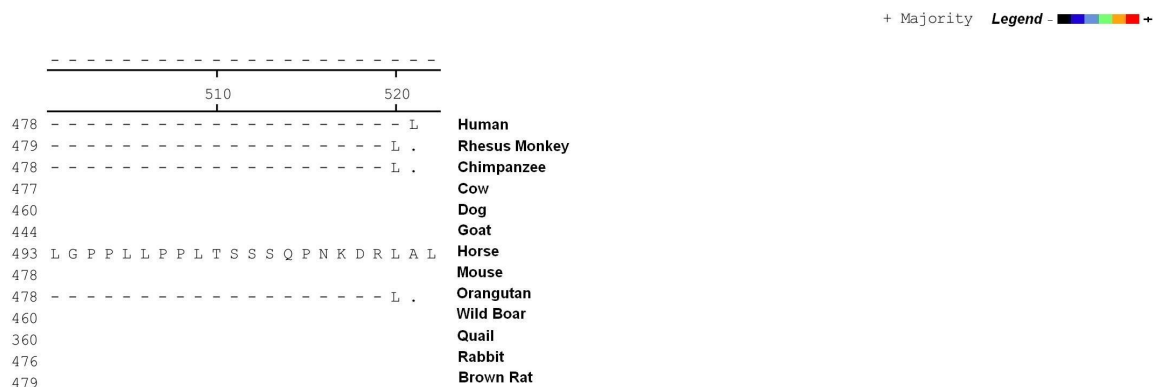
Secondly, analysis of the conservation within the Central domain region shows that peptide sequences corresponding to the β -strands that make up the 4-bladed β -propeller predicted structure are also very well conserved. Taken together, these findings point toward a functional role for the Central domain in vitronectin, which may be integral to the self-association of the protein, based on the evidence that it assumes a β -propeller fold, which facilitates aggregation.

Figure 9. The amino acid sequence alignment of vitronectin among various eukaryotes.

The legend, indicating total/strong conservation (+, red) and no/weak conservation (-, blue) across the spectrum is shown in the top right of each 100 residue fragment. The residues comprising each β -strand across the Central domain of vitronectin are outlined in black boxes. (Note – the amino acid sequence labeled ‘Majority’ does not pertain to human vitronectin, but rather a true consensus sequence based on the majority of residues for a given amino acid position across all sequences compared).







Extended sequence homology analysis between vitronectin, hemopexin and gelatinase-A.

As was shown in **Figure 1**, vitronectin contains several ‘pexin’ motifs between its Central and C-terminal domains. An early study on the sequence homology between vitronectin and hemopexin suggested that the two originated from a primordial gene, through differential gene duplication of an original peptide sequence [71]. This same study also highlighted the repeated peptide sequences common to both vitronectin and hemopexin. **Figure 10** highlights some of the data from this study. As was done in the sequence analysis of vitronectin across certain eukaryotes (**Figure 9**), the β -strands that make up each β -blade were identified and overlaid onto the results of the alignment. The results are shown in **Figure 11**.

The homology between vitronectin and gelatinase-A was noted in the threading and docking study that was done [24]. **Figure 12** shows some of the data from that study which illustrates the homology between vitronectin and gelatinase-A. As was mentioned

previously, the results from this threading study suggested that the best structural template for vitronectin was gelatinase-A.

To further examine the results outlined in the two studies above, a similar alignment-based approach was used to investigate the homology between vitronectin, hemopexin and gelatinase-A. The aim of this study was to investigate where the homology between each protein resided in terms of structure, using the 4-bladed β -propeller crystal structures of rabbit hemopexin and human gelatinase-A as references [63, 64]. **Figure 13** shows a Lipman-Pearson alignment between native full-length vitronectin and the proposed vitronectin-hemopexin chimera (hereafter denoted as VNHX). **Figure 14** shows the same alignment, only between native full-length vitronectin and the proposed vitronectin-gelatinase-A chimera (hereafter denoted as VNGA).

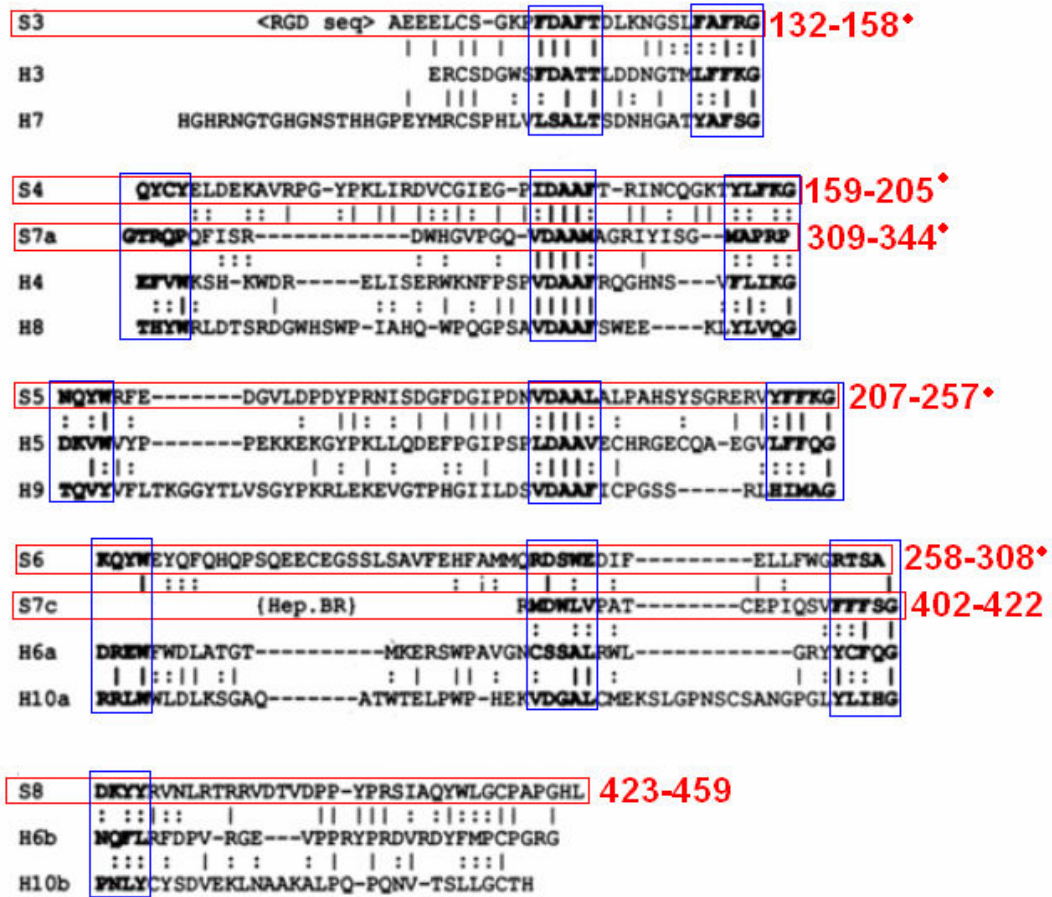


Figure 10. The alignment of exon-encoded protein segments for vitronectin and hemopexin.

[71] Exon-encoded protein segments for vitronectin (S3-S8, red boxes) and hemopexin (H3-H10). Vertical black bars show identical residues, while colons (:) show similar residues. The 'pexin' motifs homologous to both proteins are shown in bold lettering, outlined by the blue boxes. The residue ranges for each vitronectin exon are labeled in red, the red asterisks (*) denoting that the range is within the Central domain. (Note – Exon 7 of vitronectin is composed of S7a (with (**Hep.BR**) denoting the heparin binding sequence), and S7c).

```

S3      <RGD seq> AEEELCS-GKPTDAFTDLKNGSLFAFTG
              | | || | || | ||:::|
H3      ERCSDGWSFDATTLLDDNGTMLFTKG
              | || | : : | | : : |
H7      HGHRNGTGHGNSTHHGPEYMRCSPHLVLSALTS DNHGATYAFSG

S4      QYCYELDEKAVRPG-YPKLIRDVCGIEG-PIDAAFT-RINCCGKTYLFTG
              :: : : | : | || : : : | ||:::| : : | : : :
S7a     GTRQPFISR-----DWHGVPGQ-VDAAMAGRIYISG--MAPRP
              : : : : : : : : : : : : : : : :
H4      KFWKSH-KWDR-----ELISERWKNFPPVDAAFRQGHNS--VFLING
              : : : : | : : : | | |||| : : : :
H8      THYWRLDTSRDGWHSWP-IAHQ-WPQGPSAVDAAFSWE--KLYLVQG

S5      NQYWRFE-----DGVLDPDYPRNISDGFDPDNVDAALALPAHSYSGRERVYFFKG
              : : : : : ||: : | || | : ||: : : : | : || |
H5      DKVMVYP-----PEKKEGYPKLLQDEFPGIPSPLDAAVECHRGECQA-EGVLFTQG
              | : : : : | : : : : : : : : : : : : : :
H9      TVYVFLTKGGYTLVSGYPKRLEKEVGTPHGIILDSVDAAFKCPGSS----RLHIMAG

S6      NQYWEYQFQHQPSEQEECEGSSLSAVFEHFAMMORDSWEDIF-----ELLFWGRTSA
              | : : : : : : : : : : : : : : : :
S7c      (Hep. BR)      RMDNLVPAT-----CEPIQSVYFFSG
              : : : : : : : : : : : : : : : :
H6a     DRENFWDLATGT-----MKERSWPAVGNCSALRWL-----GRYYCFQG
              | : ||: : | : ||: : | : ||: : | : ||: : |
H10a    KRLRWLDLKSGAQ-----ATWTELPWP-HEKVDGALCKEKLGPNSCSANGPGLYLING

S8      DKYYRVNLRTRRVDIVDPP-YPRSIAQYWLGCAPAGHL
              : : : : : | : || | : : : : : |
H6b     NQFLRFDPV-RGE---VPPRYPRDVRDYFMPCPGRG
              : : : : | : : : : | : || : : :
H10b    FNLYCYSDVEKLNAAKALPQ-PQNV-TSLLGCTH

```

Figure 11. The alignment of exon-encoded protein segments for vitronectin and hemopexin (cont).

