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I am submitting herewith a thesis written by Jodi L. Watson entitled “Localizing Ligand Binding Sites Using Overlapping Recombinant Polypeptide Sequences 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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(Original signatures are on file with official student records.)

**LOCALIZING LIGAND BINDING SITES USING OVERLAPPING
RECOMBINANT POLYPEPTIDE SEQUENCES OF VITRONECTIN**

**A Thesis
Presented for the
Masters of Science
Degree
University of Tennessee, Knoxville**

**Jodi Watson
May 2004**

DEDICATION

This thesis is dedicated in loving memory to my mother, Judith H. Watson.

ACKNOWLEDGEMENTS

Ora et labora. Pray and work. Indeed, many times during my graduate school experience, I found myself uttering these words. Thus, as it should be, my first acknowledgement is a humble prayer of thanks to the LORD, who is a God of details and finds no concern too small, or too large. All things to His glory!

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ABSTRACT

Vitronectin is a glycoprotein involved in many cellular processes including blood coagulation, fibrinolysis, and cell/matrix binding. It interacts with a number of macromolecules including heparin, PAI-1, and the thrombin-antithrombin complex. We have studied the interaction of full-length vitronectin and the thrombin-antithrombin complex using a Far Western method. To localize the recognition site for thrombin-antithrombin binding to vitronectin, sequences for eight overlapping recombinant polypeptide sequences of vitronectin (spanning all 459 amino acids) were cloned into the pET system. Expression in BL21(DE3)pLysS *Escherichia coli* has been achieved for seven of the eight 100 amino acid fragments. Seven polypeptides (amino acids 1-100, 51-150, 101-200, 151-250, 201-300, 251-350, and 351-459) have been isolated and purified using metal-chelating affinity chromatography. These fragments have been tested for interaction with the thrombin-antithrombin pair using the Far Western technique. Results indicate interaction of the complex with vitronectin between amino acids 201-300 and possibly at another site near the C-terminal. Additionally, these expressed fragments have served as helpful reagents in mapping the epitopes of three monoclonal antibodies for vitronectin.

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LIST OF ABBREVIATIONS

ECM	extra cellular matrix
PAI-1	plasminogen activator inhibitor-1
uPA	urokinase-type plasminogen activator
TAT	thrombin-antithrombin
uPAR	urokinase-type plasminogen activator receptor
serpin	serine protease inhibitor
GPI	glycosylphosphatidyl inositol
RCL	reactive center loop
tPA	tissue-type plasminogen activator
RGD	arg-gly-asp
MAC	membrane attack complex
kDa	kilo-Dalton
PROSPECT	Protein Structure Prediction and Evaluation Computer Toolkit
DNA	deoxyribonucleic acid
RNA	ribonucleic acid
mRNA	messenger ribonucleic acid
cDNA	complementary DNA
SMB	somatomedin B
CNBr	cyanogen bromide
mAB	monoclonal antibody
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
HPD	hemopexin domain
VN	vitronectin
HPLC	high performance liquid chromatography
PDI	protein disulfide isomerase
ELISA	enzyme-linked immunosorbent assay
HRP	horse radish peroxidase
TB	Terrific® broth
amp	ampicillin
ampR	ampicillin resistance gene
chlor	chloramphenicol
PBS	phosphate-buffered saline
YNB	yeast nitrogen base
OD	optical density
IPTG	isopropylthiogalactoside
TBS	Tris-buffered saline
kb	kilobase
bp	base pairs
AT	antithrombin
Throm	thrombin
MI	Molecular Innovations

Part A

Vitronectin: An Overview of Function, Organization, and Structure

I. Introduction

Vitronectin is an adhesive glycoprotein found in both circulation and the extracellular matrix (ECM). It is involved in a number of physiological processes including blood coagulation, fibrinolysis, cellular migration, the humoral immune response, tumor metastasis, and angiogenesis (1,2). Its importance as a regulatory protein, particularly in maintaining the fine balance between thrombus formation and dissolution, cannot be over-emphasized. Disruption of this balance can result in excessive bleeding, disseminated intravascular coagulation, stroke, or myocardial infarction .

Vitronectin plays its diverse and important role *in vivo* by binding a vast array of biological ligands. These include heparin (3-5), plasminogen activator inhibitor-1 (PAI-1) (6-10), proteases such as the urokinase-type plasminogen activator (uPA) (11), constituents of the extracellular matrix such as collagen (12), the terminal complement complex (13,14), and the thrombin-antithrombin (TAT) pair (11,15-17). A number of methods have been employed to determine the number of binding sites and the region(s) or residues involved in the interaction of vitronectin with its various ligands. These methods include 1) binding studies using synthetic peptides; 2) experiments with cleavage products created via chemical and proteolytic digestion; 3) studies with monoclonal antibodies; 4) competition assays; 5) investigations involving recombinant expression of vitronectin polypeptides; 6) (photo)affinity labeling; and/or 7) a combination of these methods. The focus of this proposal centers on the recombinant expression of polypeptides to localize the vitronectin binding sites involved in the interaction of the protein with the thrombin-antithrombin complex. The polypeptides are

further exploited to epitope map a number of acquired monoclonal antibodies to vitronectin.

II. Localization and Tissue Distribution

Vitronectin was first identified in 1967 as a serum spreading factor (18). Like many other plasma proteins, it is synthesized primarily in the liver and generally identified, like fibronectin, as an acute phase protein (19). It circulates in normal plasma at concentrations of 200-400 $\mu\text{g/ml}$ (20,21), which constitutes 0.2-0.5% of total plasma proteins. Non-circulating forms of vitronectin are found in the ECM, and low levels of vitronectin expression are detected in lung, kidney, sperm, skin, and brain tissue (22,23).

Circulating vitronectin is primarily monomeric, whereas matrix vitronectin and other tissue-associated forms are predominantly multimeric (20,24). Approximately 0.8% of the circulating pool of vitronectin is contained within platelets in a rapid releasable form (25-27). Rather than being synthesized by megakaryocytes, platelet vitronectin is most likely endocytosed from plasma and incorporated into α -granules (28).

III. Vitronectin Function

Vitronectin is diverse in function. As mentioned previously, it is particularly important in the regulation of hemostasis. In the complex, highly regulated blood coagulation cascade (Figure A.1), an injury to a vessel wall initiates a series of events that leads to the eventual conversion of the zymogen prothrombin to the serine protease thrombin. Thrombin in turn cleaves soluble fibrinogen into insoluble fibrin. Together with platelet aggregation, a fibrin network works to form the hemostatic plug at the site

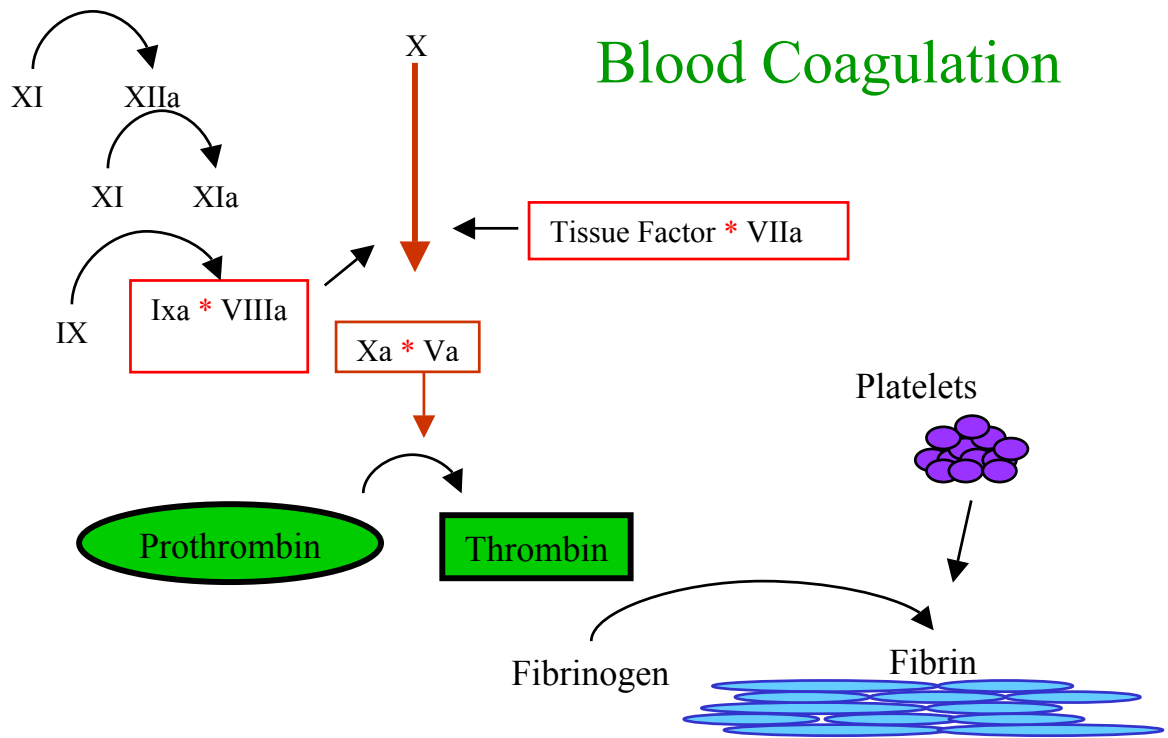


Figure A.1: The Blood Coagulation Cascade.

Schematic representing the several steps involved in the proteolytic cleavage of zymogens into their active forms that eventually lead to the formation of a hemostatic plug. Inactive prothrombin is converted to active thrombin via Factor Xa and Va. Thrombin in turn cleaves soluble fibrinogen to form insoluble fibrin, thus initiating clot formation.

of injury. Thrombin's proteolytic activity is inhibited by antithrombin, a serine protease inhibitor (serpin) that forms an inactive protease-inhibitor complex with thrombin (29). The proteoglycan heparin facilitates this complex formation. Vitronectin plays a pro-coagulant role by binding heparin, thus neutralizing the ability of heparin to initiate thrombin-antithrombin pairing (29,30). Vitronectin also binds directly to the thrombin-antithrombin complex and clears the inactivated pair from circulation (16,31,32).

Plasminogen activators enzymatically cleave precursor plasminogen to form active plasmin. Plasmin is the main protein responsible for the degradation of the fibrin network, defined as fibrinolysis (33). Inhibitors bind and inactivate plasminogen activators to regulate the fibrinolytic process. Vitronectin plays a key role in this process by maintaining the inhibitors in their active conformations and localizing them to the clot surface. The serine protease inhibitor PAI-1 is an important example of an inhibitor of fibrinolysis that binds vitronectin (9). Indeed, the vitronectin-PAI-1 interaction can be considered one of the most critical functions of vitronectin. PAI-1 binds plasminogen activators to form an inactive protease-inhibitor complex, thus blocking plasmin formation (34,35). PAI-1, however, is intrinsically unstable and converts readily into an inactive, latent form (Figure A.2) (36). Very importantly, vitronectin binds PAI-1 to keep it in an active conformation (7,37,38), thus keeping PAI-1 working as an inhibitor of the fibrinolytic process.

The urokinase plasminogen activator receptor (uPAR) is a glycosylphosphatidyl inositol (GPI) anchored receptor that has two major ligands—urokinase plasminogen activator and vitronectin (39,40). Urokinase plasminogen activator (uPA) binds uPAR with high affinity (41-43). uPA is a highly specific serine protease capable of activating

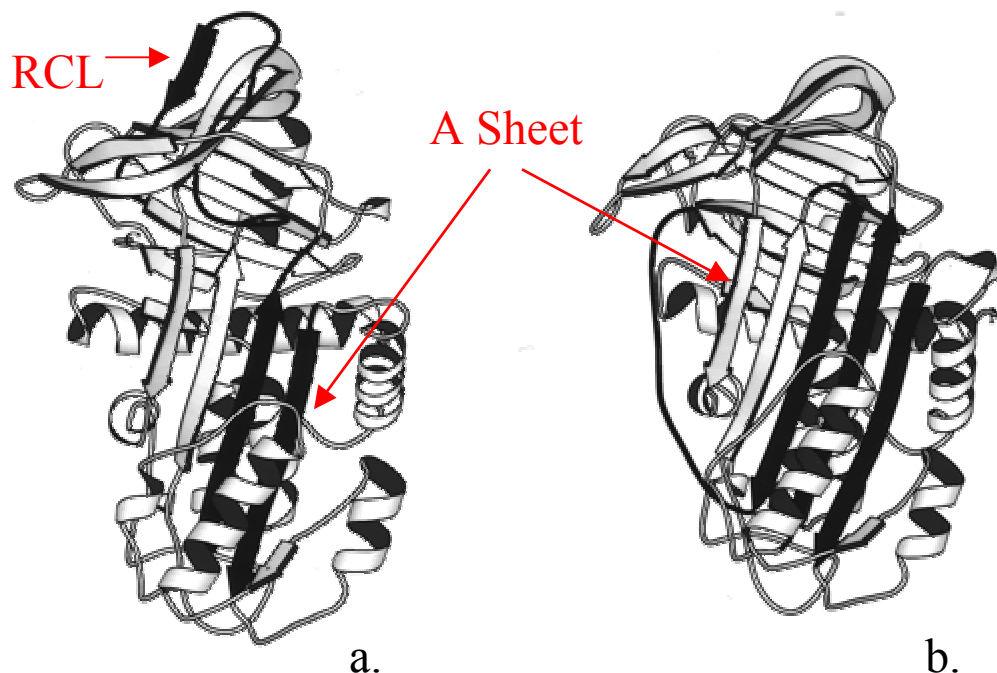


Figure A.2: Structure of Active and Latent PAI-1.

The 3D structure of the active form of PAI-1 (a.) illustrates features pertinent to the inhibitory mechanism of proteases by serpins. Serpins are folded around a large, central β -sheet (the A sheet) from which protrudes a surface-exposed loop (the reactive-center loop or RCL). Within this loop region are the residues recognized as substrates by the target protease (uPA or tPA), with the reactive center peptide bond denoted P1-P1'. Upon attack of the protease, this P1-P1' peptide bond of PAI-1 is cleaved by nucleophilic attack of the active-site serine, and an acyl-intermediate is formed. In contrast to the normal progression of proteolysis, this acyl-intermediate is extremely long-lived due to a negligible rate of deacylation. Structural rearrangements occur so that the two ends of the cleaved loop separate, and the P1 end, with the enzyme covalently coupled, is partially inserted into the central β -sheet. As such, the protease is inactivated by formation of a 1:1 stable, covalent complex with the serpin. PAI-1 is unique among the serpins because it readily relaxes from this active form (a.) to a latent, inactive form (b.). As shown, this occurs by insertion of the RCL into the central β -sheet to contribute a sixth strand to this secondary structural element. This refolding is thermodynamically driven, since the latent form of PAI-1 is more stable.

plasminogen to plasmin in the process of fibrinolysis previously described, thus it plays a very important role in tissue remodeling; PAI-1 is its major inhibitor (44). Vitronectin can bind both membrane-bound and unbound/soluble uPAR (40,45-49) with the interaction stimulated by uPA occupancy of uPAR. Additionally, uPA/uPAR binding to vitronectin has been shown to be controlled by uPAR dimerization *in vitro* (50). The association of vitronectin with uPAR appears to be partly responsible for spatial localization of uPAR on the cell surface (51). Through its interaction with matrix vitronectin, soluble uPAR may still associate with the cell surface and the ECM (52). Vitronectin can also bind uPA (11). The uPA and uPAR binding sites on vitronectin are distinct from each other. PAI-1 shares a vitronectin binding site with uPAR and competes for vitronectin binding; the binding of PAI-1 and uPAR is mutually exclusive (53-55).

Vitronectin cellular adhesion via uPAR has been observed in monocytes, fibroblasts, epithelial, and smooth muscle cells (47,56-59). Their interaction causes a “potent induction” (51) of actin cytoskeleton rearrangement and cell motility (60). Experiments demonstrate that uPAR mediated cell spreading on vitronectin necessitates integrin activation and function (61).

Integrins are so named because they “integrate” the cell with the ECM and perform receptor functions in cell interactions, as well as signal transduction. A typical integrin consists of an α/β heterodimer and usually binds a number of ligands. Integrins $\alpha_v\beta_1$, $\alpha_8\beta_1$, $\alpha_v\beta_3$, $\alpha_{IIb}\beta_3$, $\alpha_v\beta_5$, and $\alpha_v\beta_8$ have all demonstrated binding to the RGD sequence of vitronectin (amino acids 45-47) (62). Vitronectin has shown to be more adhesive than fibronectin (63), another common ligand for integrins, and essential for

integrin-dependent cellular attachment. The $\alpha_v\beta_3$ integrin is designated as the vitronectin receptor (64,65). Integrin $\alpha_v\beta_3$ has been extensively studied in the context of metastasis (62); such research was initiated by a study that found melanoma cells treated with antibodies activating this integrin would greatly increase their invasion across the reconstructed basal membrane (66).

To reiterate, vitronectin has a number of functions in the ECM. In addition to uPAR and integrin binding, vitronectin binds collagen (12) and may localize plasminogen and PAI-1 to sites of injury in the vessel wall. Such localization promotes the proteolytic events necessary for tissue remodeling. Because of these multifunctional properties of vitronectin in the extracellular matrix, along with interactions of the protein with uPAR and integrins, vitronectin is a good target for studies on tumor metastasis and angiogenesis, in that it is inherently involved in these processes.

Vitronectin is also involved in inflammation. The complement cascade is initiated to prevent and protect against the infiltration of foreign bacteria after the body suffers an injury. Complement factor C5 is proteolytically modified to C5b (67), which subsequently reacts with C6 and C7, two other complement proteins. Binding to C7 creates an amphipathic complex that can insert within the plasma membrane (68). This C5b-7 complex can also react with C8 to form C5b-8 which catalyzes the polymerization of complement factor C9. This membrane attack complex (MAC) cytolytically acts through a membrane-penetrating tubule (69). Vitronectin inhibits the cytolytic activity of the MAC, thus protecting neighboring cells from lysis (13). Vitronectin is also capable of binding a hydrophobic site on the C5b-7 complex which prevents its insertion in the plasma membrane (70).

Zheng, et al. generated a vitronectin knock-out mouse using gene displacement (71). Although the knock-out mice are viable, reproduce, and develop normally, studies with these mice indicate that vitronectin is essential for proper thrombus formation. Challenging the knock-outs under different pathologies should help to further clarify functions of vitronectin.

IV. Structure of Vitronectin

Full length human vitronectin has a molecular mass of 72 kDa by analytical ultracentrifugation (72). *In vivo*, it can exist in either a full length, single-chain form or a two-chain cleaved form; cleavage occurs at Arg-379. The residue at position 381 affects the susceptibility of the protein to cleavage; if threonine rather than methionine is present, vitronectin is more likely to be cleaved (73). Such action results in a two-chain form of vitronectin: a 62 kDa heavy chain and a 10 kDa C-terminal light chain. The two chains are linked via a single disulfide bond. Several different blood proteinases have been proposed to be responsible for the Arg³⁷⁹—Ala³⁸⁰ cleavage. Recently, however, Seger and Shaltiel (74) have reported that furin, a serine endoproteinase within the secretory pathway of hepatocytes, cleaves the bond selectively. This would thus suggest that the conversion of vitronectin to a two-chain form occurs in the liver (prior to secretion), rather than in circulation.

Currently, the structure of full-length, native vitronectin has not been determined. This can be attributed to a number of significant characteristics of vitronectin: 1) its large size and conformational lability; 2) extensive post-translational modifications (75) leading to sample heterogeneity; 3) the purification of both the single-chain and double-chain form

from human serum which are not effectively separated; 4) the hindrance of site-directed mutagenic studies due to inefficient recombinant expression systems; and 5) a high degree of adhesion to surfaces and itself. Recently, however, a prediction by Xu et al. (76) presents a model for three structural domains of vitronectin (Figure A.3). Sequence-structure alignment and domain fold recognition were performed using the threading program PROSPECT (Protein Structure Prediction and Evaluation Computer Toolkit). Vitronectin sequence was “threaded” through two sets of known structure templates and atomic structures were generated using the MODELLER program. The model corroborated a published intra-domain disulfide between C137—C161 (77) and predicts the structure of 1) an N-terminal (somatomedin B) domain; 2) a central region with a four-bladed β -propeller fold; and 3) a C-terminal heparin-binding domain. In addition to providing invaluable structural information, these predictions help to support evidence for the organization of multi-functional vitronectin into these individually folded domains, which has been tested and described in the literature.

V. Domain Organization

Several studies have been performed to evaluate the multiple functions of vitronectin and binding to various target ligands. From these investigations, a multi-domain protein model has been described. The organization of different domains provides a broad range of epitopes requisite for the binding of many target ligands. Indeed, multi-functional proteins are often found composed of independently folded domains linearly arranged with respect to amino acid, and it appears that vitronectin fits such a model.

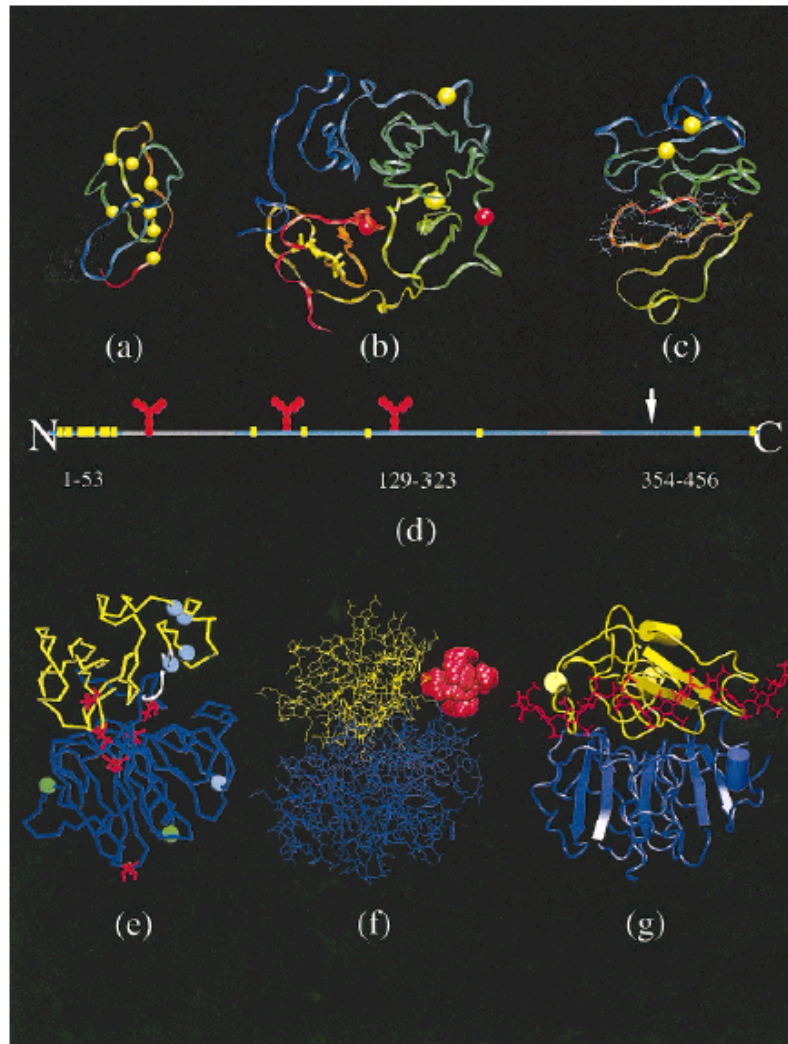


Figure A.3: Model for the Three-Dimensional Structure of Vitronectin.

The N-terminal somatomedin B domain **(a)**, central domain with a four-bladed β -propeller fold **(b)**, and C-terminal heparin-binding domain **(c)** are shown here in their predicted conformations. The color from the red to blue shows the sequence order from the N-terminus to the C-terminus. The yellow solid spheres indicate cysteines. White spheres **(a)** show the integrin attachment site; red spheres **(b)** show glycosylation sites (residues 150 and 223); and thin lines **(c)** show the heparin-binding site. **(d)** Linear representation of the sequence of vitronectin, with blue highlighting corresponding to domains modeled in (a–c), yellow dots indicating cysteines, red structures representing carbohydrate attachment sites, and the white arrow pointing to the known site of protease cleavage creating the two-chain form. **(e)** Docking structure between the central (blue) and C-terminal (yellow) domains, with cysteines (red lines), presumed heparin-binding residues (354–363 in white ribbons), sites susceptible to protease (residues 305, 361, 370, 379 and 383 with light blue spheres), and N-linked glycosylation sites (residues 150 and 223 with green spheres). Predicted docking of the N-terminal, central and heparin-binding domains **(f)**, and the predicted structure of each domain separately **(g)** (76).

In addition to the computational model, much support for a multi-domain organization springs from proteolysis experiments where isolated vitronectin fragments retain their ligand binding function. (Table I summarizes the common vitronectin digestion products and their ligand binding activity.) Vitronectin is rich in arginines and lysines, but most of the protein is resistant to cleavage by trypsin-like proteases. Vitronectin has two sites that are readily accessible to proteolysis: 1) an N-terminal sequence within residues 44 and 90, and 2) a C-terminal site spanning residues 340-370. Within the sequence spanning 340-370, vitronectin can be cleaved by plasmin (78,79), thrombin (78), and mast-cell tryptase (Cynthia B. Peterson and David Johnson, unpublished observations). Upon prolonged exposure to plasmin, vitronectin is cleaved near the N-terminus at the K⁸⁸—G⁸⁹ bond (80). Trypsin cleaves the protein between R⁴⁴ and G⁴⁵ (81). A number of potential elastase cleavage sites also exist in vitronectin, but elastase only cleaves vitronectin at residues 330 and 383 (79). Synthetic peptides and monoclonal antibodies have also been integral for the assignment of functional domains within vitronectin. Additionally, domain organization was determined through sequencing of human and rabbit cDNAs. The carboxy-terminal of vitronectin shared sequence homology with another circulatory protein, hemopexin.

The complete open reading frame of vitronectin consists of 8 exons encoding 459 amino acids for the mature protein (exons 2-8), preceded by a 19 amino acid signal peptide (exon 1). Along its linear sequence, vitronectin is organized into four regions (Figure A.4) The N-terminal (somatomedin B) domain comprises amino acids 1-44 and contains eight cysteines which form four disulfide bonds. The second region, a

Table I: Ligand Binding Activities of Vitronectin Fragments.

Treatment	Fragment	Interaction*	References
Cyanogen Bromide	1-340	+ Cells	(82)
		+ Heparin	(23,82,83)
		+ PAI-1	(83)
		- PAI-1	(23)
	51-340	- Cells	(82)
		- Heparin	(82)
		- PAI-1	(23,82,83)
		+C5b7	(84)
	340-381	- Cells	(23,82,83)
		+ Heparin	(13,23,82,83)
		+ PAI-1	(83)
		- PAI-1	(23)
		+ C5b7	(13)
Formic Acid	1-51	+ PAI-1	(23)
	1-217	+ Cells	(82)
		- Heparin	(23,82,85)
		+ PAI-1	(23)
		+ Collagen	(85)
	218-379	- Cells	(82)
		+ Heparin	(23,82,85)
		- PAI-1	(23)
		+ Collagen	(85)
Thrombin	1-306	- Heparin	(38,79)
		+ PAI-1	(38)
		- PAI-1	(79)
Trypsin	1-45	+ PAI-1	(81)
Plasmin	1-361	+ Cells	(80)
		- Heparin	(86)
		+ PAI-1	(80)
		- PAI-1	(78-80,86)
		+ Plasminogen	(79,86)
		- uPAR	(55)
	89-361	- Cells	(80)
		- PAI-1	(80)
Elastase	1-330	- Heparin	(79)
		- PAI-1	(79)

*A number of ligands were tested for interaction with the described fragments. Positive interaction is indicated by a "+," while those fragment-ligands that demonstrated no interaction are denoted with a "-."

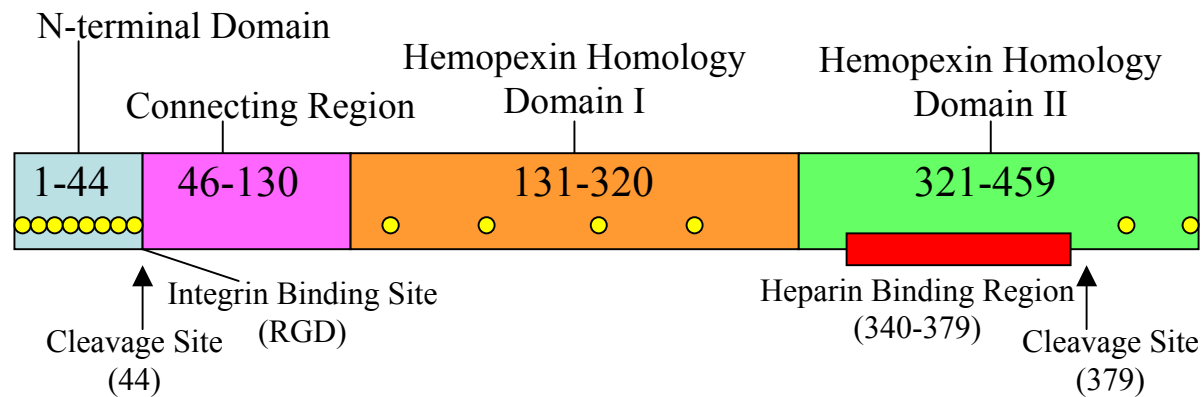


Figure A.4: The Linear Organization of Vitronectin.

Vitronectin is linearly organized into four domains or regions: an N-terminal (somatomedin B) domain created by cleavage at residue 44, a connecting region, and two hemopexin domains that share sequence homology to tandem repeats of the heme-binding protein hemopexin. Yellow circles denote cysteines residues. The integrin recognition sequence (RGD), the heparin binding site, and a second endogenous cleavage site at residue 379 are also noted.

connecting region, spans amino acids 45-130 and contains the familiar integrin binding sequence Arg-Gly-Asp (RGD). The remaining protein consists of seven hemopexin-type repeats, so named because of their sequence homology to the heme-binding protein hemopexin. The first four repeats are considered hemopexin homology domain I (aa 131-320) and contain four cysteines. The last three repeats are grouped as hemopexin homology domain II (aa 321-459), contain 2 cysteines, and house the site of heparin binding.

It is generally accepted that ligand binding to vitronectin is localized to distinct domains or linear epitopes. With this acceptance comes the possibility that vitronectin may interact with more than one ligand simultaneously. Indeed, several examples exist in the literature. 1) Via interactions with both collagen and heparan sulfate glycosaminoglycans, vitronectin is incorporated in the ECM (87-89). 2) Matrix vitronectin binds to both uPAR and integrin receptors on cells. 3) It can bind to PAI-1 and plasminogen (8,90,91), and 4) vitronectin binds glycosaminoglycans concurrently with the thrombin-antithrombin (TAT) complex (92). Elucidation of ligand epitopes is important not only for understanding the vitronectin-ligand interaction but also for appreciating the multi-functional characteristics of this diversified protein.

i. N-terminal (Somatomedin B) Domain

The N-terminal 44 amino acid sequence of vitronectin shares identical sequence homology to a reportedly naturally occurring circulating “somatomedin B” plasma protein, present in serum at concentrations of 12-14 µg/ml (93). A separate gene encoding somatomedin B has yet to be identified, so it is believed that vitronectin is

cleaved by an (as of yet) unidentified protease to release the somatomedin B (SMB) domain *in vivo* (94). The physiological role of somatomedin B has yet to be elucidated and its name now believed to be a misnomer. It was originally thought to be a member of the somatomedin/insulin-like growth factor family, but it was later discovered that the growth-promoting activity of somatomedin B was actually due to trace amounts of epidermal growth factor in the studies that assigned such activity (95).

Trypsin proteolysis cleaves the N-terminal (somatomedin B) domain from vitronectin *in vitro*, and even after prolonged protease digestion, the domain sequence remains intact. This suggests a tightly folded domain that renders protease susceptible areas inaccessible. Indeed, 8 of the 14 cysteines in vitronectin lie within this region of the protein, forming what can be described as an intradomain disulfide “knot” with all 8 cysteines participating in disulfide linkages.

A PAI-1 binding site has been localized to the N-terminal (SMB) domain by Seiffert, et al. (23). Chemically-derived fragments of vitronectin were shown to compete with full length vitronectin for PAI-1 binding. After direct binding studies, further digestion with cyanogen bromide (CNBr) identified the essential binding region as the 6-kDa N-terminal (somatomedin B) domain. In addition, monoclonal antibody (mAB) 153, which recognizes the N-terminal (somatomedin B) region of vitronectin, was used in a competition study with PAI-1 to further verify PAI-1 binding in this region (96). Polypeptides of vitronectin were also expressed in *E. coli* and tested for interaction with PAI-1. Polypeptides containing residues 1-52 and 1-40 bound to PAI-1, but the 1-30 peptide did not (96). Two additional studies (38,81) also localize PAI-1 binding to the SMB domain using thrombin-cleaved and trypsin-digested vitronectin fragments.

Deng et al. (97) generated chimeras between segments of N-terminal (SMB) domains that bound PAI-1 and segments of the N-terminal domain that did not to investigate important PAI-1 binding regions. The results of this study indicated that the essential PAI-1-binding determinant was between residues 12 and 30 on vitronectin. The importance of proper (SMB) domain folding was also demonstrated in this study. Alanine scanning mutagenesis created mutants that were screened for ability to bind and stabilize PAI-1. Results reported that all 8 cysteines and Gly¹², Asp²², Leu²⁴, Tyr²⁷, Tyr²⁸, and Asp³⁴ were necessary to preserve PAI-1 activity.

Additionally, Deng et al. (53) demonstrated that mAB 153 inhibited the binding of plasminogen activator and urokinase plasminogen activator receptor (uPAR) to vitronectin, thus also suggesting the binding sites for these ligands in the (SMB) domain. This study also generated synthetic peptides of vitronectin to confirm the localization of uPAR binding. It has also been suggested that the thrombin-antithrombin (TAT) complex binds in this region by a study using antibodies recognizing a 6 kDa CNBr amino-terminal fragment of vitronectin; the antibodies inhibited the interaction of TAT with vitronectin (16).

ii. Integrin Binding Sequence

At amino acid positions 45-47 lies the familiar integrin recognition motif of Arg-Gly-Asp (RGD). The RGD sequence is conserved in a number of other integrin-binding ECM proteins including fibronectin, fibrinogen, and von Willebrand Factor (62). When this RGD sequence is selectively mutated (KGD, RAD, RGE) or removed, the ability of vitronectin to bind cells is lost, thus making this sequence absolutely necessary for

vitronectin-promoted adhesion and spreading of cells via integrins (98-100). Also, when the RGD sequence is removed from vitronectin through thrombolytic cleavage, CNBr hydrolysis, or extended plasmin proteolysis, the cell-binding activity of vitronectin is weakened (80,82).

iii. Connecting Region

The connecting region of vitronectin extends from amino acids 45-130 and although it contains no cysteines, it does include sites for a number of modifications. Tyrosine residues 56 and 59 are modified by sulfation (101), and asparagine residue 67 is involved in N-linked glycosylation (94). Additionally, vitronectin has been identified as a substrate for transglutaminase and factor XIII A *in vitro*, with glutamine residues 73, 84, 86, and 93 believed to be the primary substrates for transaminoglutamate reactions (77). Transglutamination stimulates the formation of higher order vitronectin complexes that cannot be dissociated by either SDS or reducing agents. It is not known whether multimerization of vitronectin *in vivo* is mediated by transaminoglutaminase.

Several ligands are proposed to have binding sites within the connecting region, including a second binding site for PAI-1. Studies with *Staphylococcus aureus* protease V8-cleaved vitronectin identified an inhibitory fragment in this region (102). In the same study, a synthetic peptide corresponding to residues 115-121 inhibited PAI-1 binding to vitronectin by 50%. This same peptide also stabilized PAI-1 activity in kinetic assays.

A phage display synthetic peptide competed against immobilized vitronectin for heparin binding, suggesting the localization of a heparin binding site within the connecting region (103). Ishikawa-Sakurai et al. have shown that formic acid-hydrolyzed

vitronectin polypeptides containing the connecting regions demonstrate affinity for collagen (85). The use of mAB in competition experiments also supports a collagen binding site within this region (104).

iv. Hemopexin Repeats

The remaining portion of vitronectin (amino acids 131-459) consists of seven “hemopexin repeats” organized into two domains—hemopexin homology domain I and hemopexin homology domain II (HPD I and II). The individual domains of hemopexin are comprised of four tandem repeats. The structure of hemopexin is a 4-bladed β -propeller fold, with each repeat corresponding to a blade of the propeller. Vitronectin contains seven repeats, one less than the eight repeats requisite for two complete hemopexin domains (105). Like the N-terminal (somatomedin B) domain and the connecting region, the hemopexin homology repeats of vitronectin are highly resistant to proteolytic cleavage. Six cysteines are contained in this portion of the protein, two of which are reduced and buried (72). Two additional N-linked sites of glycosylation are found at asparagine residues 150 and 223 (94).

HPD I extends from residues 131-320 and contains the first four tandem repeats representative of a complete hemopexin structure. HPD II comprises amino acids 321-459 and contains the remaining three repeats which could result in an incomplete three-dimensional fold in regards to hemopexin. HPD II houses the important heparin binding site, discussed further below.

v. Heparin Binding Sequence

A cluster of basic amino acids (residues 345-379) lies within the second hemopexin homology domain and houses the apparent binding site of heparin. Within this region, two motifs are identified that correspond to the heparin-binding consensus sequences derived from (other) heparin-binding proteins (106). Additionally, serine 378 within this region is phosphorylated *in vivo*; *in vitro* phosphorylation has been demonstrated by protein kinase A (107-111). Mentioned previously, vitronectin circulates in both a double and single chain form resulting from proteolytic cleavage between arginine 379 and alanine 380 (112). The charged heparin-binding sequence and the residues flanking the sequence exhibit increased susceptibility to proteolytic cleavage *in vitro*, which is in contrast to a majority of the other tightly folded regions of vitronectin.

In addition to the high degree of similarity between this region and other sequences in known heparin-binding proteins, a number of studies have been performed on this region to further support a heparin binding site. CNBr digestion yields a product that competes with full-length vitronectin for heparin binding and inhibits the anticoagulant activity of heparin (82). Studies reported by Gibson et al. (113) demonstrate that heparin binding is significantly reduced upon the incorporation of an arginine-reactive probe. This probe was later localized to a CNBr fragment corresponding to residues 341-380. Further work by this group with recombinant polypeptides of the 129 C-terminal amino acids shows that heparin binding affinity of this polypeptide is comparable to that of full length vitronectin (114), thus suggesting that this region of vitronectin is responsible for heparin binding. Synthetic peptides and

mABs mapping to the heparin binding region inhibit the binding of heparin to this region of vitronectin (86). Plasmin proteolysis within this region hinders the ability of the resultant vitronectin to bind heparin (79,86). In addition, removal of sequences between residues 306-370 and 331-383 (via the addition of thrombin and elastase) also demonstrates a loss of heparin-binding activity (79).

A number of other target ligands of vitronectin have been localized to the heparin binding region including PAI-1 (78,83,115,116), plasminogen (83), uPAR (53), complement complex (69,117), collagen (85,87), and the thrombin-antithrombin (TAT) (118). Additionally, a number of microorganisms may bind vitronectin at the heparin-binding sequence (119-121). TAT, uPAR, complement complex, and collagen binding regions were elucidated using either synthetic peptides or CNBr cleavage products. In these studies, binding of the various ligands to full-length vitronectin was inhibited by the peptide or cleavage product.

More extensive experimentation has been performed to localize the binding sites for plasminogen and PAI-1 to this region. A plasmin cleavage product containing vitronectin residues 1-361 retains plasminogen-binding activity (79,86). The plasminogen activity was disrupted by treatment of the peptide with carboxypeptidase which suggests the C-terminal of the fragment is involved in the interaction with plasminogen. Kost et al. used a number of overlapping synthetic peptides derived from the heparin-binding region of vitronectin to localize the plasminogen binding site to residues 340-348 (86). Further evidence by Preissner supports this localization; *Staphylococcus* V8 protease-cleaved fragments and cathepsin D fragments bound both heparin and plasminogen in ligand-blotting assays (91)

In other studies by Preissner, it was demonstrated that active or latent PAI-1 binds to immobilized synthetic peptides corresponding to the heparin-binding region. These peptides also inhibited vitronectin binding to PAI-1 in microtiter plates (116). Kost et al. used mABs and competitive assays to localize PAI-1 binding to this region of vitronectin (86). The suggestion that PAI-1 and heparin share a binding site on vitronectin was further supported by three studies using plasmin proteolyzed vitronectin (78,80,115). Plasmin cleaves vitronectin between residues R361 and S362. Vitronectin cleaved with plasmin was unable to bind heparin or PAI-1. While intact vitronectin formed a PAI-I-VN complex that could be co-immunoprecipitated using antibodies to PAI-1, elastase- and thrombin-cleaved vitronectin did not form such a complex with PAI-1, not surprising because these proteases excise the heparin-binding domain (108). Vitronectin peptides were also generated by Preissner et al. through CNBr hydrolysis (83), and these peptides demonstrated that both PAI-1 and heparin bind the same 9 kDa fragment that includes the carboxy-terminal of vitronectin. However, Gibson et al (113) provide evidence through the use of fluorescent probes that binding determinants for PAI-1 and heparin are not shared.