[71] Similar to Figure 10 on the preceding page. Residues comprising the β -strands for both vitronectin (red boxes) and hemopexin (green) are shown, with the edge strands at the boundary of each β -blade colored in yellow.

```

Query: 123 LHPGRPQPFAEEELCSGKP-FDAFTDLKNGSLFAFRGQYCYELDEKAVRPGYPKLIRDVW 181
          |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
Templ: 461 -----LGPVTPEICKQDIVFDGIAQIR-GEIFFFKDRFIWRTVTPRDKPMGPLLVAFW 513

Query: 182 G-iegpidaafrincQGKTYLFKGSQYWRFE DGVLD PDYPRNI-SDGFDGIPDNVDAAL 239
          :  |||  :  :  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
Templ: 514 PELPEKIDAVYEAPQ-EEKAVFFAGNEYWIYSASTLERGYPKPLTSLGLPPDVQRVDAAF 572

Query: 240 ALPAHSYSGRERVYFFKKGQYWEYQFQHQPFSQEECEGSSLSAVFE-----hfammqrdsw 294
          :  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
Templ: 573 -----NWSKNKKTYIFAGDKFWRYNEVKKKMDPGFPKLIADAWNAIPDNLDAVVDLQGGG 627

Query: 295 edife----LLFWGRTSAGTRQPQFISRDWHGV 323
          |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
Templ: 628 HSYFFKGAYYKLENQSLKSVKFGSIKSDWLGC 660

```

Figure 12. Sequence homology between vitronectin and gelatinase-A.

[24] Threading/Sequence-structure alignment between the Central domain of vitronectin (red boxes, ‘**Query**’), and the C-terminal domain of gelatinase-A (‘**Templ**’). Vertical black bars show identical residues, while colons (:) show similar residues. The residue ranges are numbered to the left and right of each of the sequences. The residues with lowercase lettering indicate different alignments predicted between PROSPECT and PSI-BLAST, two of the alignment programs used in the study.

[illegible]

Figure 14. Structural-based homology between vitronectin and gelatinase-A.

Lipman-Pearson alignment using the MegAlign program (Lasergene). The amino acid sequences of both Native VN (top) and VNGA (bottom) are shown, residue numbers on the right-hand side. Each β -strand is outlined in a black box, with the outermost β -strand is colored in yellow. Residues colored in red with a vertical line between them indicate perfect matches. Data values in the top left corners refer to the default parameters that were used when doing the alignment. Favorable mismatches are denoted by a colon (:), while neutral mismatches by a period (.)

Because each blade of a β -propeller structure is composed of at least three to four β -strands [48], it is useful to see whether the homology between vitronectin, hemopexin and gelatinase-A actually resides within these structural elements. As is shown in **Figures 13 and 14**, this is predominantly the case. While some of the strands outlined share little or no homology, there is enough data from this analysis to support a distant homology between the β -propeller domain regions of vitronectin, hemopexin and gelatinase-A. This result further substantiates the replacement of the Central domain of vitronectin with the C-terminal domain regions of hemopexin and gelatinase-A.

Design of the VNHX and VNGA chimeras.

Using the crystal structures of the C-terminal domains of hemopexin and gelatinase-A [63, 64], along with the predicted Central domain structure of vitronectin [24], the residue ranges that would be used in the domain exchange were identified. Because unique restriction sites had to be engineered into the cDNA of all three proteins, the border residues were carefully selected so that the most subtle amino acid changes would be made as a result of the site-directed mutagenesis that followed. It was decided that the residue ranges between Leu136-Gln326 in vitronectin, Pro259-Tyr436 in hemopexin and Gln471-Leu658 in gelatinase-A would be used. A detailed description of the construction of both chimeras is listed in the Materials and Methods section. **Figure 15** shows the gel pictures highlighting the cDNA bands that were ligated together to form the final VNHX construct.

Designing the C-terminal deletion mutant.

As was shown in **Figure 1**, three of the ‘pexin’ motifs reside in the C-terminal domain of vitronectin. In addition to this, the predicted structure for this domain is half of a 4-bladed β -propeller fold [24]. Also mentioned was the evidence suggesting that the C-terminal domain of vitronectin was not likely to participate in the self-association of the protein when mixed with PAI-1 [58, 60]. While the C-terminal 80 amino acids do not appear to convey any functional significance to the protein [60], it was decided to test whether eliminating the C-terminal domain of vitronectin completely would abolish higher order complex formation. Therefore, a C-terminal deletion mutant (hereafter denoted as VN Δ C) was pursued.

Unlike the considerations that had to be made for the VNHX and VNGA chimeras, the only parameter to consider in the designing of the VN Δ C mutant was where to place the ‘Stop’ codon in order to terminate the sequence of vitronectin. Because it is not entirely clear where the C-terminal domain truly begins in vitronectin in terms of secondary structure, an initial point of reference was Val327. This residue marked the end-point of the region used for the domain exchanges in the VNHX and VNGA chimeras. A detailed description of the construction of this mutant is listed in the Materials and Methods section.

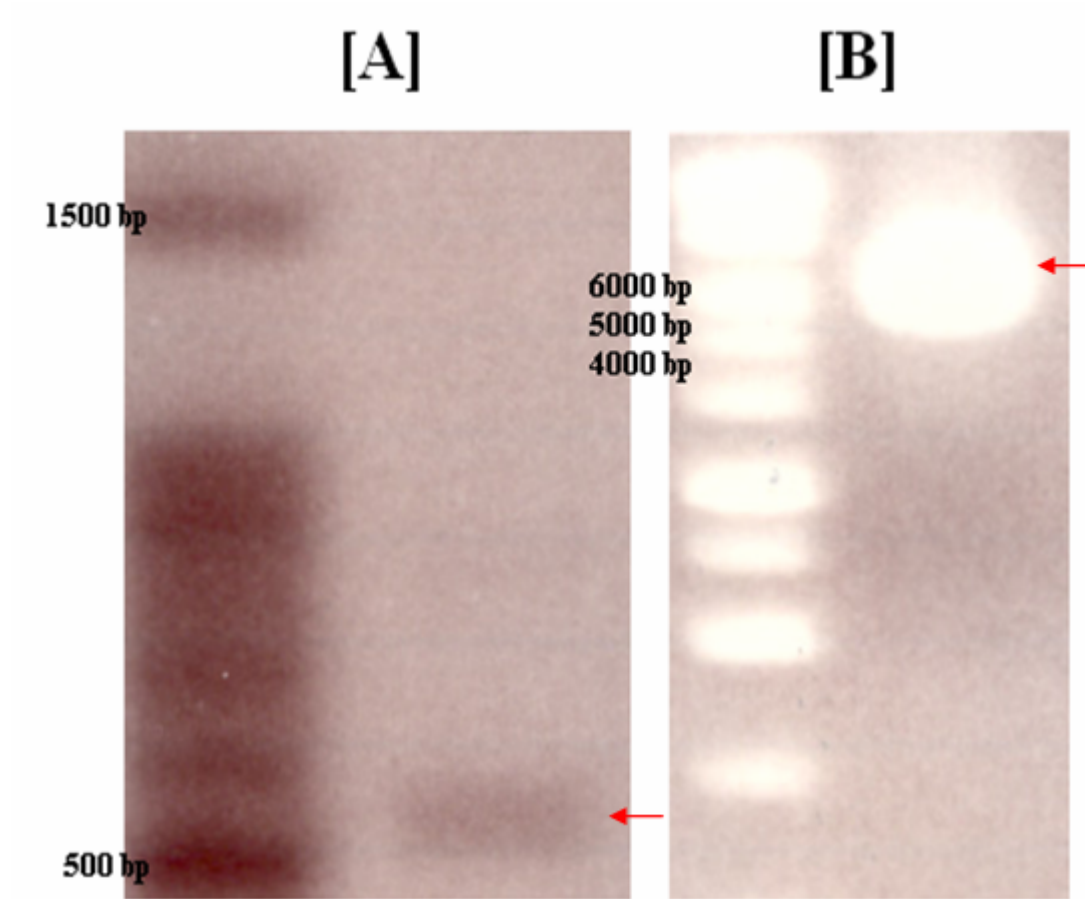


Figure 15. Gel pictures showing DNA bands used for ligation.

Gel pictures highlighting the results from the double digest of both pFASTBAC [B] and pDNR-LIB [A] mutated vectors with XhoI and NdeI. The red arrows point to the bands corresponding to the regions of DNA sequence that were used in the domain exchange.

Expression and Purification of VNHX, VNGA and VNΔC.

The elegance of the Bac-to-Bac Baculovirus expression system is that it harnesses the mechanism used by baculovirus to produce recombinant protein. The Bac-to-Bac system produces recombinant baculovirus which contains the gene corresponding to the protein of interest, and so upon infection of insect cells, produces recombinant protein. A schematic diagram of the process is shown in the Material and Methods section (**Figure 8**).

(a) Verification of successful transposition of all cDNA constructs into bacmid.

Prior to transfection into the insect Sf9 cells, PCR amplification was done using M13 primers as a final verification to ensure successful transposition before continuing onto cell transfection. **Figure 16** is a diagram showing the rationale and expected results from the PCR analysis of the bacmid DNA. As can be seen from **Figure 16**, successful transposition of the three constructs into the recombinant bacmid would correspond to a gel band size of 3919bp. As a control, empty pFASTBAC-HT was used. The empty pFASTBAC1 frozen stock was contaminated, and the pFASTBAC-HT vector differed in size by only 130bp from pFASTBAC1 with respect to the region added to the size of the insert.

Figure 17 shows the gel picture highlighting the results obtained from the PCR analysis. Since the gel shows bands at the correct size for each construct, transfection of insect cells was then done, and the P1 initial viral stock was harvested as per the Bac-to-Bac manual instructions.

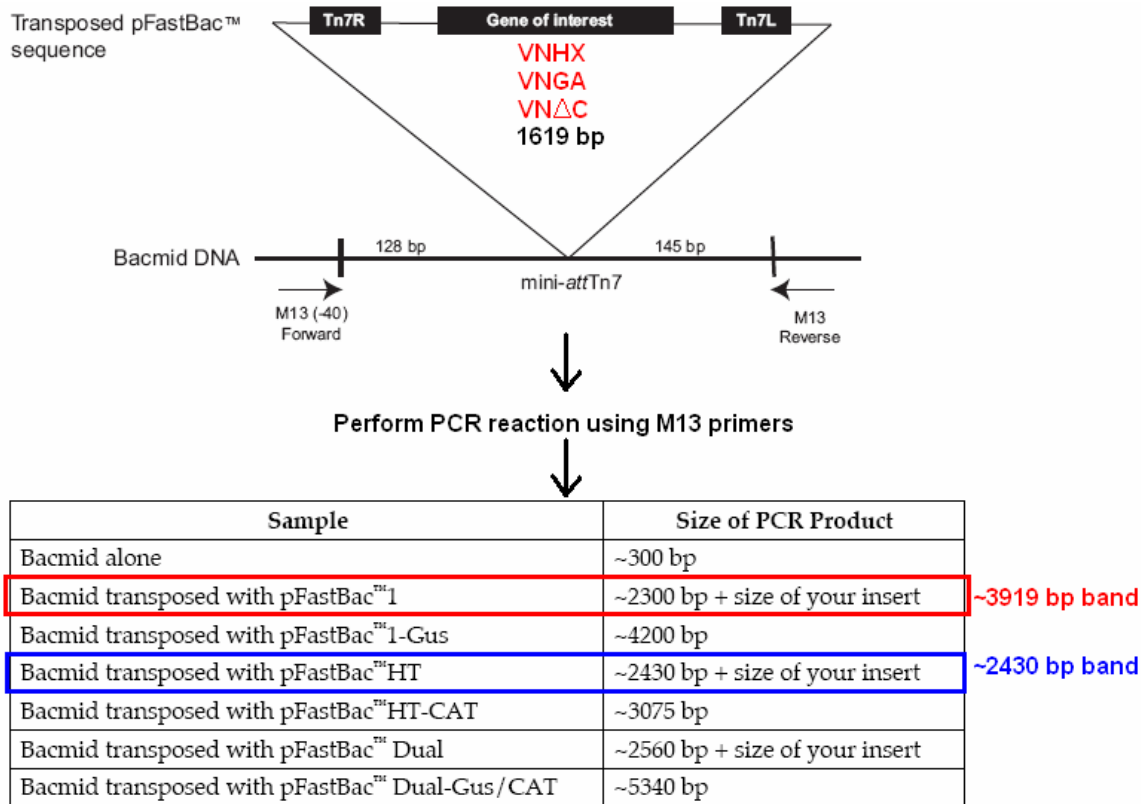


Figure 16. Verification of site-specific transposition by PCR.