VI. Considerations

The evidence suggests almost undoubtedly that vitronectin is organized to support multiple domains; multiple domains facilitate the accommodation of several ligand binding sites, many of which have been localized and described in the literature. Upon examination of vitronectin activity during different interactions and the epitopes reported, a number of discrepancies can be noted. How are these discrepancies explained?

i. Considerations of the Protein

Perhaps the most obvious thing to consider is that there may be more than one binding site on vitronectin for a particular ligand. For example, a possible resolution for the conflicting information concerning PAI-1 binding sites has been suggested by the description of two binding regions on vitronectin—one exhibiting high-affinity binding, the other demonstrating lower affinity. The well-defined high affinity binding site is located in the SMB domain and the second binding region is suggested at the C-terminal. Indeed, a model has emerged that describes PAI-1 binding first to the low affinity binding site at the C-terminal which induces a conformational change in the SMB that unmasks the high-affinity binding site. PAI-1/vitronectin complex (either the 1:1 or 2:1 form) can associate into high order oligomers via the self-association of the vitronectin component(s) (122,123).

A second consideration is the conformational lability of vitronectin. The protein can display differential binding activities depending on a specific fold. This fold-specific binding was suggested to be responsible for observations concerning the interaction of vitronectin with heparin. The heparin-binding site was first proposed to be buried within the native protein. A model prevailed suggesting that upon denaturation, the cryptic recognition site was exposed and heparin was able to bind (124).

However, Zhuang et al. found that denaturation of vitronectin is accompanied by self association into the multimeric form (125). A later study determined that heparin binding to both monomeric (native) and multimeric (denatured, renatured) vitronectin is the same (126). It is thus concluded that the heparin binding site is fully exposed and not

buried within the native fold, and a new model was proposed. In this model by Peterson (Figure A.5), the heparin binding site is fully exposed on the surface and does bind heparin. When vitronectin is refolded, it passes through a partially folded intermediate state that has a propensity to aggregate which leads to the formation of multimers. Observed enhanced heparin binding can be attributed to the alignment of multiple heparin binding sites in the multimeric vitronectin, not the exposition of an encrypted site on a monomer. This example also emphasizes the importance of monomer/multimer considerations when studying ligand interactions. Enhanced interaction may be due to a “Velcro-effect” rather than a change in fold.

Yet another consideration is enzymatic modification. Studies indicate vitronectin is susceptible to intracellular and extracellular proteolysis. Proteases like plasmin and thrombin, which are highly concentrated at injury sites, may cleave vitronectin and affect PAI-1 and heparin binding. In addition, vitronectin contains two sites of endogenous proteolytic cleavage, one at residue 44 that releases the SMB domain and one at 379 that is responsible for the two chain form of vitronectin. Endogenous removal of the SMB domain demonstrates decreased affinity for PAI-1 (81); it must be considered that other ligand interactions may be affected by endogenous cleavage at these sites.

Already mentioned are the post-translational modifications of vitronectin, another consideration. (Figure A.6 shows sites of post-translational modifications as well as a summary of proposed ligand binding sites within the domain structure of the protein.) Protein kinase A phosphorylates vitronectin within the heparin-binding sequence, both *in vitro* and *in vivo*. This phosphorylated form of vitronectin may demonstrate altered binding activities towards the ligands that interact with the arginine-rich C-terminal

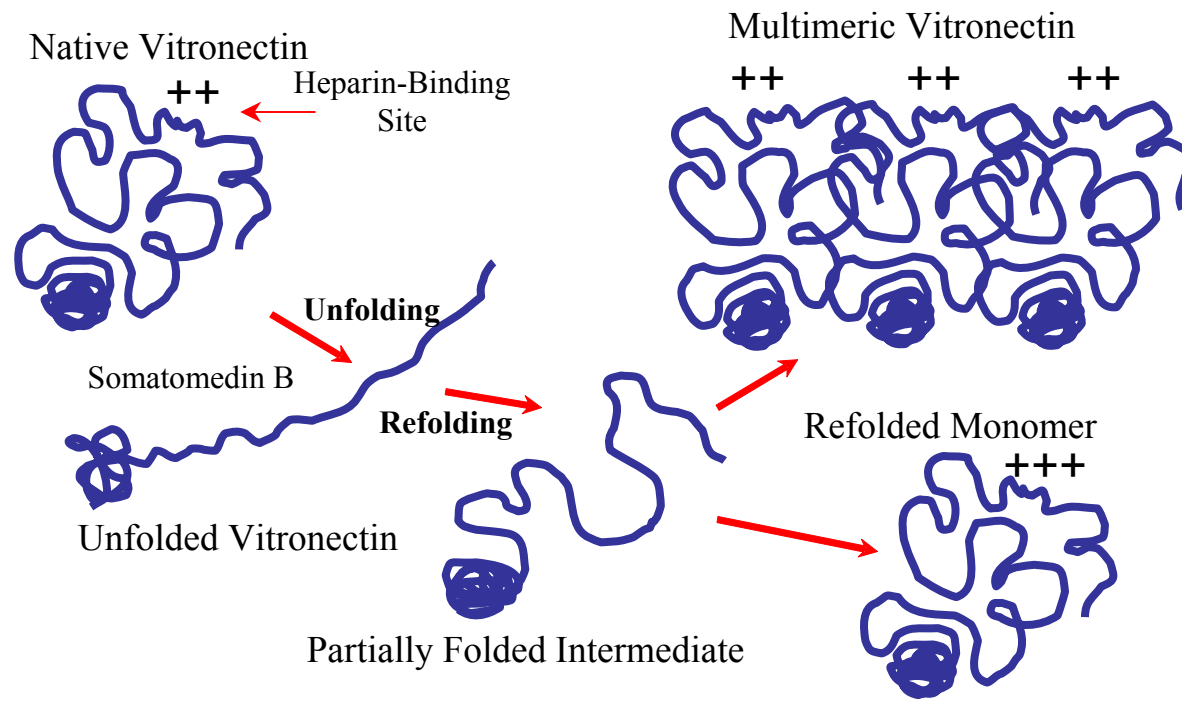


Figure A.5: Multimerization Model.

This model by Peterson suggests that plasma vitronectin is folded with the heparin-binding site exposed on the surface of the protein. During refolding, vitronectin passes through a partially folded intermediate that has a propensity to aggregate. Multimeric vitronectin results that has heparin-binding sites aligned on the surface of the molecule, providing multivalent interaction with heparin.

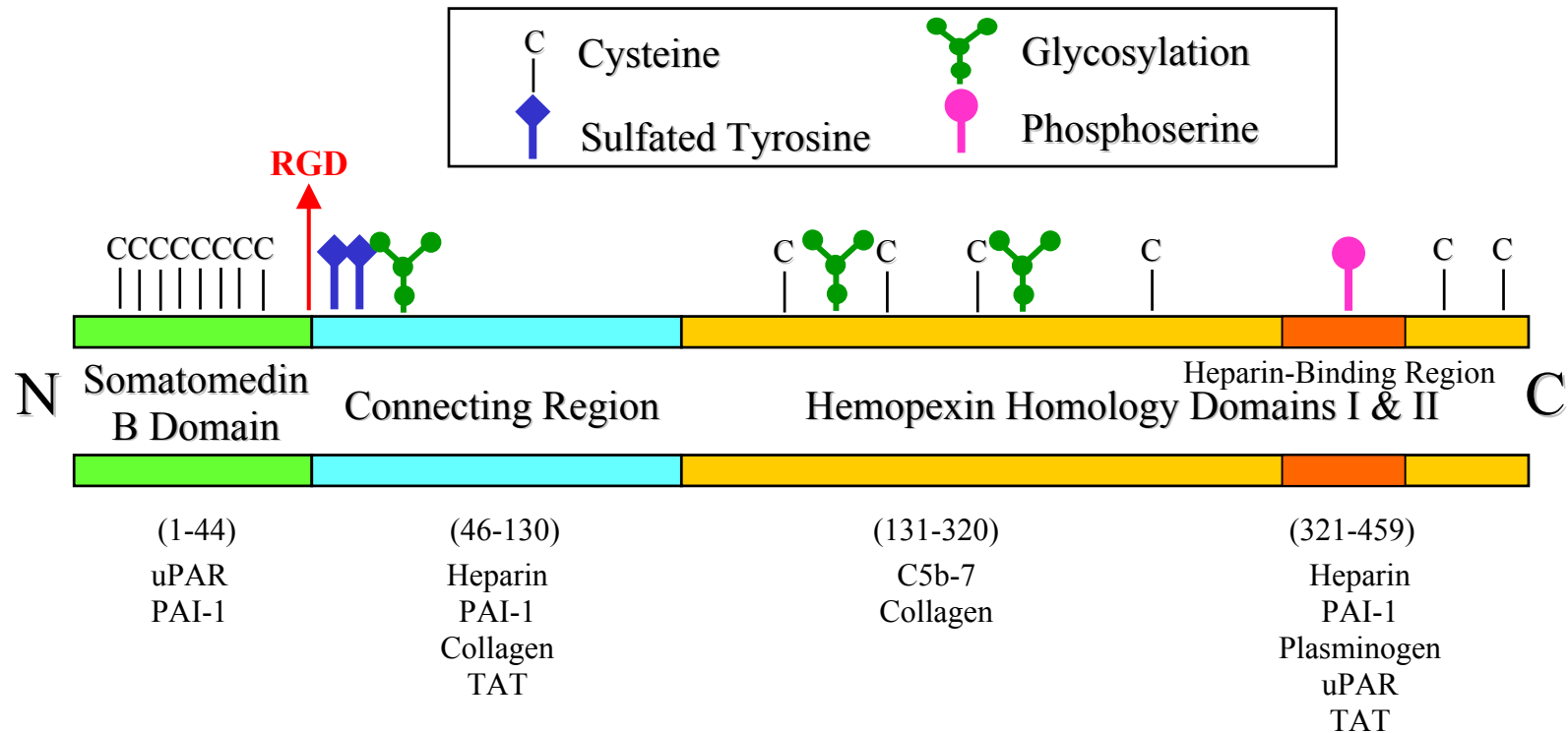


Figure A.6: Post-translational Modifications and Proposed Ligand Binding Sites Within the Domain Organization of Vitronectin.

As previously described, vitronectin is organized into four domains. This figure illustrates the sites of post-translational modifications within each domain. Also, a number of ligand binding sites have been proposed in the literature for many of the ligands with which vitronectin interacts. Some proposed ligand binding regions are represented here. Cysteine residues (C) are noted, as well the integrin recognition sequence (RGD) and the heparin binding site.

sequence of vitronectin (108,109,115,127). Other modifications could affect ligand binding in yet undetermined ways.

In summary, conformational lability, multimerization effects, enzymatic cleavages, and post-translational modifications can all contribute to discrepancies in data if not taken into consideration. A burden is placed on the researcher to select appropriate methods and preparations that will produce results that can be confidently interpreted.

ii. Considerations of Methods

A number of methods were used to localize ligand binding sites to the different domains of vitronectin. Discrepancies for binding determinants have been encountered, especially concerning the binding sites for PAI-1. In the literature, PAI-1 has been mapped to three different regions of vitronectin: the somatomedin B domain (23,81), the heparin binding region (86), and residues 115-121 in the connecting region (102). Closer examination of the methods employed by different groups reveal possible explanations for separate sites for PAI-1 binding and a number of general concerns for techniques commonly used to map ligand binding sites.

Many of the binding sites were determined using fragments of vitronectin generated through chemical and proteolytic cleavage, as well as synthetic peptides. These methods are susceptible to experimental artifact, and for many experiments, high concentrations of the fragments were required to retain function. One must keep in mind that fragments of proteins will not necessarily mimic *in vivo* interactions (full-length proteins will not necessarily, either).

Studies with monoclonal antibodies can present problems as well. Often, if a particular monoclonal is found to inhibit or compete for ligand binding to a region of vitronectin, that region of vitronectin is assumed to be a site of binding. Such assumptions must be made cautiously. In characterization of the heparin binding region, the monoclonal antibody 8E6, which supposedly mapped to the heparin binding site, was used to support the “encrypted site” model previously mentioned. It was later shown by Seiffert that 8E6 recognized a different epitope on vitronectin (128). This antibody apparently binds at the N-terminal and disrupts structure at the C-terminus, which disrupts heparin binding. This could be the case for other monoclonals. Other concerns include inconsistent preparation of the proteins involved in the assays and usage of different forms of the ligand, including PAI-1 which exists in both an active and latent form.

VII. Rationale

Much work has been done to localize PAI-1 binding to vitronectin and to understand the interaction between the protein and heparin. But another important ligand that binds vitronectin is the thrombin-antithrombin (TAT) complex. Complex interaction with vitronectin has not been characterized extensively, with the regions (and residues) on vitronectin involved in binding not conclusively identified. Using overlapping recombinantly expressed polypeptides of vitronectin, the Far Western approach has been employed to localize the binding of TAT to vitronectin. Such localization will help to further describe the vitronectin-TAT interaction and provide impetus for further study.

These polypeptides are further used to epitope map a number of murine monoclonal antibodies to vitronectin our laboratory has recently acquired.

Part B

Localization of the Thrombin-Antithrombin Binding Site(s)

I. Introduction

As previously mentioned, we know with confidence that vitronectin binds many ligands. A number of approaches have been used to determine the number of binding sites for each ligand and the region(s) of vitronectin involved in the interaction(s). The approach taken in this project involves the expression of bacterial recombinant polypeptides of vitronectin. The polypeptides are tested for interaction with TAT using a Far Western approach.

The Far Western technique (sometimes referred to as a “West-Western”) is a derivation of the traditional antigen-antibody interaction of the Western blot. Far Westerns are commonly used to screen cDNA libraries and/or to study protein-protein interactions. Here, the Far Western technique involves the incubation of a second protein of interest with an immobilized protein (electroblotted onto nitrocellulose). Interaction is visualized by detection of an antibody to the second protein.

The use of the Far Western technique with recombinant fragments to localize ligand binding sites is unique to the body of vitronectin research. The first target ligand, which is the focus of this project, is the thrombin-antithrombin complex. Thrombin, antithrombin, the complex, and interactions with vitronectin will be reviewed in the next sections.

i Thrombin

Thrombin has many diverse functions in the body, it is the ultimate activation product of the coagulation cascade. Upon injury, the blood coagulation cascade works through a series of enzymatic cleavages that activate sequential “clotting factors” that can

in turn cleave the next factor until finally soluble fibrinogen is cleaved by thrombin into insoluble fibrin. Thrombin serves as a regulator of the coagulation process through both stimulatory and inhibitory feedback mechanisms. It has thus been a popular target for anticoagulant drug therapy (129).

Thrombin is a serine protease that recognizes particular sequences and catalyzes the hydrolysis of arginine-glycine peptide bonds. After the peptide cleavage of fibrinogen, fibrin monomers are left with a specific surface charge pattern that promotes their aggregation, and this clustering together forms a fibrous blood clot (130,131). Thrombin has also been shown to trigger platelet aggregation and catalyze the activation of other blood clotting factors V, VIII, and XIII (132).

Human blood plasma contains approximately 40-50 mg/L of thrombin, and such a concentration is required for proper blood clotting to occur efficiently. Thrombin is activated by the cleavage of its zymogen prothrombin by factor Xa and factor Va. Thrombin's activity is controlled by negative regulators antithrombin III and hirudin, both of which function by binding to thrombin's active site.

Thrombin shares structural homology to serine proteases trypsin and chymotrypsin. Thrombin is a glycoprotein formed by two disulfide-linked peptide chains of 36 and 259 amino acids. Three sites of interaction have been identified on the protein, the first of which is the negatively charged catalytic site that confers thrombin's serine protease activity and contains the familiar catalytic triad. On thrombin, these residues include histidine 43, aspartic acid 99, and serine 205 (133).

The other two sites of interaction are positively charged and named exosite 1 and exosite 2. Exosite 1 borders the catalytic site and binds substrate (fibrinogen or thrombin

receptor). On the enzyme surface, exosite 2 is located proximal to exosite 1 and binds inhibitor antithrombin III responsible for thrombin's inactivation (134-136). Exosite 1 also binds Factor V and Factor VIII, while exosite 2 contains the binding site for heparin and thought to be involved in interaction with Factor V and Factor VIII (137,138). Thrombin also interacts with fibrin at either exosite 1 or exosite 2 in a manner dependent on whether heparin is present or absent, and then the concentration of heparin in the environment if heparin is indeed present. But in any binding event, thrombin remains active due to fibrin binding at a site distinct from the catalytic site on thrombin (139-141).

Fibrinolysis is the process of clot dissolution, thus thrombin is usually thought of as an inhibitor of fibrinolysis. Interestingly, however, thrombin also demonstrates stimulatory effects on the process. First, thrombin is a chemoattractant for neutrophils; neutrophils have demonstrated a role in fibrin clot degradation (142). Second, thrombin releases plasminogen activators from endothelial cells (143). These plasminogen activators generate plasmin, a key initiator of fibrinolysis and inactivator of prothrombin. Thrombin's capacity to both inhibit and enhance fibrinolysis demonstrates yet again its vital role in hemostasis and the fine balance between clot formation and clot dissolution. Disruption of this fine balance leads to either excessive hemorrhage or excessive coagulation, conditions that can be potentially fatal.

As previously mentioned, thrombin is a chemoattractant for neutrophils and monocytes. Due to this association, thrombin is also involved in inflammation. At the sites of an injury, thrombin concentrates, inducing these cells to gather and carry out their phagocytic role to protect against potential bacterial infection (144,145).

Human α -thrombin is composed of two disulfide-linked chains—a 36 amino acid long A chain and a 259 amino acid long B chain (146). β and γ -thrombin result from the limited trypsin proteolysis of α -thrombin's B chain (147). β -thrombin is formed by a cleavage at either Arg75—Tyr76 and/or Arg77—Asp78 whereas γ -thrombin has an additional cleavage at Lys149—Gly150 (148). γ -thrombin consists of three polypeptide segments that are non-covalently associated (149). Each polypeptide contains one of the catalytic triad residues: Asp-99 is on the A-B₃ chain, His-43 is on the B₁ chain, and Ser-205 is on the B₄ chain (150). Significantly, when α -thrombin is cleaved to create γ -thrombin, clotting activity is either lost or very low, although enzyme activity is retained (149,150). This is explained by disruption of the active site and also an upset of the fibrin(ogen) recognition site on γ -thrombin (151).

γ -thrombin does bind to antithrombin, but at a reportedly lower rate compared to α -thrombin (152). Electron spin resonance studies indicate that contained within the active site of γ -thrombin is a hydrophobic region not present in α -thrombin (153). This altered active site of γ -thrombin may be responsible for findings that revealed a much tighter binding of γ -thrombin to vitronectin compared to α -thrombin. Additionally, it was suggested that the α -thrombin component of thrombin-antithrombin was responsible for interaction with vitronectin, probably through a region exposed on γ -thrombin (11).

ii. Antithrombin

Antithrombin is a 432 amino acid glycoprotein that plays an important role in the regulation of blood clotting (154). In plasma, antithrombin is the major inhibitor of coagulation proteases such as thrombin, Factor Xa, and Factor IXa (155-158).

Specifically, antithrombin is a serine protease inhibitor (serpin) (159). Antithrombin deficiencies disrupt hemostasis and can lead to venous thromboses, thus demonstrating the important role it plays in maintaining the fine balance between abnormal clotting extremes.

Antithrombin shares structural homology with α_1 -proteinase inhibitor, PAI-1, heparin cofactor II, and α_1 -antichymotrypsin--other protease inhibitors (35). It contains three disulfide bonds and four sites of glycosylation (154). Two X-ray structures of antithrombin have been acquired and studied—an intact form and an inactive, cleaved form (160,161). Intact antithrombin possesses two prominent features: 1) a five-stranded β -sheet, the A sheet and 2) a large exposed loop containing the reactive bond (Arg 393—Ser-394). Inactive antithrombin is cleaved near the reactive bond, and the N-terminal portion of the reactive loop is inserted as a middle strand in the A β -sheet.

One of antithrombin's most physiologically critical targets is thrombin. Antithrombin binds thrombin in a 1:1 stoichiometric ratio whereby thrombin is inactivated in a two-step process. After the formation of an initial “encounter” complex, a conformational change occurs to form a stable serpin-enzyme pair (162-167). Enzyme inactivation is initiated upon recognition by the enzyme of antithrombin's reactive bond (168-170). Antithrombin residue Arg-393 is essential for this recognition (171-173). On thrombin, the serine of the active site (174) and Trp-60 (175,176) are required for proper interaction with antithrombin. It is believed that thrombin attacks the reactive bond of the fully exposed loop as in regular substrate binding. When this happens, the loop with thrombin bound folds into the A sheet of antithrombin and this insertion traps the enzyme in a stable complex with inhibitor whereby the enzyme is inactivated (174,177-180).