(From the Bac-to-Bac Expression Manual) Diagram showing the M13 priming sites on the bacmid DNA in relation to the region transposed from the donor plasmid. The bottom table shows the sizes of the gel bands expected for the three constructs (red, ~3919bp), and the control/empty plasmid (blue, ~2430bp).

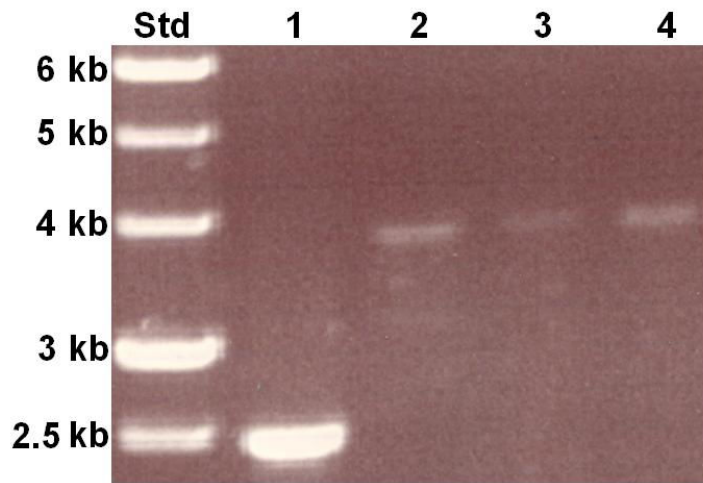


Figure 17. Results from the PCR analysis using M13 primers on the bacmid DNA for all constructs.

Gel picture showing the PCR bands that result after M13 primer analysis of bacmid DNA samples. **1** – pFASTBAC-HTA (control), **2** – VNHX, **3** – VNΔC, **4** – VNGA.

(b) Small-Scale Expression Studies.

As a starting point, 50mL insect Sf9 cell cultures were infected with 2mL and 3mL P3 viral stocks for VNHX, VNΔC and VNGA. Viral infection was allowed to proceed for 96 hours. At 24-hour time points, 1mL samples were taken from the cultures, and spun down to separate out the cells and large debris. These samples were stored at -80°C until Day 4, at which point they were thawed out and used to determine protein expression via SDS-PAGE and Western Blot analysis (using a polyclonal antibody

against vitronectin). **Figure 18** shows the result from a Western blot analysis of one expression study using P3 VNHX viral stock.

As can be seen from **Figure 18**, the bands that start to appear around Day 2 are predicted to represent the VNHX protein. However, large-scale expression and purification would have to be done to conclusively identify those bands as being the chimera. The same expression study was done using both VN Δ C and VNGA viral stocks, along with two more constructs designed by Dr.Christine Schar [45, 46]. **Figure 19** summarizes the results from both SDS-PAGE and Western blot analysis for all of the constructs.

As is shown in **Figure 19**, higher molecular weight species are present for VN Δ C, Δ SMB and full-length VN proteins expressed in Sf9 cells (bottom picture). These species are however absent in the VNHX and VNGA samples. Problematic though, is the fact that an adequate Western blot signal is not present in the VNHX or VNGA samples under non-reducing conditions. The estimated protein concentrations for each construct from quantitative Western blot analysis using dilutions of plasma purified vitronectin as a reference was then performed (data not shown). The results from this analysis predicted the protein concentrations of both the VNHX and VNGA constructs to be approximately 15 ng/mL, and the VN Δ C, SMB and FL-VN constructs to be approximately 100 ng/mL.

The optimization of the expression of these chimeras was then pursued, with several parameters being modified to try and increase protein expression. Some of these modifications included increasing the amount of time each viral stock was incubated with the cell culture, increasing the amount of viral stock added to the cell culture, and increasing the amplification level of each viral stock used to infect the cell culture. Also

considered was the amount of primary antibody used to detect the constructs in Western blot analyses, as well as the amount each construct was concentrated prior to loading on the gel. The results are summarized in **Table 1**.

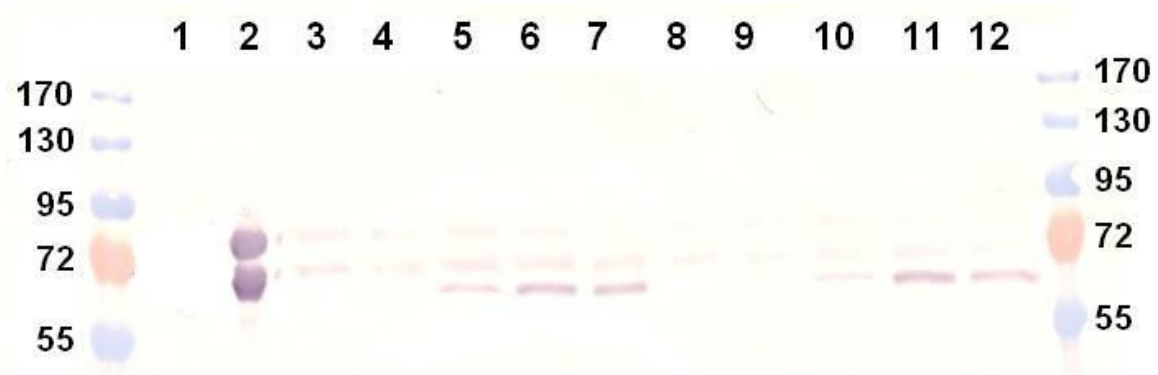


Figure 18. Small-scale expression study results using VNHX viral stock.

Western blot analysis results of samples collected during a 4-day infection of Sf9 insect cells using 2mL (lanes **3-7**) and 3mL (lanes **8-12**) of P3 VNHX viral stock. Lane **1** - Sf9 sample with no virus added, lane **2** - monomeric vitronectin, purified from plasma. All samples were mixed with SDS-PAGE loading buffer (non-reducing). Lanes **3,8** – Day 0 (no virus added), lanes **4,9** – Day 1 (24 hours virus), lanes **5,10** – Day 2 (48 hours virus), lanes **6,11** – Day 3, lanes **7,12** – Day 4. Western blot analysis was done using a polyclonal antibody against vitronectin. The same analysis was done using samples that were mixed with SDS-PAGE buffer (reducing) (data not shown).

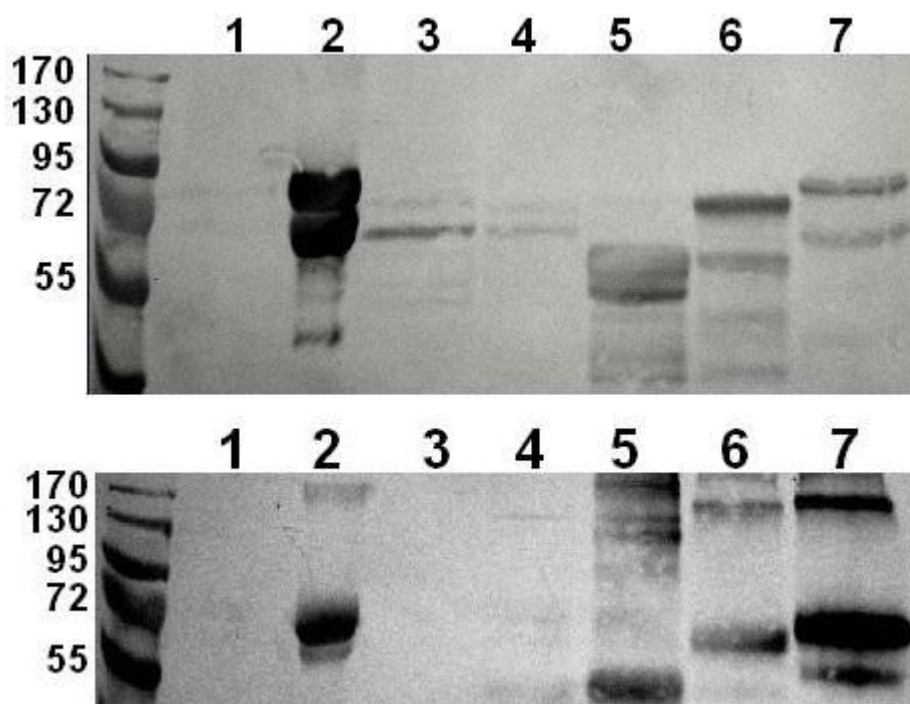


Figure 19. Summary of small-scale expression studies using all viral stocks.

Western blot analysis of all three recombinant proteins (top, under reducing conditions, bottom, under non-reducing conditions) VNHX (lane **3**), VNGA (lane **4**), and VN Δ C (lane **5**), expressed in insect Sf9 cell cultures. Also pictured are the Δ SMB (lane **6**) and full-length VN (lane **7**), also expressed in Sf9 cells. Lanes **1** and **2** are insect cell media with no virus added and plasma-purified VN, respectively.

In addition to the ‘Adjusted Quantity’ values shown in the table above, parameter values were changed in combination with one another to try and put more strain on the expression system to generate more protein. For example, the viral incubation time was increased along with the amount of viral stock used to infect the cell culture. However, no combination of modifications had any effect on the level of expressed protein detected in the Western blots.

To ascertain whether this was a problem of not enough protein getting out of the cell or simply a problem of expression, the cell pellets from each viral culture were lysed open and clarified by centrifugation. The supernatants were then examined to see if the protein was present in the cytosol. An additional lysis step was then done using 2M urea added to the lysis buffer, and the sample spun down, and both supernatant and pellet examined for the presence of protein by both SDS-PAGE and Western blot analysis. The result for a VNGA cell pellet sample is shown in **Figure 20**.

From **Figure 20**, it is clear that a large amount of the protein being synthesized is in the insoluble fraction of the cell pellet, presumably unfolded. The same result was observed for VNHX cell pellets (data not shown). Because of the better expression level observed for the VNΔC construct, a large-scale purification was attempted. However, it was abandoned after several unsuccessful attempts. As a result, the complete purification of all three constructs from large culture was temporarily put aside.

Parameter	Default Quantity	Adjusted Quantity	Result
Viral incubation time	96 hours	120 hours 144 hours 168 hours	Increased cell death, but no change in Western blot signal
Amount viral stock used	2ml/3ml P3	5 ml P3 5 ml P4 5 ml P5 10 ml P5	No change
Concentration of sample	50X	75X 100X 150X 200X	No change/loss of signal completely
Amount of antibody used (Western Blot)	1:5000 dilution	1:2500 dilution 1:1500 dilution 1:1000 dilution	No change

Table 1. Summary and results of optimization attempts done to increase expression of constructs.

Optimization attempts to increase the expression levels for VNHX and VNGA constructs. No significant change was observed upon altering any of the four parameters shown.

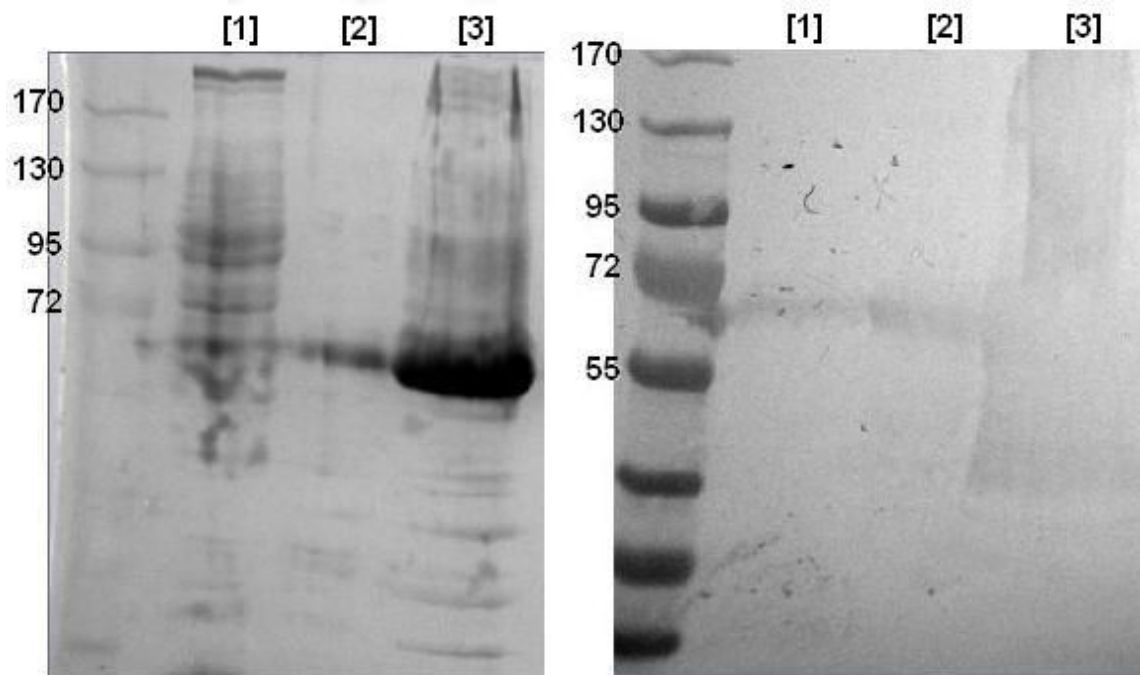


Figure 20. Cell pellet analysis of VNGA samples.

SDS-PAGE (left) and Western blot (right) analysis of cell pellet samples from a viral culture inoculated with VNGA. Lane [1] – supernatant from a cell pellet lysed using vortex/shearing+lysis buffer, spun down. Lane [2] – supernatant from the cell pellet formed in [1], lysed using vortex/shearing+lysis buffer (+ 2M urea), spun down. Lane [3] – cell pellet formed in [2].

Evaluation of the functional properties of the chimeric/recombinant proteins.

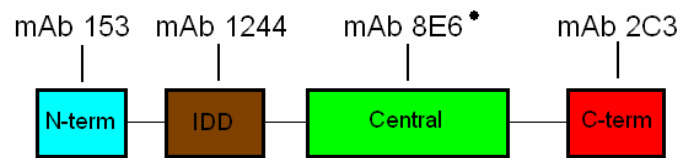
Despite the low expression levels of all of the constructs, several experiments were done to try and ascertain structural and functional data about the proteins.

(a) Examining the constructs using monoclonal antibodies.

An initial method of analysis of the proteins VNHX, VNGA and VNΔC was to test their reactivity under Western blots to a variety of monoclonal antibodies that were in our possession. The specificity of monoclonal antibodies means that they can give some indication of the structure of the area around their epitope, since if the binding site is compromised, they will not bind to another site on the protein. Because of the fact that Western blot analysis was conducted under denaturing conditions (using SDS-PAGE gels), the results of this study would only confirm that the epitopes for each antibody used were still available in the baculovirus-expressed constructs.

Additionally, this study also attempted to try and localize the binding regions for some monoclonal antibodies in our possession that were currently unknown. Because of the unique design of the constructs used, each domain knockout/replacement could be used as a screen to narrow the binding region of the monoclonal antibody on vitronectin.

The results of this experiment are summarized in **Table 2**. From the table, it is clear that no real pattern of reactivity between the monoclonal antibodies and the constructs is detectable that could be used to identify either the availability of the epitopes on a given construct or the localization of the epitope for a given antibody. Only 1244 reacted unequivocally with all the constructs, while 1E9 only reacted under non-reducing conditions.



Also tested - mAbs 1E9, 4A1, 2410, 8H1.

Figure 21. Binding regions for some monoclonal antibodies on Vitronectin.

The binding regions for the monoclonal antibodies (mAbs) 153, 1244, 8E6 and 2C3 on vitronectin (domains labeled and color-coded; IDD = intrinsically disordered domain). The antibodies listed beneath were also tested to try and localize their binding regions within vitronectin. (Note ‘-’ – 8E6 was not available to test with).

(a) Under reducing conditions.

Construct	153	1244	2C3	1E9	2410	8H1	4A1
VN(P)	+	+	+	-	-	-	-
VN(B)	-	+	-	-	-	-	-
VNΔC	-	+	-	-	-	-	-
ΔSMB	-	+	-	-	-	-	-
VNHX	-	+	-	-	-	-	-
VNGA	-	+	-	-	-	-	-
Control	-	-	-	-	-	-	-

(a) Under non-reducing conditions.

Construct	153	1244	2C3	1E9	2410	8H1	4A1
VN(P)	+	+	+	+	-	+	-
VN(B)	+	+	-	+	-	-	-
VNΔC	+	+	-	+	-	-	-
ΔSMB	-	+	-	+	-	-	-
VNHX	-	+	-	+	-	-	-
VNGA	-	+	-	+	-	-	-
Control	-	-	-	-	-	-	-

Table 2. Results from the monoclonal antibody experiments.

The results of Western blot analysis on plasma purified ‘**VN(P)**,’ and baculovirus-expressed constructs with an array of monoclonal antibodies. ‘**VN(B)**’ – full-length VN expressed in baculovirus. ‘**Control**’ – insect cell media that was not infected with virus. A ‘+’ indicates a positive signal, while a ‘-’ indicates no reaction. All constructs reacted positively using a polyclonal antibody against vitronectin.

Assuming the antibodies used were of good quality, the results from this experiment were taken to be inconclusive in both regards to try and identify epitope availability between the constructs and the monoclonal antibodies used, as well as to try and localize the binding regions of the ‘unknown’ antibodies used (1244 is believed to bind within the IDD, a region believed to be highly unstructured [26]). However, the reactivity between the constructs and monoclonal antibodies 1E9 and 1244 however, was further studied in the next set of binding experiments.

(b) Binding studies using Surface Plasmon Resonance (SPR).

Following the logic that binding is an indication of structural integrity at least in the binding vicinity of the ligand, the next set of binding experiments were performed to determine whether the three constructs (VNHX, VNGA and VNΔC) retained their ability to bind to PAI-1, the physiological co-factor of vitronectin. As was shown in **Figure 4**, the proposed mechanism of oligomerization involves the binding of two PAI-1 molecules to vitronectin in a concentration-dependant manner, which then result in the eventual formation of higher order complexes.

Because only small-scale stocks of concentrated media samples containing the VNHX, VNGA and VNΔC proteins were on-hand, the analytical method used to test this binding needed to be able to show the specific binding of each protein to PAI-1 against a background of impurities. Surface Plasmon Resonance (SPR) is a tool which nicely accommodates this requirement, as it employs the immobilization of the *analyte*, in this case, VNHX/VNGA/VNΔC via a secondary binding partner such as a specific antibody, like 1E9. The immobilization of the analyte is critical as it takes place on the sensor chip,

where binding is detected. When the *ligand* in question (PAI-1) is then injected and then binds to the analyte, the binding response detected is only between analyte and ligand [69].

As a negative control, a viral stock obtained from insect cells that had been transfected with ‘empty’ bacmid were used. These cells thus expressed the normal background of proteins seen in the other viral stocks, but did not express vitronectin whatsoever. This was verified by Western blot analysis. As a positive control, plasma-purified vitronectin was used.

The results of this experiment are shown in **Figure 22**. As expected, no binding was detected for the negative control with the antibody. As a result of this, no binding was detected when PAI-1 was subsequently injected. However, both 1E9 and PAI-1 binding was detected for all the constructs, including plasma-purified vitronectin. The Δ SMB construct seemed to bind minimally to both 1E9 and PAI-1. To find out approximately how much vitronectin and PAI-1 bound in the experiments, the molar concentrations of bound material were then calculated from the values displayed in **Figure 22**. The results are shown in **Table 3**.

As can be seen from **Table 3**, wtPAI-1 binds to each vitronectin construct in almost a 2:1 excess (PAI-1:VN). ‘Excess’ as used here simply means at a greater concentration, it is not meant to define molar stoichiometry. The exception is the Δ SMB construct, which shows more of a 1:1 molar stoichiometry of binding. This was expected since SMB domain houses the primary binding site for PAI-1, and so only one PAI-1 binding site should remain. It should be noted that these calculations do carry some error, as the molecular weight of only plasma vitronectin could be used with accuracy in

calculating the final concentrations. The other molecular weights were estimated from the migration of each construct on Western blots. Nevertheless, it can be said with good confidence that all constructs are able to bind wild-type PAI-1 in solution, and that this binding is in excess.