Heparin is a strongly negatively charged glycosaminoglycan that greatly accelerates the rate of inhibition of thrombin (and other clotting proteases) by antithrombin. Indeed, the thrombin-antithrombin pairing rate increases 2000-4000 fold in the presence of optimal concentrations of heparin (181-184). Heparin confers its effect by first binding to antithrombin (182,185). Positively charged residues on antithrombin recognize a specific pentasaccharide sequence of heparin (186-190), and upon binding, a conformational change takes place in antithrombin (Figure B.1). It is then believed that thrombin non-specifically binds the same heparin chain at any number of sites along the polysaccharide (183,191). Thrombin travels down the chain and meets the inhibitor, where the two interact. Serpin-enzyme complex formation results in a greatly diminished affinity for heparin (185,192). Thus, heparin quickly dissociates, leaving it free to bind another antithrombin molecule.

iii. Interactions of the Complex with Vitronectin

Vitronectin interacts with the thrombin-antithrombin (TAT) pair to form a ternary complex (17,20). In fact, most TAT found in human serum is complexed with vitronectin (31,118). The association of vitronectin with TAT is important for a number of reasons. 1) Vitronectin confers its heparin-binding (15) and cell-binding (193) properties to the TAT pair when the two components are bound. 2) Studies indicate a conformational change in plasma vitronectin upon binding of TAT (15). 3) It has been speculated that ternary complex formation is the primary mechanism by which circulating vitronectin is translocated to the extravascular space (1). And 4) It has been

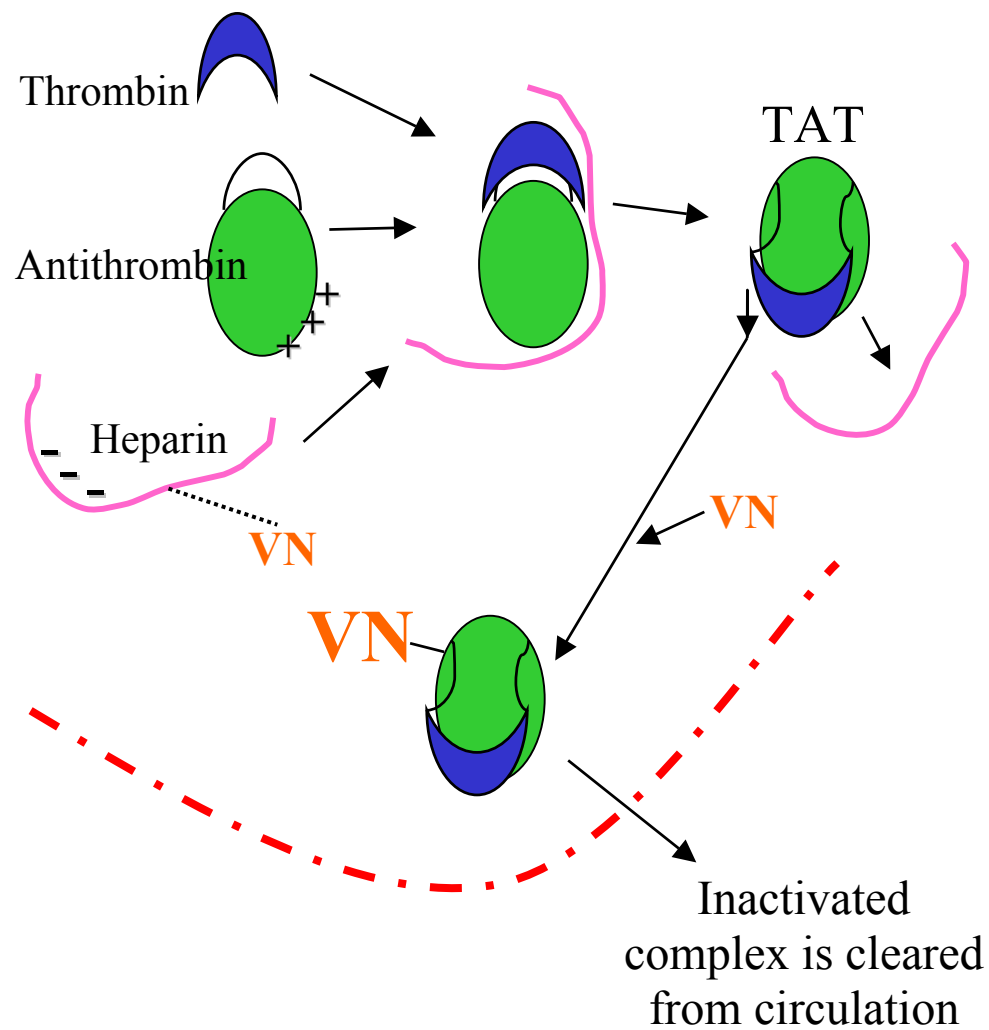


Figure B.1: Vitronectin Association with TAT and Heparin.

This schematic represents the heparin-enhanced association of thrombin with its inhibitor antithrombin. Vitronectin binds not only the complex, but the molecule that facilitates complex formation. It is believed that once vitronectin binds TAT, the ternary complex is cleared from circulation.

suggested that vitronectin binding to TAT is largely responsible for the clearance of the complex from circulation (92,194,195).

Previous work from this laboratory has contributed significantly to the understanding of the interaction between vitronectin and TAT. These studies have shown that the thrombin component of TAT is responsible for vitronectin binding, in that thrombin alone can bind vitronectin, but antithrombin is only detected bound to vitronectin when it is complexed with thrombin. Additionally, high performance liquid chromatography (HPLC) experiments demonstrated that VN-T-AT complexes lead to vitronectin multimerization over time.

The nature of the interaction between vitronectin and TAT has been reported to be a covalent interaction formed by a disulfide bond (11,15,112), catalyzed by protein disulfide isomerase (PDI) (196). Neither thrombin nor antithrombin has free sulfhydryl groups, thus a thiol-disulfide exchange is suggested, with the thiol(s) being contributed by vitronectin (112). In contrast, additional work from this laboratory reports evidence that the interaction is not disulfide mediated, but is rather an ionic interaction; in the presence of iodoacetamide, ELISA experiments demonstrated that VN-T-AT complexes were not affected.

Other subjects of speculation and study concerning the vitronectin-TAT interaction are 1) how many binding sites does vitronectin contain for the thrombin-antithrombin pair and 2) what regions of the protein are involved in the interaction with TAT? This laboratory has demonstrated that the binding site for the thrombin-antithrombin complex is separate from the PAI-1 binding site. It has also been suggested through monoclonal antibody experimentation that the amino portion of vitronectin is

involved in ternary complex formation (16), although the interaction is proposed to be disulfide-mediated, which our laboratory disputes. Early reports speculated that the heparin binding region of vitronectin could be a potential site for the vitronectin-TAT interaction (124,181). The focus of the research presented here is to use recombinantly expressed fragments of vitronectin to localize the binding region(s) for thrombin-antithrombin. The interaction will be detected using a Far Western technique.

II. Experimental Procedures

i. Proteins, Antibodies, and Protease Inhibitors

The proteins, antibodies, and protease inhibitors used throughout this study were obtained as follows. Native, monomeric vitronectin was purified from human blood plasma using a modified protocol of the method developed by Dahlback and Podack (112). Human α -thrombin was purchased from Haematologic Technologies, Inc. and human antithrombin III was obtained from the American Red Cross (manufactured by Baxter Healthcare Corporation). Polyclonal antibodies to human α -thrombin and human antithrombin III were also purchased from Haematologic Technologies, Inc. Monoclonal anti-polyhistidine clone his-1 (peroxidase conjugated) was purchased from Sigma. HRP-conjugated secondary antibodies were purchased from Vector labs (anti-mouse and anti-goat) and Santa Cruz Biotechnology (anti-sheep). All protein standards were Kaleidoscope protein or polypeptide standards purchased from Bio-Rad. Protease inhibitors Pepstatin A, Leupeptin, and Pefabloc SC were purchased from Roche.

ii. Transformation into Expression Cells

cDNAs encoding eight overlapping polypeptides of vitronectin 100 amino acids in length (aa 1-100, 51-150, 101-200, 151-250, 201-300, 251-350, 301-400, and 351-459) were obtained from D. Seiffert (DuPont Merck Research Laboratories). Figure B.2 demonstrates the domains of vitronectin each polypeptide spans. These were cloned into the multiple cloning site of pET-15b (Novagen) which carries an N-terminal His-Tag® sequence. The recombinant plasmids were transformed into BL21DE3pLysS *E. coli* (Novagen) according to company protocol.

iii. Directional Cloning of aa 251-350 and 301-400

It was determined from Christine Schar's previous work that the original DNA for polypeptide 251-350 (obtained from D. Seiffert) contained mutations at positions **** Christine worked to correct the sequence and cloned the corrected gene into the yeast vector pAS2-1 (Clontech) to pursue yeast two-hybrid studies.

For the research presented here, the 251-350 polypeptide cDNA was cloned back into pET15b. pAS2-1/251-350 was transformed into DH5α *E. coli*. The recombinant plasmid was purified using a midiprep kit (Qiagen). The 251-350 cDNA was excised using the restriction enzymes BamHI and NdeI (part of the multiple cloning sites in both plasmids of interest), available from Fisher Scientific. 251-350 cDNA was purified from 1.5% agarose gel electrophoresis using Qiagen's QiaQuick Gel Extraction Kit. pET15b was also purified from 1.0% agarose gel electrophoresis using this kit. A ligation

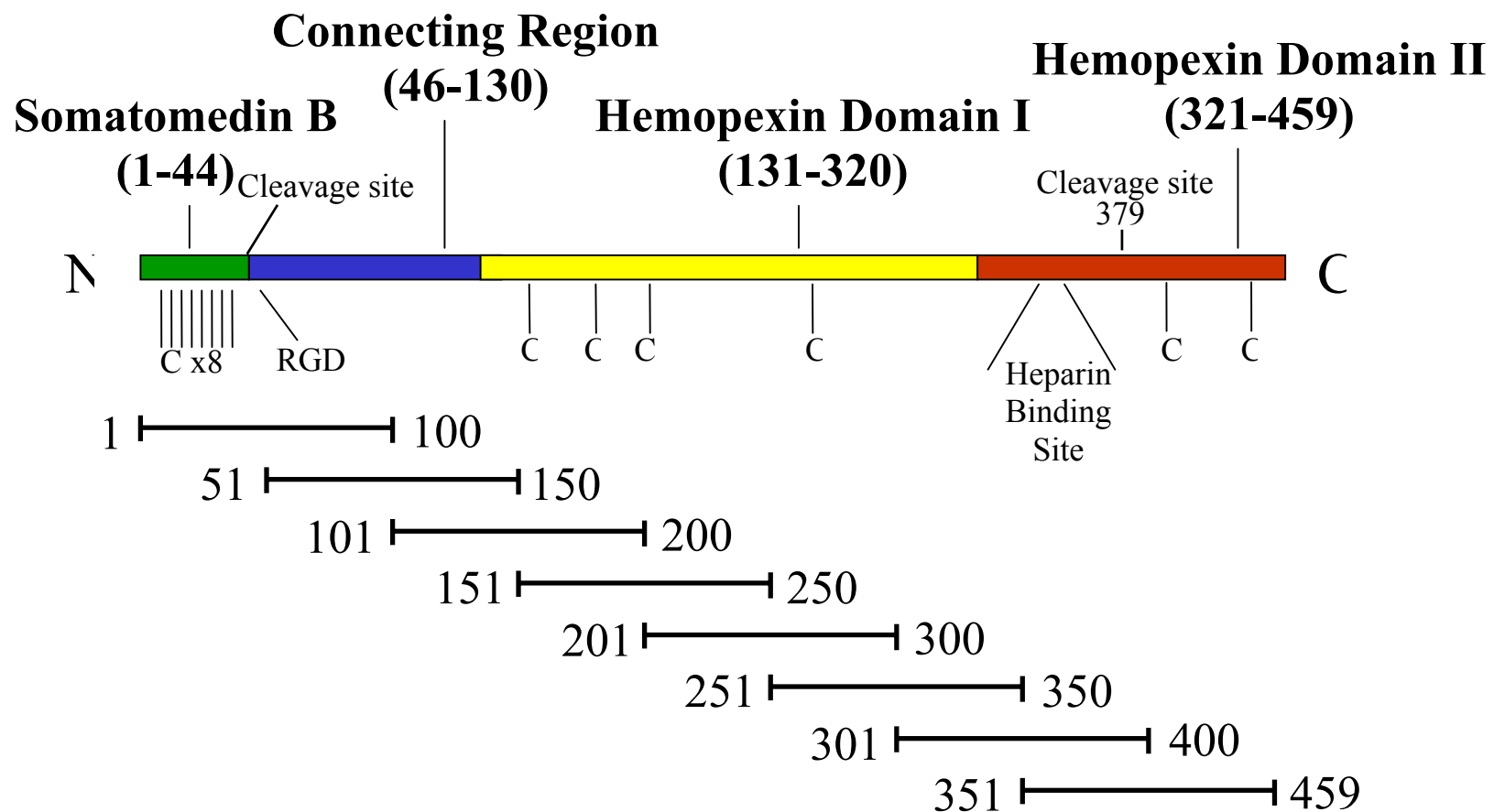


Figure B.2: Linear Organization and Corresponding 100 Amino Acid Polypeptides.

Vitronectin is (linearly) organized into 4 regions. The 14 cysteines are noted, as is the familiar integrin binding recognition sequence (RGD) at the end of the somatomedin B region, and the heparin-binding site in hemopexin domain II. Overlapping polypeptides 100 amino acids in length were engineered and cloned into the pET expression system. They are shown here to give a general idea as to which region(s) each encompasses.

reaction was performed using Invitrogen's T4 DNA ligase according to company protocol. The reaction was transformed into DH5 α cells and plated on LB/amp. Colonies were grown overnight in 2 ml TB/amp, midiprep (Qiagen), and a double digest was performed to confirm insert. Upon confirmation of insert (visualization of ~300 bp band), plasmid/insert was transformed into BL21(DE3)pLysS *E. coli* according to company protocol.

Upon DNA sequence analysis of the 301-400 recombinant, it was determined that the *lac* operator harbored a mutation. To correct the problem and obtain a functional operator, the 301-400 cDNA was excised from the mutated plasmid again using the restriction enzymes BamHI and NdeI. 301-400 was purified from 1.5% agarose gel electrophoresis using Qiagen's QiaQuick Gel Extraction Kit. The gene was ligated into the previously purified pET15b using Invitrogen's T4 DNA ligase according to company protocol and plated as described above. Confirmation of insert was visualized using agarose gel electrophoresis and DNA sequence analysis demonstrated a corrected *lac* operator.

iv. Small-scale Inductions

Isolated colonies from the previously mentioned transformations were selected for overnight growth (37°C with shaking) in 2.0 ml of Terrific® broth (TB available from Gibco) containing ampicillin (50 μ g/ml) and chloramphenicol (34 μ g/ml). Duplicate cultures of each colony were prepared and grown to mid-log phase and protein expression was induced in one of the duplicate cultures with 1.0mM IPTG (Sigma) and grown for another 4 hours. All cultures were harvested using centrifugation (13,000 rpm

5 min.) and the supernatant was decanted. Cells were resuspended in 50 μ l 2X sample loading dye, boiled, and analyzed using 15% SDS PAGE. Expression was assayed by immunoblotting to nitrocellulose at 20V for 20 minutes. Membranes were blocked for 1 hour in 10% dry milk/PBS. After washing, membranes were incubated with an anti-polyhistidine antibody (peroxidase conjugated) at a dilution of 1:2000. Expression was detected upon addition of substrate (12 μ l H₂O₂, 30ml PBS, 6 ml 4-chloro-1-naphthol).

vi. Large-scale Inductions/expression

2.0 L of enriched media (47.0 g/L TB, 5.0 g/L YNB (Difco), 4.0 ml/L glycerol, 50 μ g/ml amp, 34 μ g/ml chlor, pH 7.2) was prepared and inoculated with 100 ml of ~2.5 hour culture (TB/amp/chlor). Cells were allowed to grow with shaking at 37°C till OD₆₀₀ indicated log phase. Protein expression was induced with 1.0 mM IPTG (Sigma) and cells allowed to grow for another 8-12 hours. Cells were harvested by centrifugation (10,000 rpm at 4°C in Beckman JA-10 rotor for 30 minutes) and stored in -20°C freezer until purification.

vi. Metal-chelating Affinity Chromatography

2.0 L cells were resuspended in 160 ml resuspension buffer (5mM imidazole, 40mM Tris-HCl, 500mM NaCl, leupeptin, Pefabloc, Pepstatin A, pH 7.9). Cells were lysed using sonication. Sonication product was separated using centrifugation (10,000 rpm in Beckman JA-10 for 30 min., 4°C). Supernatant was poured off and reserved; 30 μ l of the supernatant was analyzed by 15% SDS PAGE. If the polypeptide was contained

in the pellet portion, 60 ml of solubilization buffer (5mM imidazole, 40mM Tris-HCl, 500mM NaCl, 6.0 M urea, protease inhibitor cocktail, pH 7.9) was added to the sonication pellet and the pellet resuspended. Cellular debris was separated from the solubilized pellet by centrifugation (10,000 rpm in Beckman JA-10 rotor, 40 min., 4°C) and the supernatant reserved.

Soluble or urea-solubilized polypeptide sample was loaded on an equilibrated Chelating Sepharose® Affinity column (Pharmacia) charged with 100mM NiSO₄ at a flow rate of 1.0 ml/min and 3.0 ml fractions were collected. Wash buffer (16 mM imidazole) was passed over the column till A₂₈₀ readings were ~0.00. Protein was step-wise eluted with 75 mM, 150 mM, and 300 mM imidazole buffer. Fractions were analyzed on 15% SDS-PAGE and Western blotted with fractions containing polypeptide detected using an anti-polyhistidine antibody. Protein concentrations were determined using the Bradford Assay (reagents available from Bio-Rad). Polypeptide 101-200 fractions were concentrated using an Amicon (10,000 molecular weight cut-off) 4.0 ml centrifuge concentrator. 3.0 ml of sample was loaded and centrifuged 30 minutes in a swinging bucket centrifuge (4000 rpm, 4°C).

vii. Far Western Technique

1. Full-length Vitronectin

Either 3 or 5 µg native vitronectin in PBS was loaded on a 10 % gel and SDS-PAGE was performed. Protein in the gel was transferred to nitrocellulose at 20V for 20 minutes, blocked for 1 hour in 10% dry milk/PBS, and then incubated overnight with shaking at 4°C with 100 µl thrombin-antithrombin complex in 30 ml PBS solution

(complex prepared by incubating 400 ug/ml thrombin with 1.5 mg/ml antithrombin for one hour to form a stable complex). After washing with PBS/0.1% Tween, the membrane was divided and incubated with shaking for one hour at room temperature with an antibody raised against either thrombin or antithrombin. Each membrane was washed again with PBS/Tween and peroxidase-conjugated secondary antibodies (thrombin -- anti-sheep; antithrombin -- anti-goat) were allowed to bind for one hour with shaking at room temperature. The membrane was developed upon addition of substrate.

2. Polypeptide Fragments of Vitronectin

Approximately 20µg of each pure polypeptide was loaded into 15% SDS-PAGE. Two different Western blot protocols were employed with the polypeptides involving two different transfers. In some experiments, polypeptides were transferred to nitrocellulose at 20V for 20 minutes using a BioRad Semi-dry Blotter and the same protocol was followed as for the native, full-length vitronectin. Alternatively, some polypeptide gels were transferred to Immobilon™ (Millipore) at 24V for 30 minutes in a submerged gel transfer apparatus. The membranes were blocked for 1 hour in 5% dry milk in TBS/0.1% Tween. The membranes were then incubated overnight at 4°C with the thrombin-antithrombin complex (prepared as described above) in TBS/Tween. The membrane was washed 3X for 10 minutes in TBS/Tween. Membranes were incubated with either an anti-thrombin or anti-antithrombin antibody for 40 minutes in 2.5% dry milk in TBS/Tween. Again, the membranes were washed and appropriate peroxidase conjugated secondary antibodies were allowed to bind for 40 minutes. The membranes were washed as described and then bathed in a 1:1 solution of SuperSignal® Chemiluminescent

Substrate ULTRA Stable Peroxide Solution and Chemiluminescent Luminol/Enhancer Solution (Pierce) for 1 minute. The membranes were exposed on a film sheet for 5 minutes and developed in the dark room.