The same SPR experiments were then repeated with another monoclonal antibody to see if any change in binding would be observed. Using mAb 1244, another series of experiments were conducted, in the same manner as was outlined for 1E9 above. The results are shown in **Figure 23**.

The results from **Figure 23** are a bit different from those shown in **Figure 22**. Firstly, while both binding to 1244 and PAI-1 could be detected with all of the constructs, the level of binding is somewhat offset by the large error values, especially for VN Δ C and Δ SMB. However, this is believed to be due to the inconsistency found in the raw data values between each experiment. This inconsistency is most likely a result of the degradation of the target proteins in their viral stock solutions over time. Therefore, while the first set of experiments with 1244 showed higher Δ RU values than the following two, this was probably not because the integrity of the instrument was compromised, but rather that the target constructs were being degraded. It is also possible that some constructs, such as Δ SMB and VN Δ C were relatively more oligomeric, and thus were not binding as strongly to the antibody.

The binding concentrations for each construct and PAI-1 are shown in the **Table**

4. As is seen in the table, PAI-1 seems to bind in excess to the vitronectin samples. The exception is of course VNΔC. However, due to the large errors calculated for the binding to both 1244 and PAI-1, these values may be more skewed than those calculated in the 1E9 experiments.

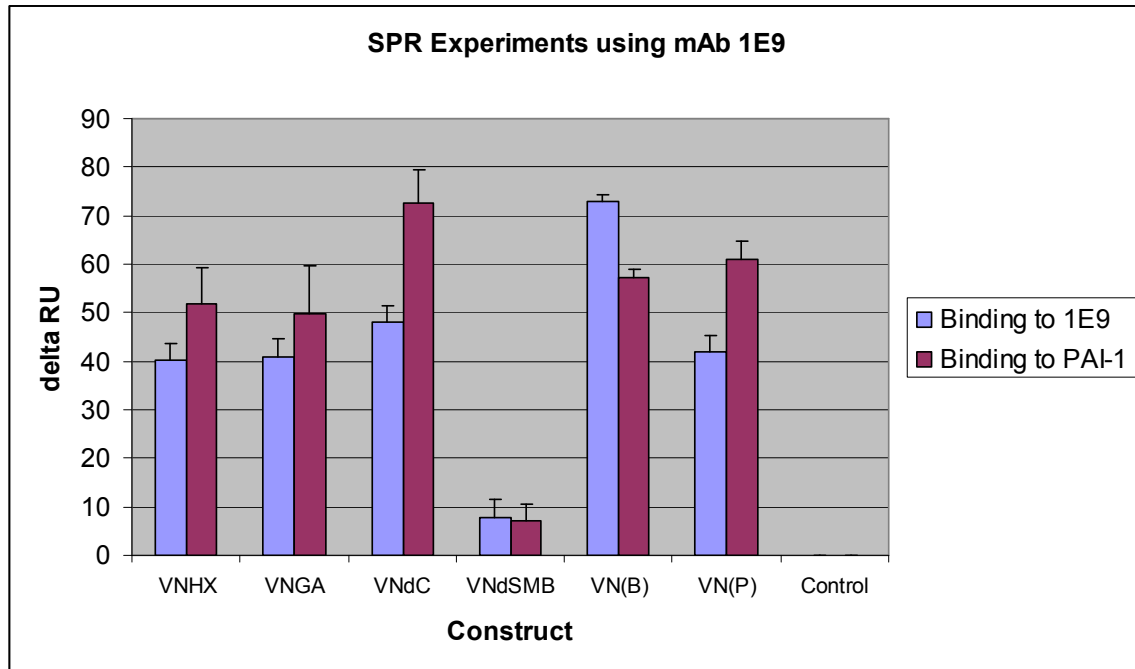


Figure 22. SPR Experiments testing PAI-1 binding to constructs using mAb 1E9.

Results from the SPR experiments in which binding between baculovirus expressed constructs and PAI-1 (via mAb 1E9) was tested. As a positive control, plasma-purified vitronectin was used. As a negative control, a viral stock from insect cells transfected with ‘empty’ bacmid was used. Each series (bar) is the average ΔRU measured for all three trials of the experiment. Standard error calculations are also shown.

Construct	Concentration bound to 1E9 (nM)	Concentration of PAI-1 bound (nM)
VNHX	1.44	3.04
VNGA	1.79	2.91
VN Δ C	2.19	4.24
Δ SMB	0.287	0.409
FL-VN (bac)	2.53	3.34
FL-VN (plasma)	1.46	3.56
Control	0	0

Table 3. Molar concentrations of bound vitronectin and PAI-1 using 1E9.

Concentrations of both vitronectin samples (as a response to 1E9 binding), and PAI-1 (as a response to vitronectin binding) bound to one another.

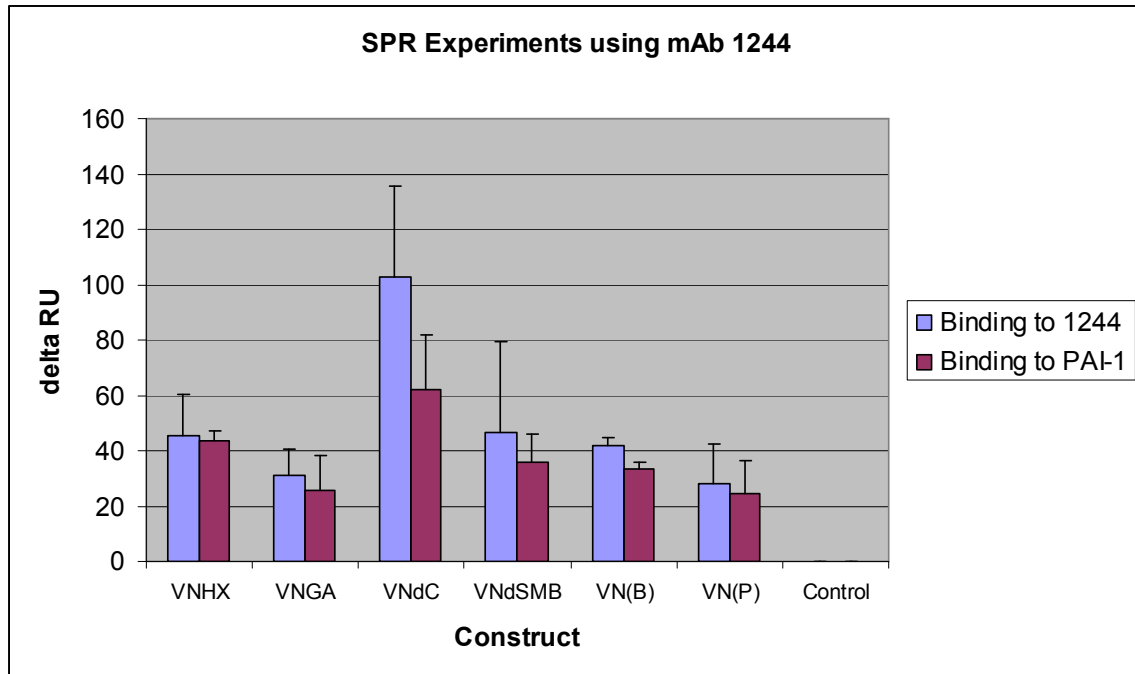


Figure 23. SPR Experiments testing PAI-1 binding to constructs using mAb 1244.

Results from SPR experiments (a series of three total) using mAb 1244. Error bars are shown.

Construct	Concentration bound to 1244 (nM)	Concentration of PAI-1 bound (nM)
VNHX	1.61	2.54
VNGA	1.12	1.50
VN Δ C	4.71	3.62
Δ SMB	1.71	2.10
FL-VN (bac)	1.46	1.97
FL-VN (plasma)	0.980	1.42
Control	0	0

Table 4. Molar concentrations of bound vitronectin and PAI-1 using 1244.

Concentrations of vitronectin samples (as a response to binding to 1244) and PAI-1 (as a response to binding to vitronectin) bound to one another.

Characterization of the Central Domain using limited proteolysis.

The rationale behind this study was to use limited proteolysis to try and isolate and identify the Central domain region of vitronectin. One benefit envisioned for this study was that the isolated Central domain would be more amenable to structural analysis, in the same way the isolated N-terminal domain was [19]. Early research done on defining the domain boundaries within vitronectin utilized limited proteolysis experiments [18, 72-74]. **Figure 24** shows a sampling of the sites of proteolytic cleavage gathered from these experiments.

Since the target region of this study was the Central domain, it was decided that plasmin would be the most appropriate enzyme to use since it is expected to cut outside this region in vitronectin (Figure 23). The parameters for this experiment were parallel to an earlier limited proteolysis study on vitronectin [75]. The products generated in this study however would be analyzed using reverse-phase HPLC and mass spectrometry. **Figure 25** shows the SDS-PAGE gel used to visualize the digest products of the proteolysis reaction over time.

The estimated molecular weight of the fragment containing the Central domain from the plasmin digest was calculated to be 31057.18 Da (Monoisotopic mass, http://ca.expasy.org/tools/pi_tool.html). Because there are two glycosylation sites in this region of vitronectin, the actual mass was expected to be higher. Therefore, the bands above 34 kDa in the 1H, 3H and 24H lane samples under reducing conditions, along with the bands below 43 kDa at the same time points under non-reducing conditions were candidates for this region.

Samples from each of the time points were then injected onto a RP-HPLC column, so that the individual peptide fragments could be separated and collected. **Figure 26** shows the HPLC chromatograms generated for all of the samples, showing the profile of the digest over time.

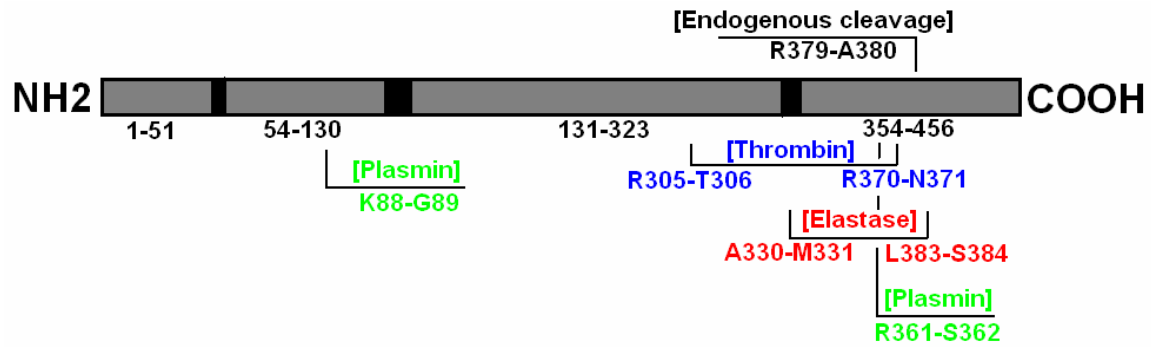


Figure 24. Known protease cleavage sites on vitronectin.