III. Results

i. Cloning of 251-350 and 301-400

The cDNA encoding polypeptide 251-350 obtained by our laboratory included mutations that were corrected by another member of our lab and cloned into a yeast vector. For expression in the pET system, it was essential that the corrected cDNA sequence be cloned into pET15b. Figure B.3a is a schematic representing the cloning procedure. The cDNA was excised by a double digest with BamHI/NdeI, which would also linearize the plasmid. After successful purification of cDNA 251-350 and pET15b from an agarose gel, T4 DNA ligase was used to ligate the cDNA into the plasmid and the reaction was plated. Colonies were analyzed by agarose gel electrophoresis and as is obvious from the gel picture in Figure B.3b, a successful clone resulted.

The cDNA encoding polypeptide 301-400 was originally in pET15b and the cDNA was shown to be correct by DNA sequence analysis, yet no polypeptide was expressed when transformed into BL21(DE3)pLysS cells. Upon sequence analysis of the promoter region, a mutation was found in the *lac* operator, which was believed to be the culprit of no expression. Thus the cDNA was excised and purified from the deficient plasmid and cloned in a similar procedure as 251-350 into the already successfully purified pET15b. After several attempts, a successful ligation was obtained, seen in Figure B.4, and sequence analysis confirmed a correct sequence in the promoter region.

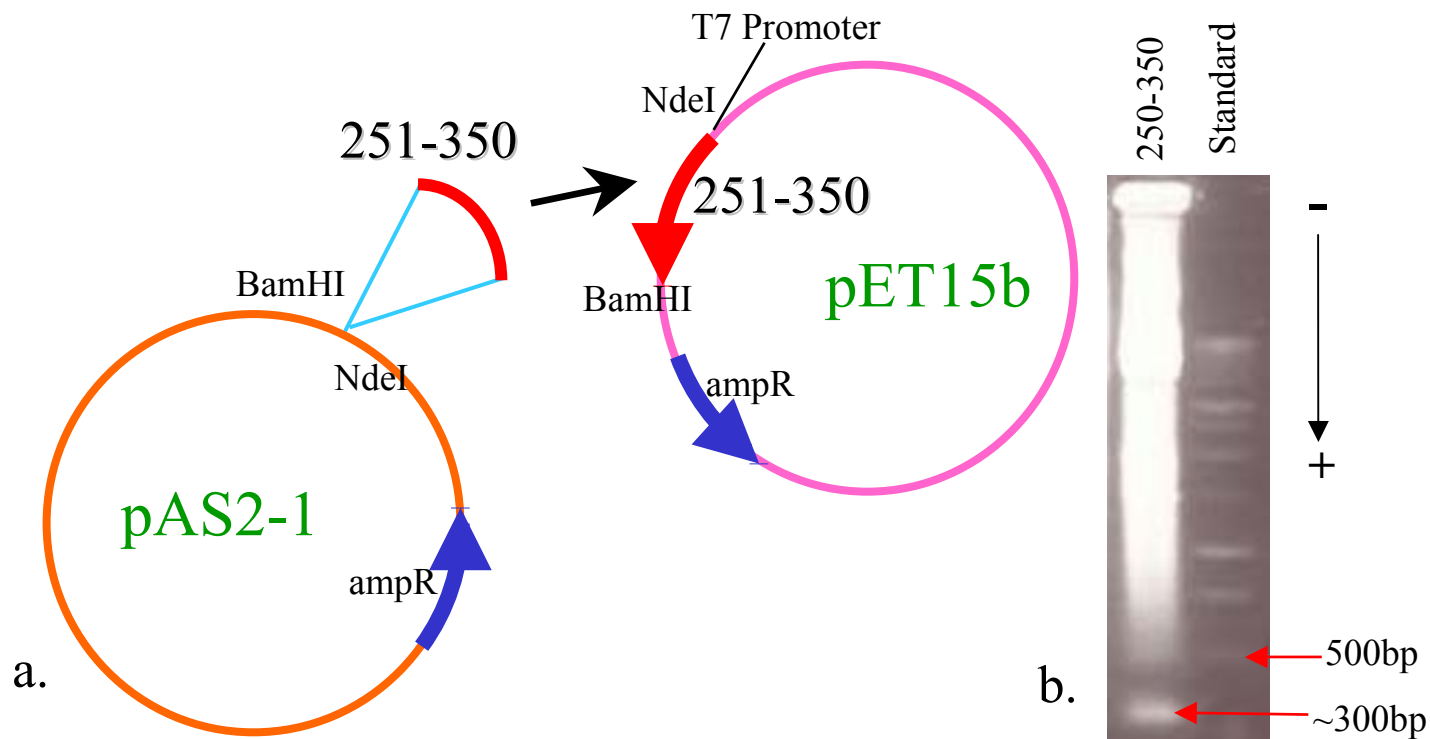


Figure B.3: Cloning of 251-350.

- Schematic representing the cloning strategy for 251-350. cDNA was excised from the yeast plasmid pAS2-1 using restriction enzymes BamHI and NdeI. It was then ligated into the expression plasmid pET15b which contains an inducible T7 promoter and confers ampicillin resistance.
- A 1.5% agarose gel picture of double digested recombinant plasmid. After the cDNA was transformed into DH5 α cells, the recombinant was purified, double-digested with BamHI/NdeI and analyzed by electrophoresis. The 251-350 cDNA is shown at ~300bp.

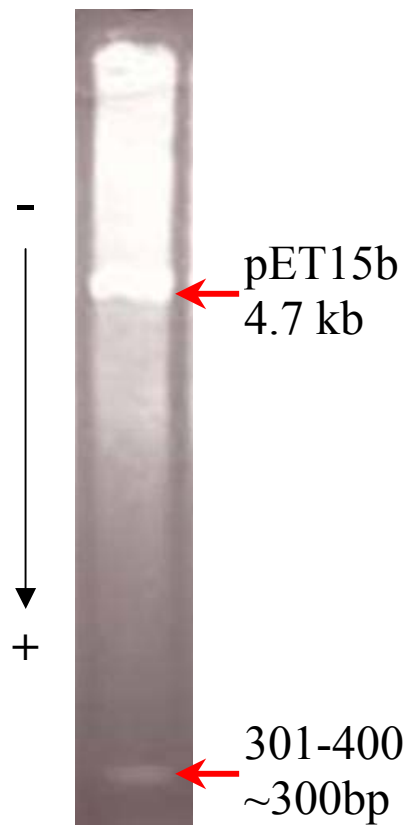


Figure B.4: Agarose Gel Picture of 301-400.

cDNA coding for polypeptide 301-400 was cloned into pET15b, double digested with BamHI/NdeI and analyzed by agarose gel electrophoresis. The 301-400 cDNA does appear around bp 300, but subsequent attempts to express this polypeptide by transformation into BL21DE3pLysS cells did not prove successful.

ii. Seven of the Polypeptides Were Successfully Expressed

To express the polypeptides using the pET expression system, recombinant plasmids were transformed into BL21(DE3)pLysS *E. coli* cells. This strain was selected because of its successful history for inducible expression of protein/polypeptides in pET plasmids, the chloramphenicol selection capability, and because this strain codes for lysosyme, assisting in efficient lysis of cells when purifying a protein of interest. Before the polypeptides were expressed on a large scale, transformants were screened for expression on a small scale. 4-6 colonies were selected and grown in 2.0 ml of media and induced with 1.0mM IPTG. Expression was successfully achieved for seven of the eight polypeptides: 1-100, 51-150, 101-200, 151-251, 201-300, 251-350, and 351-459, shown in Figure B.5, which does include the new 251-350 recombinant. The colony from each recombinant polypeptide that demonstrated the best expression (by visual assessment) was selected for growth/expression on a large scale in 2.0 L as described. Expression was detected using an antibody to the His-tag at the N-terminal of the expressed polypeptide.

Small scale expression of the polypeptides was performed multiple times throughout the course of this project. Expression levels of each polypeptide were somewhat variable from one transformation to another, but each demonstrated a (general) repeatable level of expression in comparison to the other polypeptides. For example, polypeptides 201-300 and 351-459 consistently expressed at higher levels than either 1-100 or 51-150. Inconsistencies in expression are most easily explained by loading variabilities. Cells are grown in slightly different conditions and post-induction growth times do vary for individual induction studies. It is very difficult to assess the

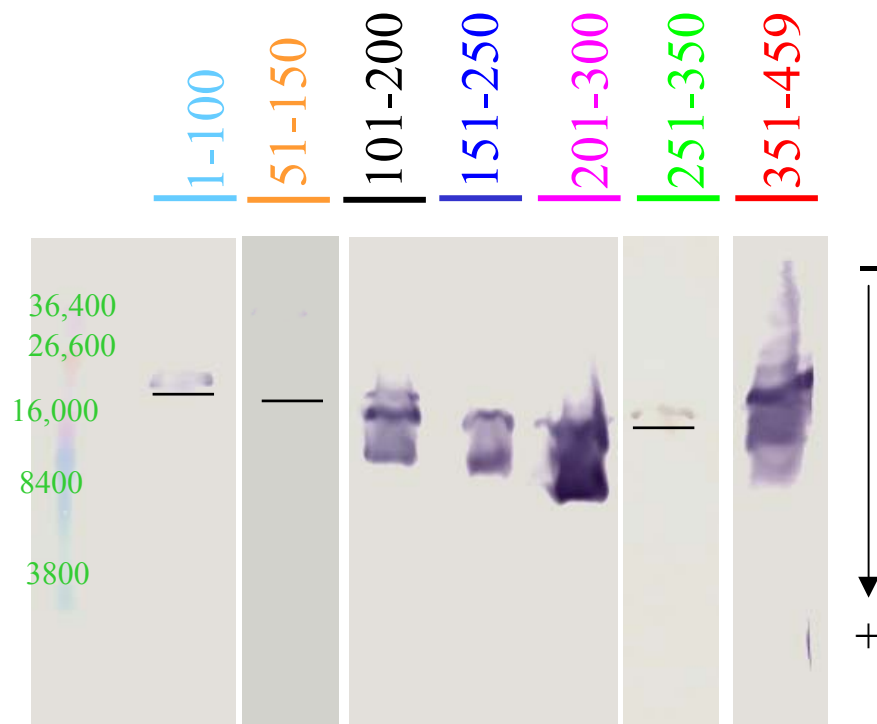


Figure B.5: Expression of Overlapping 100aa Polypeptides

BL21(DE3)pLysS cells containing the recombinant plasmids were induced to express using 1.0mM IPTG. Crude extracts were loaded on 15% SDS PAGE and transferred to nitrocellulose. Expression was detected using an anti-His tag antibody and Western blots are pictured here. Expression was achieved for all the polypeptides except 301-400. The newly cloned 251-350, which had eluded expression previously, did express as shown here.

concentration of the target polypeptide for each experiment, so a fixed microliter amount is loaded and inevitably variable expression levels are detected.

Even after successfully cloning 301-400, multiple attempts to express the polypeptide failed. When the recombinant plasmid was digested with BamHI/NdeI, an “insert” band was visualized by gel electrophoresis. DNA sequence analysis shows correct promoter sequence, and the cells supposedly containing the recombinant plasmids are viable. But this recombinant continues to resist expression.

iii. Purification Was Achieved for Seven Polypeptides

To exploit the N-terminal His-tag on each expressed polypeptide, nickel affinity chromatography was selected to purify the recombinants. Centrifuged cells were resuspended and the cells were lysed by sonication. Upon Western analysis, polypeptides 1-100 and 51-150 were found to be present primarily in the “soluble” supernatant, thus the chromatography described was conducted in the absence of urea. Polypeptides 101-200, 151-250, 201-300, 251-350, and 351-459 were resolubilized in 6.0 M urea buffer and further centrifuged to separate cellular debris. Polypeptide presence was confirmed by SDS-PAGE/Western blot in the urea-solubilized sonication pellet and the chromatography performed for these polypeptides included buffers containing 6.0 M urea. Affinity chromatography was performed at 4°C to hinder degradation per company suggestion. Polypeptide samples were loaded and washed at a flow rate of 1.0 ml/min and 3.0 ml fractions were collected and monitored at 280nm till the absorbance was close to 0.0.

First attempts at purification with polypeptide 151-250 (found to be a high-yield recombinant compared to the other polypeptides) employed a protocol with only one elution step with 300 mM imidazole. This resulted in a polypeptide in solution with several contaminants. Subsequent purifications were performed with a step-wise elution of 75, 150, and finally 300 mM imidazole. This yielded much purer polypeptides. SDS-PAGE gels for the purification of each polypeptide are shown in Figure B.6. Protein concentrations for the elution fractions with highest absorbance (280 nm) were determined using the Bradford Assay, the values of which are listed in Table II. The expression of 101-200 was low compared to other polypeptides, thus a 3.0 ml fraction of 101-200 was concentrated down to 1.0 ml using an Amicon centrifuge concentrator.

These polypeptides tend to be susceptible to degradation. It was observed that even 4-5 days after purification, the concentration of a particular polypeptide was dramatically reduced, probably due to protease degradation. Because of this observation, a portion of all the eluted polypeptides was immediately reserved, mixed 1:1 with reducing sample loading buffer, boiled 5 minutes and frozen in the -80°C storage freezer. Figure B.7 is a 15% SDS-PAGE gel picture of the seven purified polypeptides, along with relative molecular weight values.

iv. Far Western Conditions Were Optimized

To determine if this assay could feasibly be used with the protein components involved and also to determine effective Far Western conditions to eventually use in experiments with the polypeptides, a series of Far Western blots was performed with full-length vitronectin. Different amounts of vitronectin—0.5µg, 1.0µg, 2.0µg, 3.0µg, 5.0 µg,

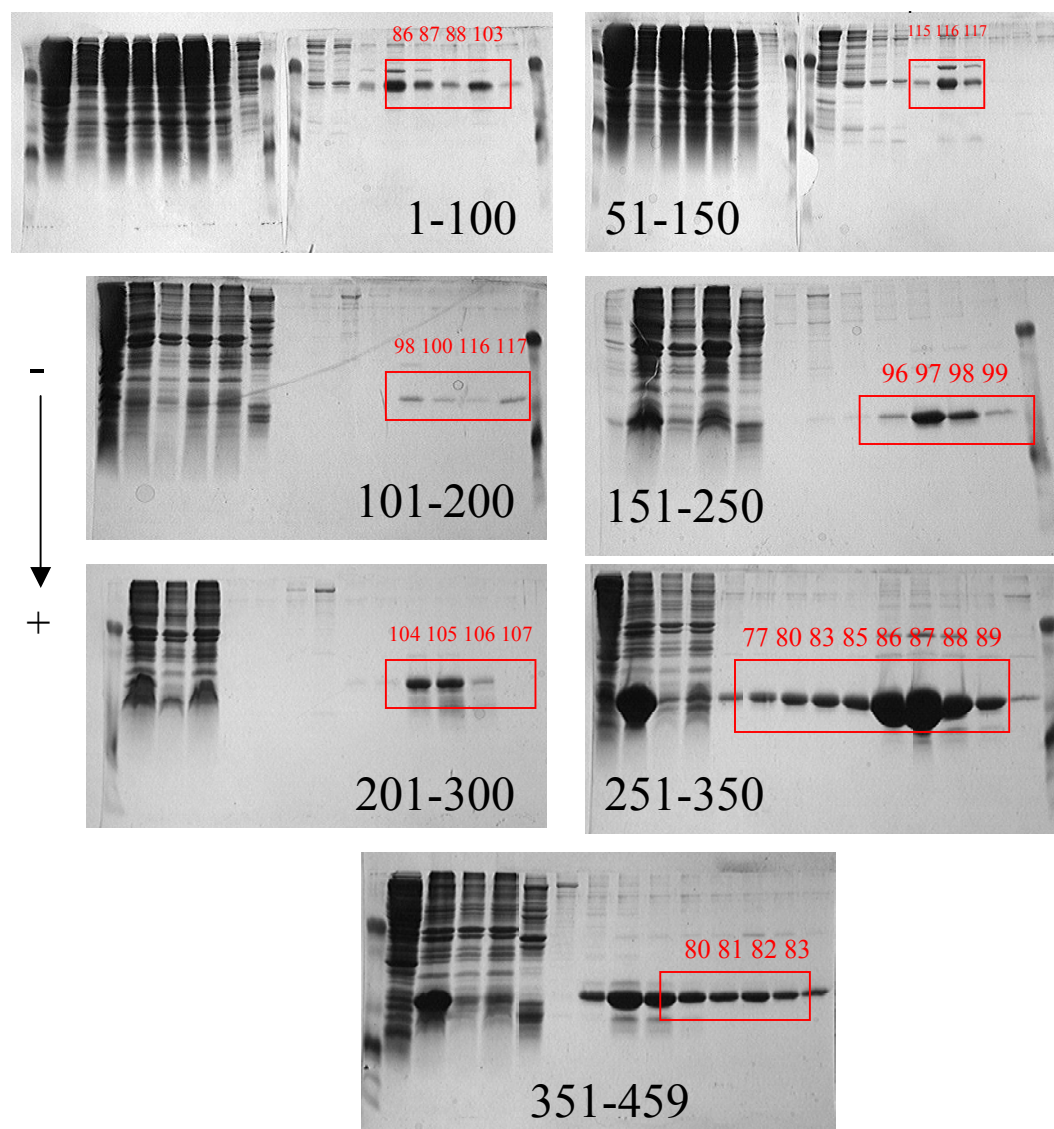


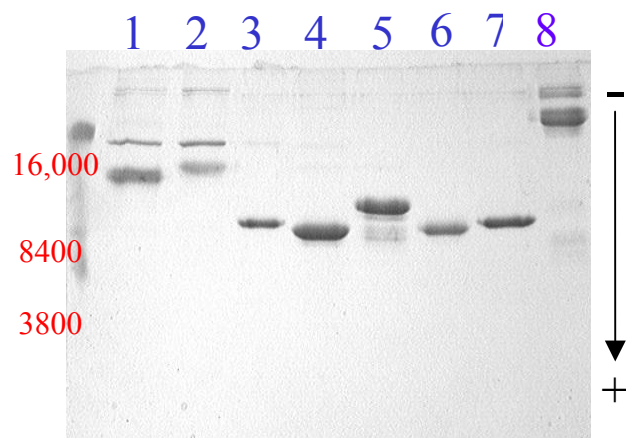
Figure B.6: SDS-PAGE of Polypeptide Purifications.

Original supernatants and fractions from different steps of the purification process were analyzed by SDS-PAGE for each of the polypeptides and gel pictures are presented here. Purification was achieved using nickel affinity chromatography. The red squares indicate the polypeptide of interest in each gel picture. Relative concentrations of pure polypeptides in the noted fractions (red) were assayed using the Bradford method and a summary is reported in Table II.

Table II: Relative Concentrations of Purified Polypeptides.

Polypeptide	Fraction #	Concentration (µg/ml)*
1-100	86	97
	87	348
	88	176
	103	211
51-150	115	71
	116	212
	117	81
101-200	98	5
	100	2
	116	19
	117	53
151-250	96	63
	97	1034
	98	259
	99	64
201-300	104	378
	105	457
	106	106
	107	23
251-350	77	167
	80	258
	83	310
	85	374
	86	2459
	87	2763
	88	1432
	89	562
351-459	80	1323
	81	924
	82	421
	83	311

*Concentrations determined using a standard Bradford assay.



Lane	Polypeptide	Molecular Weight
1	1-100	14,000
2	51-150	13,450
3	101-200	13,500
4	151-250	14,000
5	201-300	14,500
6	251-350	14,400
7	351-459	15,600
8	full length vitronectin	

Figure B.7: Purified Polypeptides of Vitronectin.