Some known protease sites on vitronectin. The linear sequence of vitronectin is represented by the grey rectangle, with the residue ranges indicated. The endogenous cleavage site which yields the two-chain form of vitronectin is shown (**R379-A380**), along with known **Plasmin** (green), **Thrombin** (blue) and **Elastase** (red) sites.

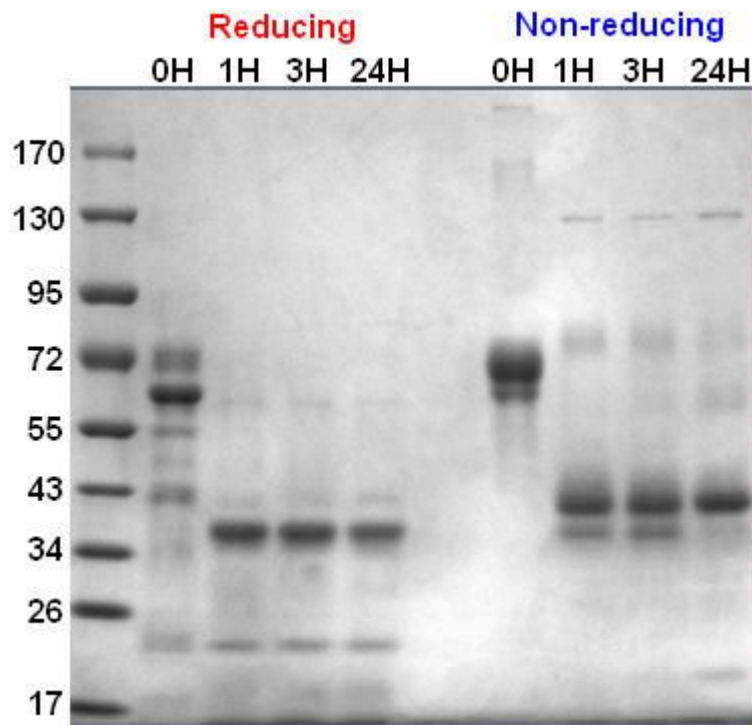
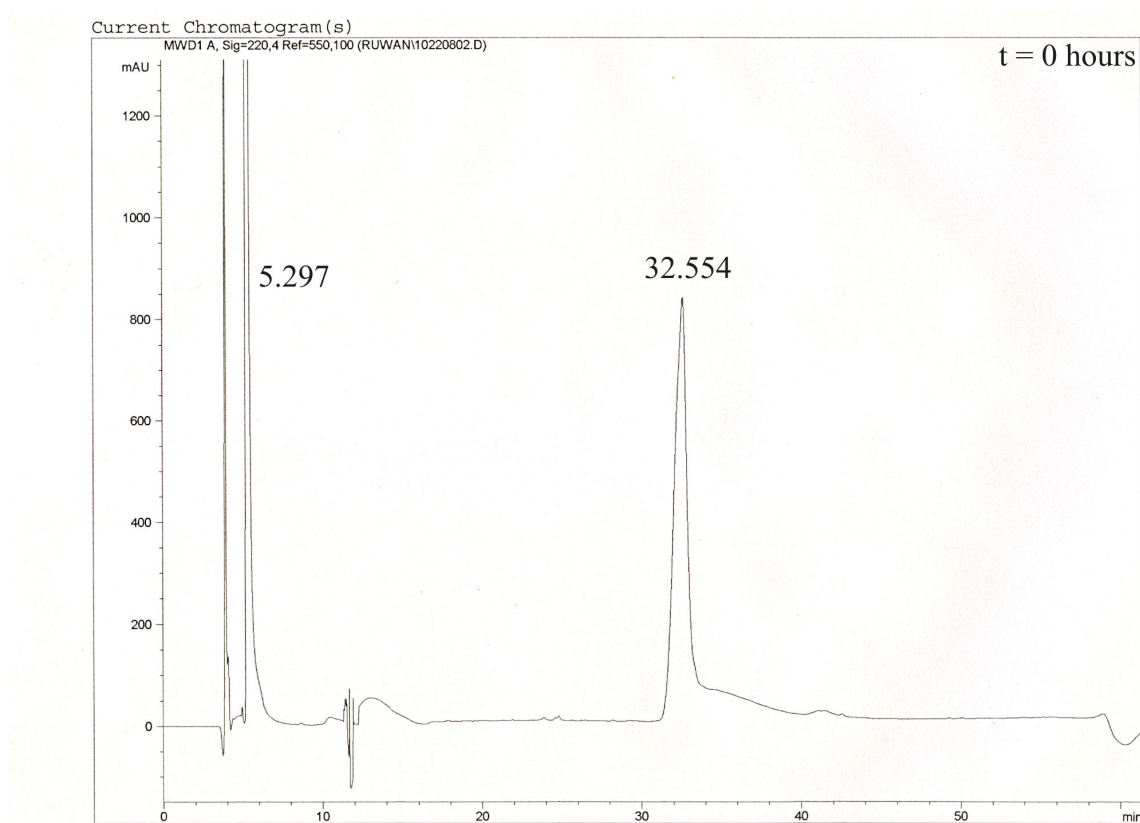


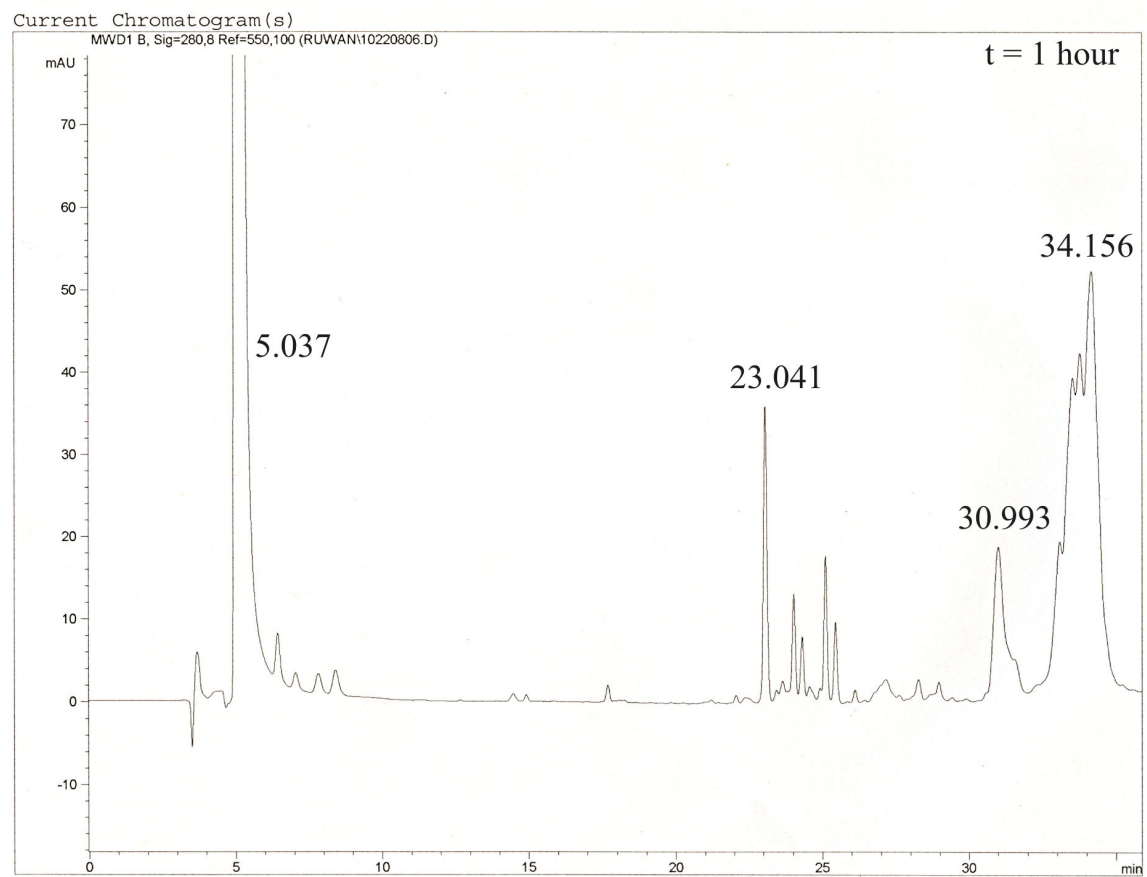
Figure 25. SDS-PAGE analysis of the proteolysis reaction over time.

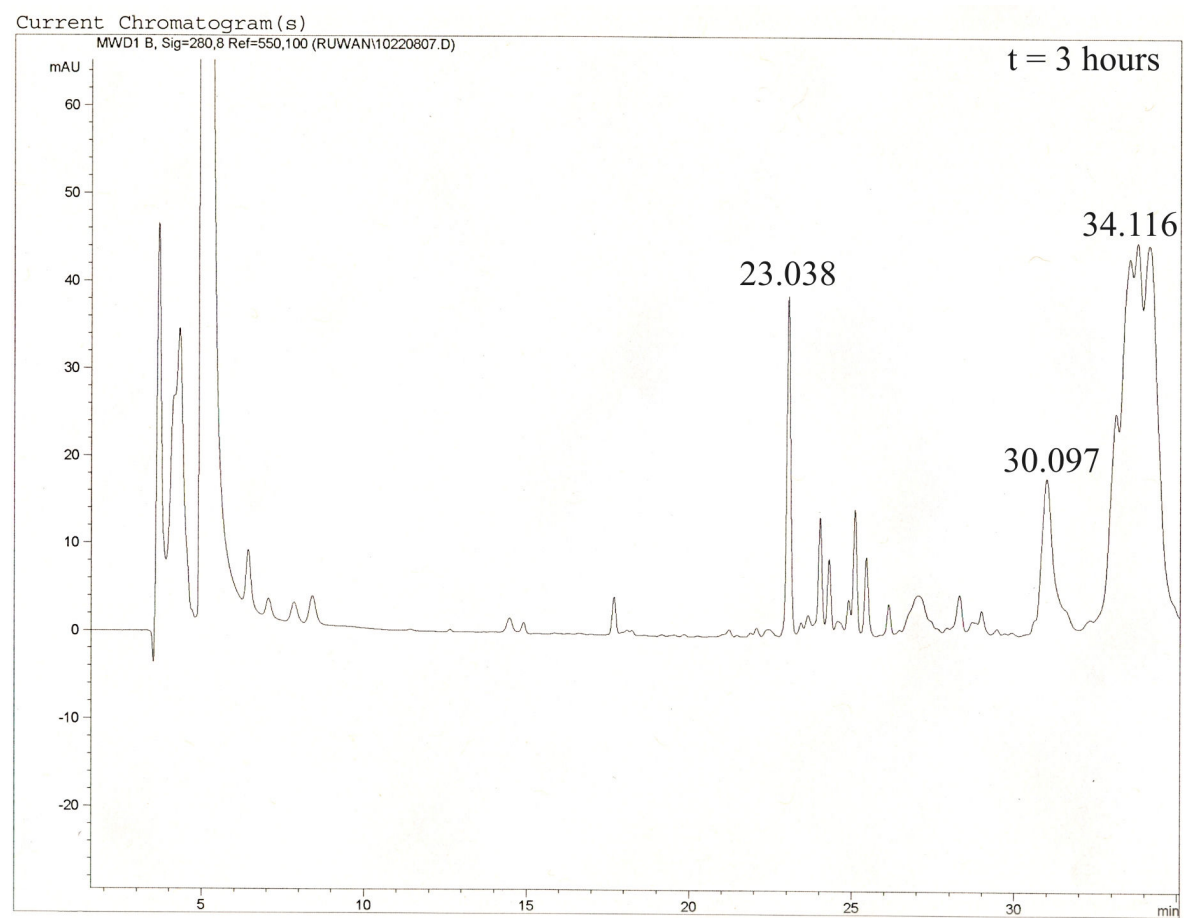
The SDS-PAGE gel, showing digest bands under reducing (red) and non-reducing (blue) conditions. Time points are labeled. (0H = just before plasmin addition).

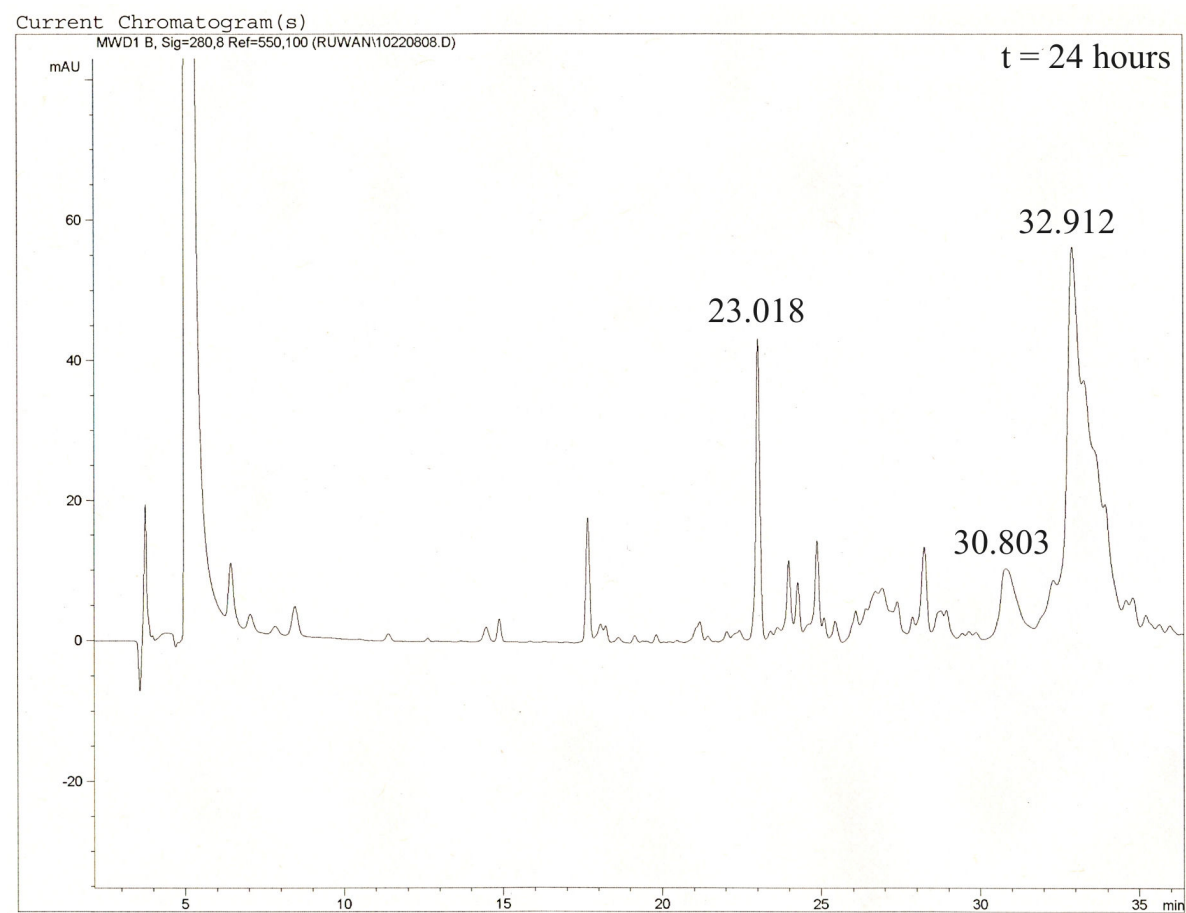
Figure 26. HPLC chromatogram profiles of plasmin digest.

HPLC chromatograms (A280 scans), showing the profile of the plasmin digest of vitronectin over time. Samples are listed in the top right-hand corners. Certain retention times are noted as well.









As is shown in **Figure 26**, the major peak at around 32 minutes for the 't = 0 hours' sample is full-length vitronectin. This was identified in previous trials of this experiment using Western blot analysis (data not shown). The early peak at around 5 minutes is believed to be leftover iodoacetamide in the reaction mixture. As can be seen in the t = 1, 3 and 24 hour chromatograms, the peak shape corresponding to full-length vitronectin begins to change over the time-course of the reaction. This is coupled with the emergence of peaks at approximately 24 and 30 minutes. Sample fractions corresponding to the retention times mentioned above were collected and analyzed by mass spectrometry. The mass spectra for a fraction collected from the 24-hour time point sample, corresponding to the peak at around 30 minutes in Figure 25 (preceding page) is shown in **Figure 27**.

As is shown in **Figure 27**, the peak identified at m/z 35316.7 is in range of the 31057.8 g/mol predicted mass for the Central domain region calculated. The difference in mass is likely due to the two glycosylation sites present in the Central domain, which may be adding to the mass of the fragment. The peak at 35316.7 m/z most likely represents the singly charged species of the fragment peptide, while the peak at 17690.0 m/z represents the doubly-charged species. However, because the mass/charge ratio is not exactly one-half (i.e. 17658), this peak may represent another fragment that co-eluted with the piece at mass/charge 35316.7.

Many of the other fractions analyzed using mass spectrometry did not display spectra of good resolution as is shown in Figure 26 (data not shown). This was attributed largely to the amount of material left over after RP-HPLC, but may also be a result of a need to optimize the method of analysis.

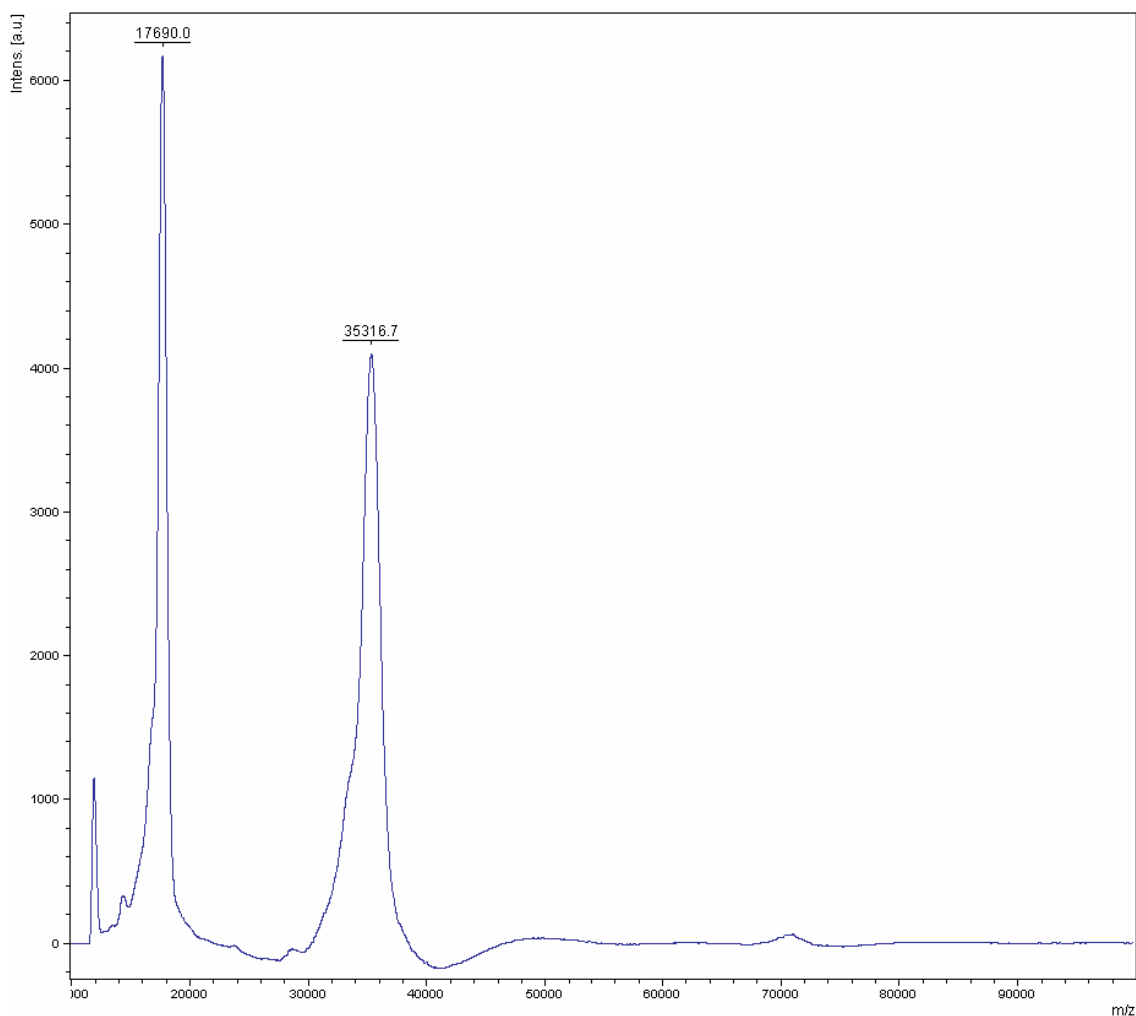


Figure 27. Mass spectrum of a 24-hour time point sample collected from RP-HPLC.

The y-axis is labeled 'Intensity', the x-axis is labeled 'm/z' (mass/charge). The taller of the two peaks shown is at 17690.0 m/z. The smaller one is at m/z 35316.7.

Conclusions and Future Directions.