This is a 15% SDS-PAGE gel picture of the purified polypeptides. In addition, their relative molecular weights are listed; these weights include the N-terminal his-tag. Approximately 10 μ g total protein was loaded in each lane. Full length vitronectin was also run in *lane 8*.

and 10 μ g—were loaded on 10% SDS-PAGE and then transferred to nitrocellulose. Blocking conditions were consistently 10% dry milk in PBS. Different concentrations/molarities of TAT complex were incubated with the membranes and allowed to bind at room temperature for 1 hour or overnight at 4°C to determine the better binding conditions. After incubation with the complex, the membranes were allowed to bind primary antibodies to thrombin and antithrombin (separate membranes). Dilutions of the primaries were varied in these optimization experiments to assay both 1:2000 and 1:1000 dilutions. Secondary peroxidase-conjugated antibodies were consistently 1:2000 dilutions. From the optimization experiments (Figure B.8), the best conditions were determined to be 3 μ g protein (or comparable ratio of polypeptide), overnight binding of the complex (200 μ l in 30 ml 1% dry milk/PBS) at 4°C, and 1:2000 dilution of the primaries. Such conditions were necessary to elicit a visual colormetric signal. A positive result also supported the usage of such a method to study interaction between vitronectin (or polypeptides) and the thrombin-antithrombin complex.

v. The TAT Complex Localizes to Two Regions on Vitronectin

With an optimized protocol, Far Western experiments were attempted with the purified polypeptides. Approximately 10-20 μ g total polypeptide were loaded on 15% SDS-PAGE with appropriate controls (full-length vitronectin and either thrombin or antithrombin). The conditions used to produce a positive signal with full-length vitronectin initially did not give a positive signal with the polypeptides. Vitronectin was serving as a positive control and it was visualized on each blot, but the polypeptides failed to indicate any interaction. More Far Western blots were performed, each blot

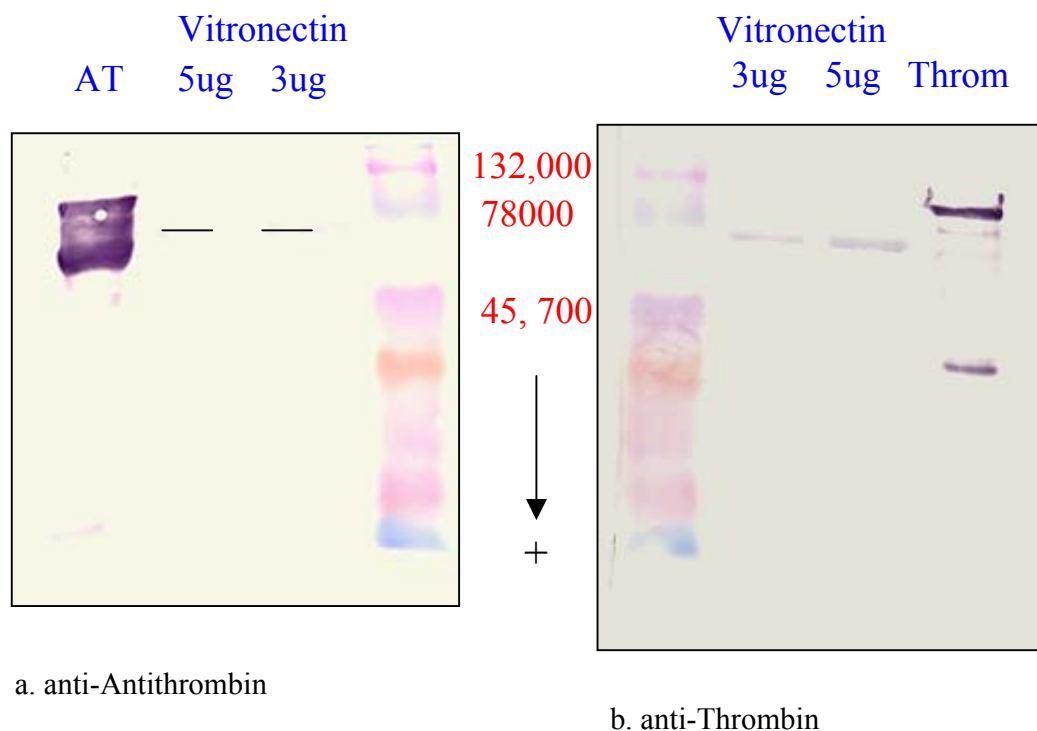
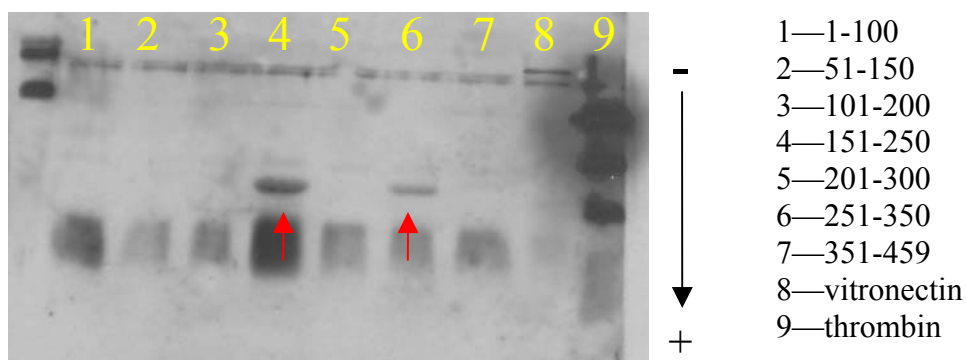


Figure B.8: Far Western Results for Native Vitronectin and TAT Interaction. 3µg and 5µg of vitronectin was loaded on 10% SDS-PAGE and transferred to nitrocellulose. The membrane was blocked for 1 hour in 10% dry milk in PBS and then allowed to incubate with the thrombin-antithrombin complex overnight at 4°C. After washing, the membranes were allowed to incubate with either anti-antithrombin (a.) or anti-thrombin (b.) primary antibodies diluted 1:2000. Peroxidase-conjugated secondary antibodies were allowed to bind and then chromagenic detection was used to visualize the interaction.

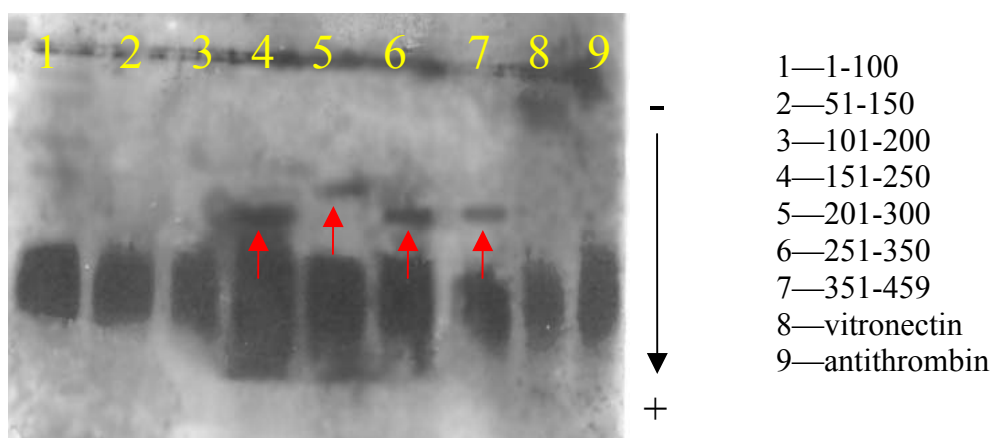
varying one condition: greater amount of total polypeptide was loaded, blocking conditions were reduced to 5% dry milk in PBS for 45 minutes instead of an hour, dilution of the primary antibody (to both thrombin and antithrombin) was increased to 1:1000. Still, an interaction was never indicated with the chromagenic development method routinely employed in this laboratory.

Seeking a more sensitive detection method, Far Western experiments were performed employing chemiluminescent development techniques. Approximately 10 μ g total of each polypeptide was loaded on 10% SDS-PAGE and transferred to Immobilon™ (Millipore) via a submerged transfer apparatus. The blot was blocked 40 minutes in 5% dry milk TBS/Tween and allowed to incubate with the complex (50 μ l 400 μ g/ml thrombin:50 μ l 1.5 mg/ml antithrombin in 30 ml 2.5% dry milk TBS/Tween) overnight at 4°C. After careful washing for 30 minutes total, primary antibodies were diluted 1:25,000 in 2.5% dry milk TBS/Tween and allowed to bind 40 minutes. The membranes were washed and peroxidase-conjugated secondaries were allowed to bind 40 minutes (1:25,000 dilution). After careful washing, the membranes were bathed in a chemiluminescent-specific substrate available from Pierce. The membranes were exposed to film for 5 minutes, 10 minutes, and 30 minutes and then developed in the dark room.

The Far Westerns performed and developed with this more sensitive detection method did reveal some interactions (Figure B.9). The thrombin Far Western (primary antibody to thrombin) indicates the TAT complex binds to the 151-250 polypeptide and the 251-350 polypeptide. The antithrombin Far Western gave a signal for four of the



a. Thrombin Far Western



b. Antithrombin Far Western

Figure B.9: Far Western Results of the Interaction Between Vitronectin Polypeptides and TAT.

These are 5 minute exposure films of Far Westerns of vitronectin polypeptides incubated with the thrombin-antithrombin complex. Approximately 10 μ g of each polypeptide was loaded on 15% SDS-PAGE and transferred to Immobilon™. Complex was allowed to bind overnight and then the membrane was incubated with primary antibodies to both thrombin (a.) and antithrombin (b.) diluted 1:25,000 in 2.5% dry milk in TBS/Tween. An interaction between the polypeptides and the thrombin-antithrombin complex is detected by chemilluminescence between polypeptides 151-250 and 251-350 on both blots. In addition, the antithrombin blot indicates an interaction at 201-300 and 351-459. (These are X-ray films after 5 minutes of exposure.)

polypeptides: 151-250, 201-300, 251-350, and 351-459. The Far Westerns were repeated and revealed the same interactions.

Even though thrombin and antithrombin form a complex that should be identified by a primary antibody to either component of the pair, both an antibody to thrombin and an antibody to antithrombin were employed in the Far Westerns. This was done to be more thorough in evaluation of TAT interaction and also to corroborate data generated by each antibody. The thrombin Far Western gave a signal with only two polypeptides, while the antithrombin Far Western gave a signal with four polypeptides. There are a number of possibilities to explain this result. The primary antibodies are both polyclonals that are known to recognize their respective proteins *and* the TAT complex. The two additional interactions indicated with the antithrombin antibody could have been a result of non-specific binding or genuine interactions of vitronectin with the TAT complex. Perhaps the interaction was not detected with the thrombin antibody due to antibody interference from association of TAT with the polypeptide.

Thrombin-antithrombin complex formation was achieved by mixing together a 1:1.5 molar ratio of thrombin:antithrombin. Although the thrombin component is responsible for the interaction of the TAT complex with vitronectin, excess free antithrombin could have interacted with the polypeptides and then given a false positive signal upon the addition of antithrombin antibody, although this possibility seems highly unlikely.

In any event, the 151-250 and 251-350 interactions are corroborated by both thrombin antibody and antithrombin antibody Far Westerns. The 151-250 polypeptide lies within the first hemopexin homology domain. The 251-351 spans both hemopexin

domains and does include part of the heparin binding region where TAT has been speculated to interact.

IV. Discussion

Vitronectin performs many physiological roles due to its interactions with multiple ligands. Many of these interactions have been studied extensively to elucidate vitronectin function. Binding determinants have been concurrently localized, giving credence to a domain organization of the protein. Ligand-binding regions have been assigned with a relative degree of confidence from various groups/techniques reporting similar findings. Heparin-binding to vitronectin is such an example, as well as a high-affinity binding site in the SMB domain for PAI-1. However, the binding region(s) for the thrombin-antithrombin complex have not been elucidated. The ternary complex formation of TAT with vitronectin is a very important event that warrants closer examination. The association of thrombin with antithrombin has been well characterized, but much is still unknown about what happens when the TAT complex encounters multi-functional vitronectin.

Work with mABs and cleavage products of vitronectin by de Boer et al. suggests the region on vitronectin responsible for interaction with TAT is the N-terminal region (16). Earlier studies by the same group demonstrated that the ternary complex bound endothelial cells via the heparin-binding region of vitronectin, thus this region could not be involved in the formation of the VN-TAT complex (92). Another site had to be responsible.

Although recombinant polypeptides have been used to localize ligand binding sites on vitronectin for some molecules, they have not been employed in assays with the thrombin-antithrombin complex. Using bacterial recombinants, this project reports the possibility of two TAT binding regions on vitronectin, one spanning amino acids 151-250, the other amino acids 251-350. Interactions were detected by Far Western, a technique utilized by many groups to study protein-protein interactions. Two other points of interaction are reported from the antithrombin antibody Far Western, but these interactions are not seen in the thrombin Far Western. This does not throw out their validity as genuine points of interaction, but neither does it strengthen their case.

Far Westerns are derivations of Western blots, and certain limitations exist in these assays. Results from experiments that capitalize on immobilized protein must be interpreted carefully. Conformationally-dependent binding determinants are often undetectable in such assays due to fold disruption. It is also assumed many times when conducting these experiments that epitopes are independent; multivalency effects are often lost. Additionally, when electroblotting proteins, it is difficult to determine accurate amounts or concentrations of proteins that actually end up being assayed.

Furthermore, preparations of the polypeptides and proteins are subject to consideration. Recombinant expression in *E. coli* renders a polypeptide devoid of any post-translational modifications. In some cases, phosphorylation of vitronectin has proven important for ligand binding. Such knowledge must be noted in any ligand-binding study that utilizes polypeptides devoid of such modifications, such as this one. Also, when using “pieces” of proteins, whether recombinantly expressed polypeptides, synthetic peptides, or proteolytic fragments, there is always the possibility that a binding

site has been disrupted by the separation technique (cleavage or engineering). Assays with prepared peptides are also often highly susceptible to experimental artifact.

Still, the data collected and reported here has value. The interaction of two polypeptides suggests two possible binding sites. The stoichiometry of the ternary complex of VN-T-AT appears to be 1:1:1, although vitronectin has demonstrated interesting binding stoichiometries with thrombin alone (197). (The ternary complex migrates at approximately 160kDa in non-reducing conditions.) Detection of two regions of interaction could suggest a different binding stoichiometry, but it is highly unlikely.

Polypeptide 151-250 spans hemopexin homology domain I. No reports of TAT binding have been mapped here or ever speculated. The 251-350 polypeptide spans part of the heparin-binding region, which has been eliminated experimentally as a region for binding. However, this polypeptide spans 90 amino acids that are not part of the heparin binding region, so another epitope could very possibly lie within that region.

V. Future Experiments

This work poses two possible regions for TAT interaction with vitronectin. Further experiments should include competitive binding experiments with monoclonals of known epitope, a method extensively used and reported in the literature. The polypeptides should also be examined for binding to thrombin alone. Working under the assumption that thrombin is the binding component of the TAT pair that interacts with vitronectin, it would be interesting to see if similar results are discovered. Eventually, once a polypeptide binds consistently in both Far Westerns and competitive binding

experiments, work should be done to elucidate specific residues important for binding using site-directed mutagenesis or synthetic peptides.

A successful purification protocol has been written and tested for each of the recombinant polypeptides. Pure polypeptides have even reserved and placed in long-term storage and can be used in any number of subsequent experiments. These polypeptides have served and will continue to serve as very useful reagents in elucidating information about vitronectin activity and function.

Part C

Monoclonal Antibody Characterization and Mapping

I. Introduction

Monoclonal antibodies are invaluable tools of biochemistry. They are used in a wide variety of assays to ascertain various kinds of information. Monoclonal antibodies have proven very important in vitronectin research. They have been used generally to detect the presence of protein and more specifically to propose and localize the ligand binding sites of PAI-1 (86,96,102), plasminogen (86), TAT (16,92), collagen (104,198), complement complex (104), and heparin (104) to vitronectin. For localization of regions of ligand interaction, it is often advantageous and sometimes necessary to know the specific region of vitronectin to which a certain antibody maps, such as competition assays. Thus, when a new monoclonal has been generated, it must be further characterized to be fully appreciated.

Our laboratory has recently acquired eight murine monoclonal antibodies, one from Green Mountain, one from Quidel and six from Molecular Innovations (MI). Cindy Brown, a graduate student in the lab, has performed preliminary experiments with these antibodies to generally characterize their binding to vitronectin. ELISA results indicate binding titers for this type of solid-phase assay and help to eliminate weakly-binding monoclonals. HPLC analysis is being conducted to resolve vitronectin-antibody complexes that can ultimately be isolated and utilized in crystallography trials. Vitronectin has been titrated at different ratios to antibody and preliminary results for two of the monoclonals (Quidel and MI-1D1) look promising.

II. Experimental Procedures

Murine monoclonal antibodies to vitronectin were obtained from Green Mountain, Quidel, and Molecular Innovations (1D1, 1E9, 2C3, 8H1, 2A10, 4A1). Secondary peroxidase-conjugated anti-mouse antibodies were purchased from Vector Laboratories. As a first step, the antibodies were tested for interaction with vitronectin in Western blotting. 5 µg native vitronectin was loaded on 10% SDS-PAGE and transferred to nitrocellulose via a BioRad Semi-dry Blotter at 20V for 20 minutes. The blots were blocked for 1 hour in 10% dry milk in PBS/0.1% Tween and then incubated with the monoclonals at ELISA-determined titers (as a starting dilution) for 1 hour. After washing, secondary peroxidase-conjugated anti-mouse antibodies were allowed to bind and detection was visualized upon addition of peroxide substrate.

Epitope mapping of the antibodies was performed by loading 10 µg of each polypeptide on 15% SDS-PAGE and performing a Western blot as previously described; both chromagenic and chemiluminescent development were used. Epitope mapping experiments were repeated.

III. Results

As a first step in characterizing these monoclonals, it was important to establish that they could be used in immunoblotting experiments, namely Westerns. A chart summarizing the ELISA and Western data for the eight monoclonal antibodies is found in Table III. Three of the eight monoclonals (4A1, 2A10, 8H1) were determined to bind poorly to solid phase vitronectin based on the ELISA experiments. Western blots were performed with all the antibodies with antibody dilutions determined from the effective

Table III: Summary of ELISA and Western Results for Monoclonals.

Antibody	ELISA effective concentration ($\mu\text{g/ml}$)*	Western concentration tested ($\mu\text{g/ml}$)	Signal [§]
Green Mountain	0.052	0.052	+
Quidel	0.6	0.6	+
MI-1D1	0.3	0.3	+
MI-1E9	0.32	0.32	+
MI-2C3	0.86	0.86	+
MI-4A1	---	0.4	+
MI-2A10	---	0.2	---
MI-8H1	---	0.1	---

Eight monoclonal antibodies were assays with native vitronectin in both ELISA experiments and Western blots. *Cindy Brown performed the ELISA experiments, while Western blots were incubated with dilutions of the monoclonals based on the effective titers calculated from the ELISAs. [§]A “+” denotes a positive interaction between native vitronectin and the antibody tested, while “---” indicates no interaction.

ELISA titers or a 1:1000 dilution of the company provided stock. Native vitronectin was immunoblotted onto nitrocellulose and incubated with the monoclonals. Six (Green Mountain, Quidel, MI-1D1, MI-1E9, MI-2C3, MI-4A1) of the eight monoclonals gave a positive signal on the Western blot (Figure C.1a). These six were further characterized by Western blot with the vitronectin polypeptides.

Approximately 10 µg of each polypeptide was loaded, along with a native vitronectin control, on 15% SDS-PAGE. The polypeptides were electroblotted first onto nitrocellulose and incubated with one of the monoclonals. Polypeptide 51-150 gave a positive signal on the Quidel blot (Figure C.1b), and polypeptides 101-200 and 351-459 gave a positive signal on the MI-2C3 blot (Figure C.1c). 2C3 is a monoclonal antibody, so the detection of two potential binding sites for 2C3 is suspicious and probably caused by non-specific binding, although anything is possible when testing “uncharacterized” tools.

On the blots incubated with Green Mountain, MI-1D1, MI-1E9, and MI-4A1, no color development resulted except for the full length vitronectin control. A concern arose that blocking conditions were too severe, so the Westerns were repeated and blocked only for 45 minutes in 5% milk/PBS, but similar blots resulted with no development. As was the case for the Far Westerns, a more sensitive detection method was sought after, and again chemiluminescent development was employed for subsequent Westerns. When this detection method was used, results for Green Mountain, 1D1, and 1E9 were the same. No interaction with the polypeptides was detected. This suggests that these monoclonals recognize epitopes that are conformationally-dependent and structural binding requirements are disrupted when the polypeptides are prepared or immobilized.