To restate, the objectives of this study were as follows –

1. To generate chimeric proteins of vitronectin, in which the Central domain of vitronectin was replaced with the homologous domains from hemopexin and gelatinase-A (a third chimera using the homologous domain from collagenase-3 may also be pursued).
2. To generate a recombinant form of vitronectin, which lacked the C-terminal domain.
3. To express and purify all three of the constructs mentioned above.
4. To use limited proteolysis in an attempt to isolate the Central domain of vitronectin.
5. To test the structural and functional properties of the chimeras, recombinant and isolated Central domain in comparison to native, full-length vitronectin, with emphasis being placed on the ability to form higher order complexes when mixed with PAI-1.

The data presented in the previous sections show that both goals 1 and 2 were completed in full. With respect to goals 3, 4 and 5 however, some compromises had to be made. Firstly, while it was shown that all of the desired constructs were being expressed, purification could not be achieved due to the apparently low expression levels observed. This may have been the result of the fact that much of the synthesized protein was found to be insoluble, stuck inside the cells. Low expression may also have been a codon optimization issue, and so it may be worthwhile to try and modify the cDNAs for each of

the constructs so that they are codon optimized for expression in Sf9 cells. However, previous research has also shown that proteins which have had β -propeller domains substituted do not express well, if at all [76]. This latter piece of information further supports the inherent challenges that were met in this study.

Certain factors pertaining to the design of the VNHX and VNGA chimeras, as well as the VN Δ C mutant that may be worth re-evaluating should also be mentioned here. Firstly, the domain exchange using hemopexin and gelatinase-A removed two of the three glycosylation sites on vitronectin. In addition to this, four cysteine residues were substituted in the hemopexin chimera, and removed in the gelatinase-A chimera. The importance of both the glycosylation sites and the disulfide bond arrangement in vitronectin should be carefully considered when substituting an entire region of the protein. **Figure 28** shows then cysteine residues within the Central domain of vitronectin, as well as their bonding arrangements.

Secondly, with respect to the VN Δ C mutant, the entire deletion of the C-terminal domain may carry serious consequences when considering that the docked structures of the Central and C-terminal domains cover a predicted area of almost 3500 Å² (**Figure 29**). Furthermore, any interaction between the C-terminal and N-terminal domains that may have served to stabilize the structure of the protein may have been lost.

Future experiments should thus optimize the constructs if possible to alleviate these potential structural flaws. This may in result improve expression levels, or simply lead to a more straightforward comparison of the construct to native, plasma vitronectin.

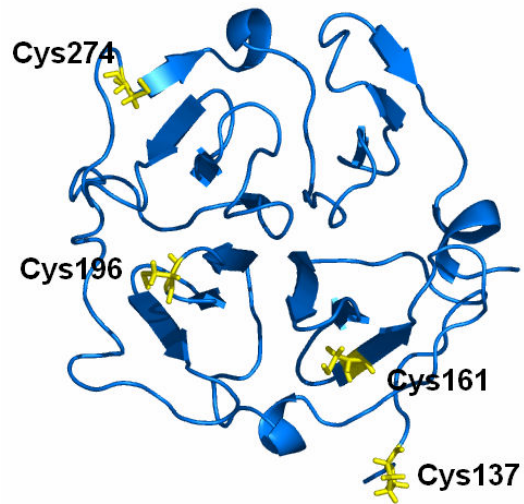


Figure 28. Cysteine residues in the Central domain of vitronectin.

The 4 Cys residues (shown in yellow, labeled), within the Central domain of vitronectin (blue chain). Cys274 provides an inter-domain linkage to Cys453 in the C-terminal domain in the two-chain (heavy and light) form of vitronectin. Cys137-161 provide an intra-domain disulfide bridge [77].



Figure 29. The predicted docked structures of the Central and C-terminal domains in Vitronectin.

The predicted docked structures of the Central (green) and C-terminal (blue) domains of vitronectin. The residues lining part of the interface are shown in red. The Cys274-Cys453 inter-domain disulfide linkage is shown in yellow. Using the computer program GRASP [78], the total surface accessible area buried between the Central and C-terminal domains was calculated to be 3498.89 Å².

The limited proteolysis study provided a unique approach to try and characterize the Central domain of vitronectin. The SDS-PAGE gel picture clearly shows the digestion of vitronectin by plasmin over time (**Figure 25**). The digestion profile seen in the gel correlated nicely in the HPLC chromatograms (**Figure 26**) for each time point sample. Future experiments should use increased quantities of both vitronectin and plasmin, so that a larger amount of material is left over for analyses following RP-HPLC, which can then be used for N-terminal sequencing to identify the peptide fragments collected, so that further structural characterization, e.g. CD spectroscopy to verify β -sheet content, can be done.

Lastly, the characterization of the constructs was also limited in scope due to the inability to purify them. The results observed in the monoclonal antibody study may have been due to the inability to obtain a consistent level of protein for each construct for Western blot analysis. Conversely, the concentrations of some of the antibodies tested were unknown, and so an inadequate amount may have been added. The optimization of the former parameter was attempted and met with no success. The latter, however, might still be optimized for future studies.

The monoclonal antibody study was able to show that the constructs retained their epitopes to monoclonal antibodies 1E9 and 1244, and the SPR experiments done using these antibodies showed that the constructs retained structural conformity around these epitopes, as well as retaining their ability to bind wild-type PAI-1 in solution. Perhaps an extension of the SPR experiments could be done using other ligands known to bind to vitronectin, for example heparin or the thrombin:antithrombin complex.

With respect to the underlying goal of this study – *to determine the region(s) within vitronectin that are responsible for its self-association upon binding PAI-1* – the question still remains unanswered. However, the aggregation of proteins is an intensely debated topic, and as yet no conclusive answers have emerged as to why or how some proteins aggregate and others do not. Some researchers believe it is due to a conformational change that then initiates aggregation (like in this study), while others believe that aggregation itself drives conformational change [79]. In either case, most researchers try and tackle protein oligomerization using mini-peptides that are easy to work with, unlike globular/multi-domain proteins [80, 81].

A potential model for the self-association of vitronectin is shown in **Figure 30**. In this model, the self-association is allowed via a ‘domain exchange’ between the N-terminal domains of two vitronectin molecules [81]. As stated earlier, the linker region between the N-terminal and Central domains of vitronectin is thought to be highly unstructured [26]. This flexibility would allow the N-terminal domain from one vitronectin molecule to swap with another, thus completing the exchange. Added stability may be donated by hydrophobic interactions between the β -strands in the Central domain regions between the two vitronectin molecules. This would result in a 4:2 complex of PAI-1:vitronec tin, which would then associate with other 4:2, ultimately resulting in the large-scale oligomeric complex formation noted in the earlier studies [16, 82].

Regarding the focus of this study also on the proposed β -propeller domain structure of vitronectin, much evidence was presented to suggest that despite a crystal structure of the protein, the Central domain does in fact assume this fold. However, because β -propeller domains can show a variety of functional roles despite a common

structural fold [48], it is difficult to say at this time whether in the case of vitronectin facilitating large scale aggregation is one of them. It may be one of many functions. In the course of this study, some new information published by other researchers shed light on other potential key role players in the oligomerization of vitronectin – the glycosylation sites. Vitronectin contains three N-linked glycosylation sites, two of which are within the Central domain region. One group found that the trimming of these glycans seemed to increase multimer formation, as well as collagen binding [83]. Another group found that removing the O-linked glycosylation sites from the β -propeller domain of Gelatinase-B inhibited the dimerization/multimerization of the protein [84]. Thus, modulation of glycan residues on β -propeller domain-containing proteins may play a role in their observed aggregative behavior, but it may not be so straightforward.

The oligomerization of proteins like vitronectin is no doubt a complex process, but unveiling the mechanism would be a great achievement in the field of healthcare research. As mentioned earlier, it is unlikely that the oligomerization of vitronectin proceeds unchecked – aggregation can result in plaque deposition, which is most always associated with severe pathological/debilitating conditions ranging from atherosclerosis to Alzheimer's [85]. The curing of these diseases in humans most certainly lies in the fundamental understanding of their arising, on the molecular level, and so must be pursued with the utmost diligence and resolve.

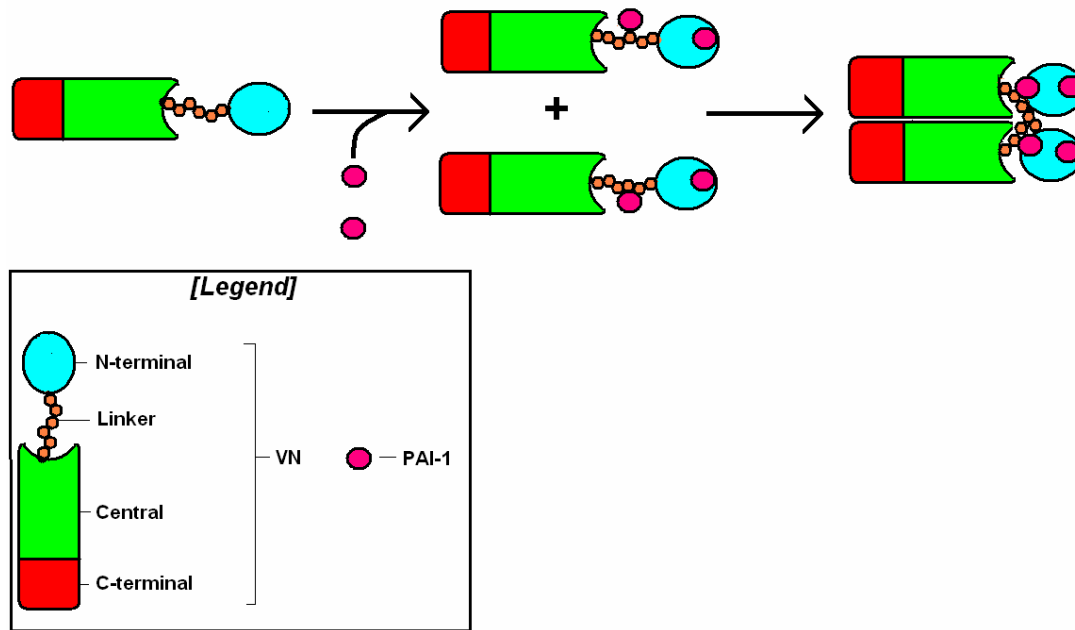


Figure 30. Model for the self-association of vitronectin.

The figure legend is shown in the box (*left, inset*). As illustrated, monomeric vitronectin binds with two molecules of PAI-1 to form the 2:1 complex (PAI-1:VN) [16]. Two of these 2:1 complexes then associate with one another via the ‘domain exchange’ method, resulting in a 4:2 stable complex. PAI-1 in this figure is shown binding to vitronectin at its primary site in the N-terminal/SMB domain [15-17], as well as the postulated secondary binding site in the linker region between the N-terminal and Central domains [17, 45, 46]. (Note – the final 4:2 complex illustration is not attempting to show the second PAI-1 molecule binding to both the linker region and the N-terminal domain).

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Vita.

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