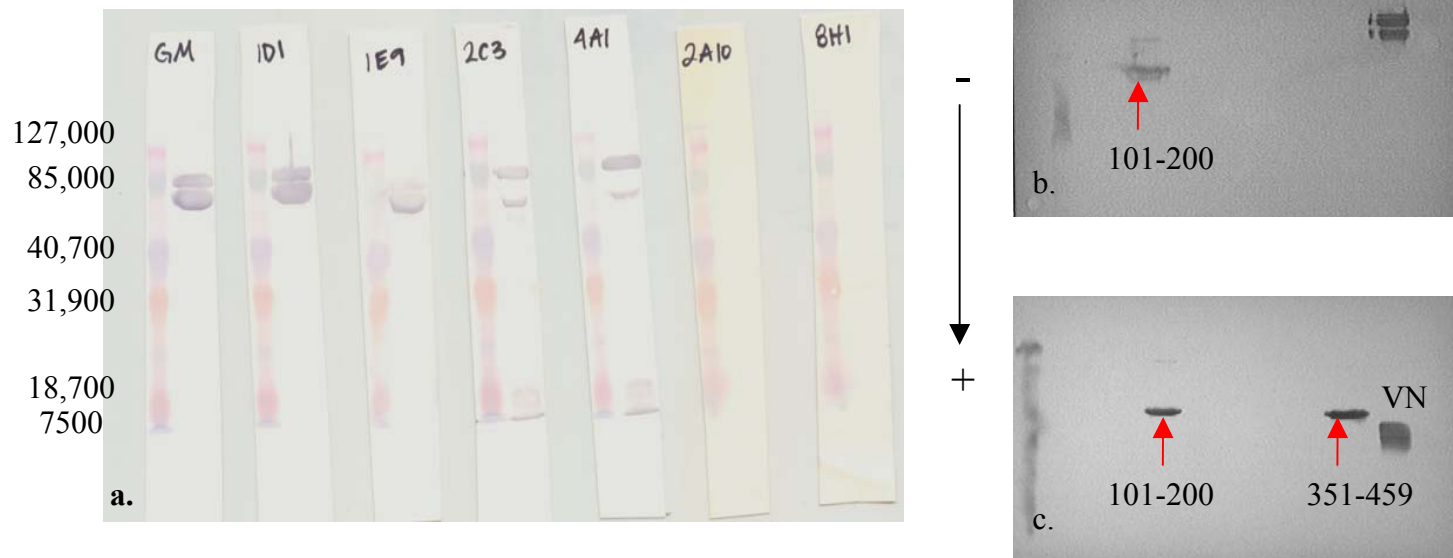


Figure C.1: Antibody Mapping.

In (a.), 5.0 μ g of full length vitronectin was loaded on 10% SDS-PAGE and Western blotted to nitrocellulose. The membrane was cut into seven strips and incubated with seven different murine monoclonal antibodies to vitronectin. The first five pictured indicated interaction, while the last two do not. Quidel (b.), MI-2C3 (c.) and MI-4A1 (d.) antibodies were incubated with blotted polypeptides. Quidel maps to polypeptide 51-150. 2C3 maps to polypeptides 101-200 and 351-459. 4A1 maps to polypeptides 101-250 and 151-251. (b.) and (c.) are pictures of nitrocellulose developed using chromagenic detection. (d.) is a picture of X-ray film exposed for one minute to Immobilon™ as part of a chemiluminescent detection protocol.

MI-4A1, however, gave a positive result in binding to polypeptides 101-200 and 151-250 (Figure C.1d). The binding of two consecutive polypeptides is very encouraging. These polypeptides overlap, and such a pattern of binding could be interpreted as two binding sites or the same binding within the overlapping region. For 4A1, one binding site could potentially be between 151-200, which localizes the binding to 50 amino acids instead of 100. Interestingly, on the full length vitronectin control for 2C3, the antibody recognizes the low molecular weight band of the two chain form of vitronectin, which is usually not visualized by Coomassie staining. This band is a 10kDa peptide that results from the cleavage at residue 379. I would expect an antibody that recognizes the low molecular weight band to bind the 351-459 polypeptide, but that is not the case here.

IV. Discussion

There are several published techniques that have been used to map monoclonal antibodies to vitronectin, some very direct and some very elegantly designed. The approach used here is a direct one and capitalizes on the recombinant polypeptides of vitronectin previously expressed and purified. Eight monoclonal antibodies have been acquired by this laboratory. Six were found to bind full-length vitronectin in Western immunoblotting experiments. Green Mountain, MI-1D1, and MI-1E9 do not appear to have linear epitopes that can be mapped by the method employed here. Three others—Quidel, MI-2C3, and MI-4A1—seem to bind the polypeptides with high affinity and recognize linear epitope(s) that have been successfully localized. Quidel maps to 101-

200; 2C3 interacts with two non-consecutive polypeptides, 101-200 and 351-49; 4A1 maps to 101-200 and 151-250, or is perhaps localized more specifically to 151-200.

V. Future Experiments

In order to acquire epitope information for Green Mountain, 1D1, and 1E9 monoclonal antibodies, native gels should be run with polypeptides or cleavage products to gain insight on the recognition regions for these monoclonals that did not give a signal on SDS-PAGE Westerns.

Any of the monoclonals that map with some degree of confidence and consistency becomes a candidate for co-crystallization trials. These will also be used to study interactions of vitronectin with its many ligands and generally to detect presence of protein.

References

1. Preissner, K. T. (1991) *Annu Rev Cell Biol* **7**, 275-310. "Structure and biological role of vitronectin"
2. Preissner, K. T., and Jenne, D. (1991) *Thromb Haemost* **66**, 189-194. "Vitronectin: a new molecular connection in haemostasis"
3. Barnes, D. W., Reing, J. E., and Amos, B. (1985) *J Biol Chem* **260**, 9117-9122. "Heparin-binding properties of human serum spreading factor"
4. Hayashi, M., Akama, T., Kono, I., and Kashiwagi, H. (1985) *J Biochem (Tokyo)* **98**, 1135-1138. "Activation of vitronectin (serum spreading factor) binding of heparin by denaturing agents"
5. Lane, D. A., Flynn, A. M., Pejler, G., Lindahl, U., Choay, J., and Preissner, K. (1987) *J Biol Chem* **262**, 16343-16348. "Structural requirements for the neutralization of heparin-like saccharides by complement S protein/vitronectin"
6. Deng, G., Curriden, S. A., Hu, G., Czekay, R. P., and Loskutoff, D. J. (2001) *J Cell Physiol* **189**, 23-33. "Plasminogen activator inhibitor-1 regulates cell adhesion by binding to the somatomedin B domain of vitronectin"
7. Declerck, P. J., De Mol, M., Alessi, M. C., Baudner, S., Paques, E. P., Preissner, K. T., Muller-Berghaus, G., and Collen, D. (1988) *J Biol Chem* **263**, 15454-15461. "Purification and characterization of a plasminogen activator inhibitor 1 binding protein from human plasma. Identification as a multimeric form of S protein (vitronectin)"
8. Owensby, D. A., Morton, P. A., Wun, T. C., and Schwartz, A. L. (1991) *J Biol Chem* **266**, 4334-4340. "Binding of plasminogen activator inhibitor type-1 to extracellular matrix of Hep G2 cells. Evidence that the binding protein is vitronectin"
9. Lawrence, D. A., Palaniappan, S., Stefansson, S., Olson, S. T., Francis-Chmura, A. M., Shore, J. D., and Ginsburg, D. (1997) *J Biol Chem* **272**, 7676-7680. "Characterization of the binding of different conformational forms of plasminogen activator inhibitor-1 to vitronectin. Implications for the regulation of pericellular proteolysis"
10. Arroyo De Prada, N., Schroeck, F., Sinner, E. K., Muehlenweg, B., Twellmeyer, J., Sperl, S., Wilhelm, O. G., Schmitt, M., and Magdolen, V. (2002) *Eur J Biochem* **269**, 184-192. "Interaction of plasminogen activator inhibitor type-1 (PAI-1) with vitronectin"
11. Tomasini, B. R., Owen, M. C., Fenton, J. W., 2nd, and Mosher, D. F. (1989) *Biochemistry* **28**, 7617-7623. "Conformational lability of vitronectin: induction of an antigenic change by alpha-thrombin-serpin complexes and by proteolytically modified thrombin"
12. Gebb, C., Hayman, E. G., Engvall, E., and Ruoslahti, E. (1986) *J Biol Chem* **261**, 16698-16703. "Interaction of vitronectin with collagen"
13. Tschopp, J., Masson, D., Schafer, S., Peitsch, M., and Preissner, K. T. (1988) *Biochemistry* **27**, 4103-4109. "The heparin binding domain of S-protein/vitronectin binds to complement components C7, C8, and C9 and perforin from cytolytic T-cells and inhibits their lytic activities"

14. Hogasen, K., Mollnes, T. E., and Harboe, M. (1992) *J Biol Chem* **267**, 23076-23082. "Heparin-binding properties of vitronectin are linked to complex formation as illustrated by in vitro polymerization and binding to the terminal complement complex"
15. Tomasini, B. R., and Mosher, D. F. (1988) *Blood* **72**, 903-912. "Conformational states of vitronectin: preferential expression of an antigenic epitope when vitronectin is covalently and noncovalently complexed with thrombin-antithrombin III or treated with urea"
16. de Boer, H. C., de Groot, P. G., Bouma, B. N., and Preissner, K. T. (1993) *J Biol Chem* **268**, 1279-1283. "Ternary vitronectin-thrombin-antithrombin III complexes in human plasma. Detection and mode of association"
17. Jenne, D., Hugo, F., and Bhakdi, S. (1985) *Thromb Res* **38**, 401-412. "Interaction of complement S-protein with thrombin-antithrombin complexes: a role for the S-protein in haemostasis"
18. Holmes, R. (1967) *J Cell Biol* **32**, 297-308. "Preparation from human serum of an alpha-one protein which induces the immediate growth of unadapted cells in vitro"
19. Preissner, K. T., and Seiffert, D. (1998) *Thromb Res* **89**, 1-21. "Role of vitronectin and its receptors in haemostasis and vascular remodeling"
20. Preissner, K. T., Wassmuth, R., and Muller-Berghaus, G. (1985) *Biochem J* **231**, 349-355. "Physicochemical characterization of human S-protein and its function in the blood coagulation system"
21. Barnes, D. W., and Silnutzer, J. (1983) *J Biol Chem* **258**, 12548-12552. "Isolation of human serum spreading factor"
22. Seiffert, D., Keeton, M., Eguchi, Y., Sawdey, M., and Loskutoff, D. J. (1991) *Proc Natl Acad Sci U S A* **88**, 9402-9406. "Detection of vitronectin mRNA in tissues and cells of the mouse"
23. Seiffert, D., and Loskutoff, D. J. (1991) *J Biol Chem* **266**, 2824-2830. "Evidence that type 1 plasminogen activator inhibitor binds to the somatomedin B domain of vitronectin"
24. Barnes, D. W., Silnutzer, J., See, C., and Shaffer, M. (1983) *Proc Natl Acad Sci U S A* **80**, 1362-1366. "Characterization of human serum spreading factor with monoclonal antibody"
25. Parker, C. J., Stone, O. L., White, V. F., and Bernshaw, N. J. (1989) *Br J Haematol* **71**, 245-252. "Vitronectin (S protein) is associated with platelets"
26. Preissner, K. T., Holzhuter, S., Justus, C., and Muller-Berghaus, G. (1989) *Blood* **74**, 1989-1996. "Identification of and partial characterization of platelet vitronectin: evidence for complex formation with platelet-derived plasminogen activator inhibitor-1"
27. Stockmann, A., Hess, S., Declerck, P., Timpl, R., and Preissner, K. T. (1993) *J Biol Chem* **268**, 22874-22882. "Multimeric vitronectin. Identification and characterization of conformation-dependent self-association of the adhesive protein"
28. Hill, S. A., Shaughnessy, S. G., Joshua, P., Ribau, J., Austin, R. C., and Podor, T. J. (1996) *Blood* **87**, 5061-5073. "Differential mechanisms targeting type 1

- plasminogen activator inhibitor and vitronectin into the storage granules of a human megakaryocytic cell line"
29. Rosenberg, R. D., and Damus, P. S. (1973) *J Biol Chem* **248**, 6490-6505. "The purification and mechanism of action of human antithrombin-heparin cofactor"
 30. Peterson, C. B., and Blackburn, M. N. (1987) *J Biol Chem* **262**, 7559-7566. "Antithrombin conformation and the catalytic role of heparin. II. Is the heparin-induced conformational change in antithrombin required for rapid inactivation of thrombin?"
 31. Ill, C. R., and Ruoslahti, E. (1985) *J Biol Chem* **260**, 15610-15615. "Association of thrombin-antithrombin III complex with vitronectin in serum"
 32. Shifman, M. A., and Pizzo, S. V. (1982) *J Biol Chem* **257**, 3243-3248. "The in vivo metabolism of antithrombin III and antithrombin III complexes"
 33. Buluk, K., Januszko, T., and Olbromski, J. (1962) *Postepy Hig Med Dosw* **16**, 343-349. "[On the role of plasmin in the activation of fibrinolysis with the aid of tissue plasminogen activator]"
 34. Masson, C., and Angles-Cano, E. (1988) *Biochem J* **256**, 237-244. "Kinetic analysis of the interaction between plasminogen activator inhibitor-1 and tissue-type plasminogen activator"
 35. Huber, R., and Carrell, R. W. (1989) *Biochemistry* **28**, 8951-8966. "Implications of the three-dimensional structure of alpha 1-antitrypsin for structure and function of serpins"
 36. Mottonen, J., Strand, A., Symersky, J., Sweet, R. M., Danley, D. E., Geoghegan, K. F., Gerard, R. D., and Goldsmith, E. J. (1992) *Nature* **355**, 270-273. "Structural basis of latency in plasminogen activator inhibitor-1"
 37. Wun, T. C., Palmier, M. O., Siegel, N. R., and Smith, C. E. (1989) *J Biol Chem* **264**, 7862-7868. "Affinity purification of active plasminogen activator inhibitor-1 (PAI-1) using immobilized anhydrourokinase. Demonstration of the binding, stabilization, and activation of PAI-1 by vitronectin"
 38. Seiffert, D., and Loskutoff, D. J. (1991) *Biochim Biophys Acta* **1078**, 23-30. "Kinetic analysis of the interaction between type 1 plasminogen activator inhibitor and vitronectin and evidence that the bovine inhibitor binds to a thrombin-derived amino-terminal fragment of bovine vitronectin"
 39. Ronne, E., Behrendt, N., Ploug, M., Nielsen, H. J., Wollisch, E., Weidle, U., Dano, K., and Hoyer-Hansen, G. (1994) *J Immunol Methods* **167**, 91-101. "Quantitation of the receptor for urokinase plasminogen activator by enzyme-linked immunosorbent assay"
 40. Wei, Y., Waltz, D. A., Rao, N., Drummond, R. J., Rosenberg, S., and Chapman, H. A. (1994) *J Biol Chem* **269**, 32380-32388. "Identification of the urokinase receptor as an adhesion receptor for vitronectin"
 41. Bajpai, A., and Baker, J. B. (1985) *Biochem Biophys Res Commun* **133**, 475-482. "Cryptic urokinase binding sites on human foreskin fibroblasts"
 42. Vassalli, J. D., Baccino, D., and Belin, D. (1985) *J Cell Biol* **100**, 86-92. "A cellular binding site for the Mr 55,000 form of the human plasminogen activator, urokinase"

43. Stoppelli, M. P., Corti, A., Soffientini, A., Cassani, G., Blasi, F., and Assoian, R. K. (1985) *Proc Natl Acad Sci U S A* **82**, 4939-4943. "Differentiation-enhanced binding of the amino-terminal fragment of human urokinase plasminogen activator to a specific receptor on U937 monocytes"
44. Wun, T. C., and Reich, E. (1987) *J Biol Chem* **262**, 3646-3653. "An inhibitor of plasminogen activation from human placenta. Purification and characterization"
45. Rolli, M., Fransvea, E., Pilch, J., Saven, A., and Felding-Habermann, B. (2003) *Proc Natl Acad Sci U S A* **100**, 9482-9487. "Activated integrin alphavbeta3 cooperates with metalloproteinase MMP-9 in regulating migration of metastatic breast cancer cells"
46. Hoyer-Hansen, G., Behrendt, N., Ploug, M., Dano, K., and Preissner, K. T. (1997) *FEBS Lett* **420**, 79-85. "The intact urokinase receptor is required for efficient vitronectin binding: receptor cleavage prevents ligand interaction"
47. Waltz, D. A., and Chapman, H. A. (1994) *J Biol Chem* **269**, 14746-14750. "Reversible cellular adhesion to vitronectin linked to urokinase receptor occupancy"
48. Kanse, S. M., Kost, C., Wilhelm, O. G., Andreasen, P. A., and Preissner, K. T. (1996) *Exp Cell Res* **224**, 344-353. "The urokinase receptor is a major vitronectin-binding protein on endothelial cells"
49. Sidenius, N., and Blasi, F. (2000) *FEBS Lett* **470**, 40-46. "Domain 1 of the urokinase receptor (uPAR) is required for uPAR-mediated cell binding to vitronectin"
50. Sidenius, N., Andolfo, A., Fesce, R., and Blasi, F. (2002) *J Biol Chem* **277**, 27982-27990. "Urokinase regulates vitronectin binding by controlling urokinase receptor oligomerization"
51. Sidenius, N., and Blasi, F. (2003) *Cancer Metastasis Rev* **22**, 205-222. "The urokinase plasminogen activator system in cancer: recent advances and implication for prognosis and therapy"
52. Chavakis, T., Kanse, S. M., Yutzy, B., Lijnen, H. R., and Preissner, K. T. (1998) *Blood* **91**, 2305-2312. "Vitronectin concentrates proteolytic activity on the cell surface and extracellular matrix by trapping soluble urokinase receptor-urokinase complexes"
53. Deng, G., Curriden, S. A., Wang, S., Rosenberg, S., and Loskutoff, D. J. (1996) *J Cell Biol* **134**, 1563-1571. "Is plasminogen activator inhibitor-1 the molecular switch that governs urokinase receptor-mediated cell adhesion and release?"
54. Kjoller, L., Kanse, S. M., Kirkegaard, T., Rodenburg, K. W., Ronne, E., Goodman, S. L., Preissner, K. T., Ossowski, L., and Andreasen, P. A. (1997) *Exp Cell Res* **232**, 420-429. "Plasminogen activator inhibitor-1 represses integrin- and vitronectin-mediated cell migration independently of its function as an inhibitor of plasminogen activation"
55. Waltz, D. A., Natkin, L. R., Fujita, R. M., Wei, Y., and Chapman, H. A. (1997) *J Clin Invest* **100**, 58-67. "Plasmin and plasminogen activator inhibitor type 1 promote cellular motility by regulating the interaction between the urokinase receptor and vitronectin"

56. Devy, L., Blacher, S., Grignet-Debrus, C., Bajou, K., Masson, V., Gerard, R. D., Gils, A., Carmeliet, G., Carmeliet, P., Declerck, P. J., Noel, A., and Foidart, J. M. (2002) *Faseb J* **16**, 147-154. "The pro- or antiangiogenic effect of plasminogen activator inhibitor 1 is dose dependent"
57. Ragno, P., Montuori, N., Covelli, B., Hoyer-Hansen, G., and Rossi, G. (1998) *Cancer Res* **58**, 1315-1319. "Differential expression of a truncated form of the urokinase-type plasminogen-activator receptor in normal and tumor thyroid cells"
58. Chang, A. W., Kuo, A., Barnathan, E. S., and Okada, S. S. (1998) *Arterioscler Thromb Vasc Biol* **18**, 1855-1860. "Urokinase receptor-dependent upregulation of smooth muscle cell adhesion to vitronectin by urokinase"
59. Kjoller, L., and Hall, A. (2001) *J Cell Biol* **152**, 1145-1157. "Rac mediates cytoskeletal rearrangements and increased cell motility induced by urokinase-type plasminogen activator receptor binding to vitronectin"
60. Degryse, B., Orlando, S., Resnati, M., Rabbani, S. A., and Blasi, F. (2001) *Oncogene* **20**, 2032-2043. "Urokinase/urokinase receptor and vitronectin/alpha(v)beta(3) integrin induce chemotaxis and cytoskeleton reorganization through different signaling pathways"
61. Ossowski, L., and Aguirre-Ghiso, J. A. (2000) *Curr Opin Cell Biol* **12**, 613-620. "Urokinase receptor and integrin partnership: coordination of signaling for cell adhesion, migration and growth"
62. Berman, A. E., and Kozlova, N. I. (2000) *Membr Cell Biol* **13**, 207-244. "Integrins: structure and functions"
63. Underwood, P. A., and Bennett, F. A. (1989) *J Cell Sci* **93 (Pt 4)**, 641-649. "A comparison of the biological activities of the cell-adhesive proteins vitronectin and fibronectin"
64. Pytela, R., Pierschbacher, M. D., and Ruoslahti, E. (1985) *Proc Natl Acad Sci U S A* **82**, 5766-5770. "A 125/115-kDa cell surface receptor specific for vitronectin interacts with the arginine-glycine-aspartic acid adhesion sequence derived from fibronectin"
65. Horton, M. A. (1997) *Int J Biochem Cell Biol* **29**, 721-725. "The alpha v beta 3 integrin "vitronectin receptor""
66. Montgomery, A. M., Reisfeld, R. A., and Cheresch, D. A. (1994) *Proc Natl Acad Sci U S A* **91**, 8856-8860. "Integrin alpha v beta 3 rescues melanoma cells from apoptosis in three-dimensional dermal collagen"
67. Muller-Eberhard, H. J. (1986) *Annu Rev Immunol* **4**, 503-528. "The membrane attack complex of complement"
68. Podack, E. R., Kolb, W. P., and Muller-Eberhard, H. J. (1978) *J Immunol* **120**, 1841-1848. "The C5b-6 complex: formation, isolation, and inhibition of its activity by lipoprotein and the S-protein of human serum"
69. Tschopp, J., Podack, E. R., and Muller-Eberhard, H. J. (1985) *J Immunol* **134**, 495-499. "The membrane attack complex of complement: C5b-8 complex as accelerator of C9 polymerization"
70. Podack, E. R., Kolb, W. P., and Muller-Eberhard, H. J. (1977) *J Immunol* **119**, 2024-2029. "The SC5b-7 complex: formation, isolation, properties, and subunit composition"

71. Zheng, X., Saunders, T. L., Camper, S. A., Samuelson, L. C., and Ginsburg, D. (1995) *Proc Natl Acad Sci U S A* **92**, 12426-12430. "Vitronectin is not essential for normal mammalian development and fertility"
72. Zhuang, P., Blackburn, M. N., and Peterson, C. B. (1996) *J Biol Chem* **271**, 14323-14332. "Characterization of the denaturation and renaturation of human plasma vitronectin. I. Biophysical characterization of protein unfolding and multimerization"
73. Tollefsen, D. M., Weigel, C. J., and Kabeer, M. H. (1990) *J Biol Chem* **265**, 9778-9781. "The presence of methionine or threonine at position 381 in vitronectin is correlated with proteolytic cleavage at arginine 379"
74. Seger, D., and Shaltiel, S. (2000) *FEBS Lett* **480**, 169-174. "Evidence showing that the two-chain form of vitronectin is produced in the liver by a selective furin cleavage"
75. Ogawa, H., Yoneda, A., Seno, N., Hayashi, M., Ishizuka, I., Hase, S., and Matsumoto, I. (1995) *Eur J Biochem* **230**, 994-1000. "Structures of the N-linked oligosaccharides on human plasma vitronectin"
76. Xu, D., Baburaj, K., Peterson, C. B., and Xu, Y. (2001) *Proteins* **44**, 312-320. "Model for the three-dimensional structure of vitronectin: predictions for the multi-domain protein from threading and docking"
77. Skorstengaard, K., Halkier, T., Hojrup, P., and Mosher, D. (1990) *FEBS Lett* **262**, 269-274. "Sequence location of a putative transglutaminase cross-linking site in human vitronectin"
78. Chain, D., Kreizman, T., Shapira, H., and Shaltiel, S. (1991) *FEBS Lett* **285**, 251-256. "Plasmin cleavage of vitronectin. Identification of the site and consequent attenuation in binding plasminogen activator inhibitor-1"
79. Gechtman, Z., Belleli, A., Lechpammer, S., and Shaltiel, S. (1997) *Biochem J* **325** (Pt 2), 339-349. "The cluster of basic amino acids in vitronectin contributes to its binding of plasminogen activator inhibitor-1: evidence from thrombin-, elastase- and plasmin-cleaved vitronectins and anti-peptide antibodies"
80. Kost, C., Benner, K., Stockmann, A., Linder, D., and Preissner, K. T. (1996) *Eur J Biochem* **236**, 682-688. "Limited plasmin proteolysis of vitronectin. Characterization of the adhesion protein as morpho-regulatory and angiostatin-binding factor"
81. Sigurdardottir, O., and Wiman, B. (1994) *Biochim Biophys Acta* **1208**, 104-110. "Identification of a PAI-1 binding site in vitronectin"
82. Suzuki, S., Pierschbacher, M. D., Hayman, E. G., Nguyen, K., Ohgren, Y., and Ruoslahti, E. (1984) *J Biol Chem* **259**, 15307-15314. "Domain structure of vitronectin. Alignment of active sites"
83. Preissner, K. T., Grulich-Henn, J., Ehrlich, H. J., Declerck, P., Justus, C., Collen, D., Pannekoek, H., and Muller-Berghaus, G. (1990) *J Biol Chem* **265**, 18490-18498. "Structural requirements for the extracellular interaction of plasminogen activator inhibitor 1 with endothelial cell matrix-associated vitronectin"
84. Sheehan, M., Morris, C. A., Pussell, B. A., and Charlesworth, J. A. (1995) *Clin Exp Immunol* **101**, 136-141. "Complement inhibition by human vitronectin involves non-heparin binding domains"

85. Ishikawa-Sakurai, M., and Hayashi, M. (1993) *Cell Struct Funct* **18**, 253-259. "Two collagen-binding domains of vitronectin"
86. Kost, C., Stuber, W., Ehrlich, H. J., Pannekoek, H., and Preissner, K. T. (1992) *J Biol Chem* **267**, 12098-12105. "Mapping of binding sites for heparin, plasminogen activator inhibitor-1, and plasminogen to vitronectin's heparin-binding region reveals a novel vitronectin-dependent feedback mechanism for the control of plasmin formation"
87. Ishikawa, M., and Hayashi, M. (1992) *Biochim Biophys Acta* **1121**, 173-177. "Activation of the collagen-binding of endogenous serum vitronectin by heating, urea and glycosaminoglycans"
88. Sane, D. C., Moser, T. L., Parker, C. J., Seiffert, D., Loskutoff, D. J., and Greenberg, C. S. (1990) *J Biol Chem* **265**, 3543-3548. "Highly sulfated glycosaminoglycans augment the cross-linking of vitronectin by guinea pig liver transglutaminase. Functional studies of the cross-linked vitronectin multimers"
89. Thiagarajan, P., Le, A., Snuggs, M. B., and VanWinkle, B. (1996) *Cell Adhes Commun* **4**, 317-325. "The role of carboxy-terminal glycosaminoglycan-binding domain of vitronectin in cytoskeletal organization and migration of endothelial cells"
90. Mimuro, J., Schleef, R. R., and Loskutoff, D. J. (1987) *Blood* **70**, 721-728. "Extracellular matrix of cultured bovine aortic endothelial cells contains functionally active type 1 plasminogen activator inhibitor"
91. Preissner, K. T. (1990) *Biochem Biophys Res Commun* **168**, 966-971. "Specific binding of plasminogen to vitronectin. Evidence for a modulatory role of vitronectin on fibrin(ogen)-induced plasmin formation by tissue plasminogen activator"
92. de Boer, H. C., Preissner, K. T., Bouma, B. N., and de Groot, P. G. (1992) *J Biol Chem* **267**, 2264-2268. "Binding of vitronectin-thrombin-antithrombin III complex to human endothelial cells is mediated by the heparin binding site of vitronectin"
93. Wachenburg, B. L., Liberman, B., Gomes, E., and Pieron, R. R. (1980) *Horm Metab Res* **12**, 516-519. "Radioimmunoassayable serum somatomedin B in normal subjects and in patients with acromegaly and pituitary dwarfism: effects of human growth hormone therapy"
94. Tomasini, B. R., and Mosher, D. F. (1991) *Prog. in Hemostasis and Thrombosis* (Coller, B. S., Ed.), 10, W.B. Saunders Company
95. Heldin, C. H., Wasteson, A., Fryklund, L., and Westermark, B. (1981) *Science* **213**, 1122-1123. "Somatomedin B: mitogenic activity derived from contaminant epidermal growth factor"
96. Seiffert, D., Ciambrone, G., Wagner, N. V., Binder, B. R., and Loskutoff, D. J. (1994) *J Biol Chem* **269**, 2659-2666. "The somatomedin B domain of vitronectin. Structural requirements for the binding and stabilization of active type 1 plasminogen activator inhibitor"
97. Deng, G., Royle, G., Wang, S., Crain, K., and Loskutoff, D. J. (1996) *J Biol Chem* **271**, 12716-12723. "Structural and functional analysis of the plasminogen activator inhibitor-1 binding motif in the somatomedin B domain of vitronectin"

98. Sane, D. C., and Zhao, Y. (1993) *Biology of Vitronectins and Their Receptors* (Preissner, K. T., Rosenblatt, S., Kost, C., Wegerhoff, J., and Mosher, D. F., Eds.), Elsevier, Amsterdam
99. Cherny, R. C., and Thiagarajan, P. (1993) *Vitronectins and Their Receptors* (Preissner, K. T., Rosenblatt, C., Kost, C., Wegerhoff, C., and Mosher, D., Eds.), Elsevier, Amsterdam
100. Cherny, R. C., Honan, M. A., and Thiagarajan, P. (1993) *J Biol Chem* **268**, 9725-9729. "Site-directed mutagenesis of the arginine-glycine-aspartic acid in vitronectin abolishes cell adhesion"
101. Jenne, D., Hille, A., Stanley, K. K., and Huttner, W. B. (1989) *Eur J Biochem* **185**, 391-395. "Sulfation of two tyrosine-residues in human complement S-protein (vitronectin)"
102. Mimuro, J., Muramatsu, S., Kurano, Y., Uchida, Y., Ikadai, H., Watanabe, S., and Sakata, Y. (1993) *Biochemistry* **32**, 2314-2320. "Identification of the plasminogen activator inhibitor-1 binding heptapeptide in vitronectin"
103. Liang, O. D., Rosenblatt, S., Chhatwal, G. S., and Preissner, K. T. (1997) *FEBS Lett* **407**, 169-172. "Identification of novel heparin-binding domains of vitronectin"
104. Morris, C. A., Underwood, P. A., Bean, P. A., Sheehan, M., and Charlesworth, J. A. (1994) *J Biol Chem* **269**, 23845-23852. "Relative topography of biologically active domains of human vitronectin. Evidence from monoclonal antibody epitope and denaturation studies"
105. Jenne, D., and Stanley, K. K. (1987) *Biochemistry* **26**, 6735-6742. "Nucleotide sequence and organization of the human S-protein gene: repeating peptide motifs in the "pexin" family and a model for their evolution"
106. Cardin, A. D., and Weintraub, H. J. (1989) *Arteriosclerosis* **9**, 21-32. "Molecular modeling of protein-glycosaminoglycan interactions"
107. McGuire, E. A., Peacock, M. E., Inhorn, R. C., Siegel, N. R., and Tollefsen, D. M. (1988) *J Biol Chem* **263**, 1942-1945. "Phosphorylation of vitronectin by a protein kinase in human plasma. Identification of a unique phosphorylation site in the heparin-binding domain"
108. Gechtman, Z., and Shaltiel, S. (1997) *Eur J Biochem* **243**, 493-501. "Phosphorylation of vitronectin on Ser362 by protein kinase C attenuates its cleavage by plasmin"
109. Chain, D., Korc-Grodzicki, B., Kreizman, T., and Shaltiel, S. (1990) *FEBS Lett* **269**, 221-225. "The phosphorylation of the two-chain form of vitronectin by protein kinase A is heparin dependent"
110. Korc-Grodzicki, B., Tauber-Finkelstein, M., Chain, D., and Shaltiel, S. (1988) *Biochem Biophys Res Commun* **157**, 1131-1138. "Vitronectin is phosphorylated by a cAMP-dependent protein kinase released by activation of human platelets with thrombin"
111. Korc-Grodzicki, B., Chain, D., Kreizman, T., and Shaltiel, S. (1990) *Anal Biochem* **188**, 288-294. "An enzymatic assay for vitronectin based on its selective phosphorylation by protein kinase A"

112. Dahlback, B., and Podack, E. R. (1985) *Biochemistry* **24**, 2368-2374. "Characterization of human S protein, an inhibitor of the membrane attack complex of complement. Demonstration of a free reactive thiol group"
113. Gibson, A., Baburaj, K., Day, D. E., Verhamme, I., Shore, J. D., and Peterson, C. B. (1997) *J Biol Chem* **272**, 5112-5121. "The use of fluorescent probes to characterize conformational changes in the interaction between vitronectin and plasminogen activator inhibitor-1"
114. Gibson, A. D., Lamerdin, J. A., Zhuang, P., Baburaj, K., Serpersu, E. H., and Peterson, C. B. (1999) *J Biol Chem* **274**, 6432-6442. "Orientation of heparin-binding sites in native vitronectin. Analyses of ligand binding to the primary glycosaminoglycan-binding site indicate that putative secondary sites are not functional"
115. Sane, D. C., Moser, T. L., and Greenberg, C. S. (1991) *Thromb Haemost* **66**, 310-314. "Limited proteolysis of vitronectin by plasmin destroys heparin binding activity"
116. Gechtman, Z., Sharma, R., Kreizman, T., Fridkin, M., and Shaltiel, S. (1993) *FEBS Lett* **315**, 293-297. "Synthetic peptides derived from the sequence around the plasmin cleavage site in vitronectin. Use in mapping the PAI-1 binding site"
117. Milis, L., Morris, C. A., Sheehan, M. C., Charlesworth, J. A., and Pussell, B. A. (1993) *Clin Exp Immunol* **92**, 114-119. "Vitronectin-mediated inhibition of complement: evidence for different binding sites for C5b-7 and C9"
118. Preissner, K. T., Zwicker, L., and Muller-Berghaus, G. (1987) *Biochem. J.* **243**, 105-111. "Formation, characterization and detection of a ternary complex between S protein, thrombin, and antithrombin III in serum"
119. Liang, O. D., Flock, J. I., and Wadstrom, T. (1994) *J Biochem (Tokyo)* **116**, 457-463. "Evidence that the heparin-binding consensus sequence of vitronectin is recognized by *Staphylococcus aureus*"
120. Wisniowski, P., and Martin, W. J., 2nd. (1995) *J Lab Clin Med* **125**, 38-45. "Interaction of vitronectin with *Pneumocystis carinii*: evidence for binding via the heparin binding domain"
121. Limper, A. H., and Standing, J. E. (1994) *Immunol Lett* **42**, 139-144. "Vitronectin interacts with *Candida albicans* and augments organism attachment to the NR8383 macrophage cell line"
122. Podor, T. J., Shaughnessy, S. G., Blackburn, M. N., and Peterson, C. B. (2000) *J Biol Chem* **275**, 25402-25410. "New insights into the size and stoichiometry of the plasminogen activator inhibitor type-1.vitronectin complex"
123. Minor, K. H., and Peterson, C. B. (2002) *J Biol Chem* **277**, 10337-10345. "Plasminogen activator inhibitor type 1 promotes the self-association of vitronectin into complexes exhibiting altered incorporation into the extracellular matrix"
124. Preissner, K. T., and Muller-Berghaus, G. (1987) *J Biol Chem* **262**, 12247-12253. "Neutralization and binding of heparin by S protein/vitronectin in the inhibition of factor Xa by antithrombin III. Involvement of an inducible heparin-binding domain of S protein/vitronectin"

125. Zhuang, P., Li, H., Williams, J. G., Wagner, N. V., Seiffert, D., and Peterson, C. B. (1996) *J Biol Chem* **271**, 14333-14343. "Characterization of the denaturation and renaturation of human plasma vitronectin. II. Investigation into the mechanism of formation of multimers"
126. Zhuang, P., Chen, A. I., and Peterson, C. B. (1997) *J Biol Chem* **272**, 6858-6867. "Native and multimeric vitronectin exhibit similar affinity for heparin. Differences in heparin binding properties induced upon denaturation are due to self-association into a multivalent form"
127. Peake, P. W., Greenstein, J. D., Pussell, B. A., and Charlesworth, J. A. (1996) *Clin Exp Immunol* **106**, 416-422. "The behaviour of human vitronectin in vivo: effects of complement activation, conformation and phosphorylation"
128. Seiffert, D. (1995) *FEBS Lett* **368**, 155-159. "Evidence that conformational changes upon the transition of the native to the modified form of vitronectin are not limited to the heparin binding domain"
129. Fenton, J. W., 2nd, Ofosu, F. A., Moon, D. G., and Maraganore, J. M. (1991) *Blood Coagul Fibrinolysis* **2**, 69-75. "Thrombin structure and function: why thrombin is the primary target for antithrombotics"
130. Blomback, B., Blomback, M., Hessel, B., and Iwanaga, S. (1967) *Nature* **215**, 1445-1448. "Structure of N-terminal fragments of fibrinogen and specificity of thrombin"
131. Mosesson, M. W. (1998) *Semin Thromb Hemost* **24**, 169-174. "Fibrinogen structure and fibrin clot assembly"
132. Davie, E. W., Fujikawa, K., and Kisiel, W. (1991) *Biochemistry* **30**, 10363-10370. "The coagulation cascade: initiation, maintenance, and regulation"
133. Church, F. C., Pratt, C. W., Noyes, C. M., Kalayanamit, T., Sherrill, G. B., Tobin, R. B., and Meade, J. B. (1989) *J Biol Chem* **264**, 18419-18425. "Structural and functional properties of human alpha-thrombin, phosphopyridoxylated alpha-thrombin, and gamma T-thrombin. Identification of lysyl residues in alpha-thrombin that are critical for heparin and fibrin(ogen) interactions"
134. Fenton, J. W., 2nd, Olson, T. A., Zabinski, M. P., and Wilner, G. D. (1988) *Biochemistry* **27**, 7106-7112. "Anion-binding exosite of human alpha-thrombin and fibrin(ogen) recognition"
135. Stubbs, M. T., and Bode, W. (1995) *Trends Biochem Sci* **20**, 23-28. "The clot thickens: clues provided by thrombin structure"
136. Bode, W., Turk, D., and Karshikov, A. (1992) *Protein Sci* **1**, 426-471. "The refined 1.9-A X-ray crystal structure of D-Phe-Pro-Arg chloromethylketone-inhibited human alpha-thrombin: structure analysis, overall structure, electrostatic properties, detailed active-site geometry, and structure-function relationships"
137. Stubbs, M. T., and Bode, W. (1993) *Thromb Res* **69**, 1-58. "A player of many parts: the spotlight falls on thrombin's structure"
138. Esmon, C. T., and Lollar, P. (1996) *J Biol Chem* **271**, 13882-13887. "Involvement of thrombin anion-binding exosites 1 and 2 in the activation of factor V and factor VIII"
139. Vali, Z., and Scheraga, H. A. (1988) *Biochemistry* **27**, 1956-1963. "Localization of the binding site on fibrin for the secondary binding site of thrombin"

140. Kaminski, M., and McDonagh, J. (1983) *J Biol Chem* **258**, 10530-10535. "Studies on the mechanism of thrombin. Interaction with fibrin"
141. Berliner, L. J., Sugawara, Y., and Fenton, J. W., 2nd. (1985) *Biochemistry* **24**, 7005-7009. "Human alpha-thrombin binding to nonpolymerized fibrin-Sepharose: evidence for an anionic binding region"
142. Yamaguchi, Y., Okabe, K., Liang, J., Ohshiro, H., Ishihara, K., Uchino, S., Zhang, J. L., Hidaka, H., Yamada, S., and Ogawa, M. (2000) *J Surg Res* **92**, 96-102. "Thrombin and factor Xa enhance neutrophil chemoattractant production after ischemia/reperfusion in the rat liver"
143. Fenton, J. W., 2nd, Ofosu, F. A., Breznjak, D. V., and Hassouna, H. I. (1998) *Semin Thromb Hemost* **24**, 87-91. "Thrombin and antithrombotics"
144. Esmon, C. (2000) *Crit Care Med* **28**, S44-48. "The protein C pathway"
145. Becker, R. C., Bovill, E. G., Seghatchian, M. J., and Samama, M. M. (1998) *Am Heart J* **136**, S19-31. "Pathobiology of thrombin in acute coronary syndromes"
146. Degen, S. J., MacGillivray, R. T., and Davie, E. W. (1983) *Biochemistry* **22**, 2087-2097. "Characterization of the complementary deoxyribonucleic acid and gene coding for human prothrombin"
147. Chang, J. Y. (1986) *Biochem J* **240**, 797-802. "The structures and proteolytic specificities of autolysed human thrombin"
148. Hofsteenge, J., Braun, P. J., and Stone, S. R. (1988) *Biochemistry* **27**, 2144-2151. "Enzymatic properties of proteolytic derivatives of human alpha-thrombin"
149. Fenton, J. W., 2nd, Fasco, M. J., and Stackrow, A. B. (1977) *J Biol Chem* **252**, 3587-3598. "Human thrombins. Production, evaluation, and properties of alpha-thrombin"
150. Chang, T. L., Bauer, R. S., and Berliner, L. J. (1980) *J Biol Chem* **255**, 5904-5906. "Refolding of a three (noncovalently linked)-domain enzyme. Human gamma-thrombin"
151. Lewis, S. D., Lorand, L., Fenton, J. W., 2nd, and Shafer, J. A. (1987) *Biochemistry* **26**, 7597-7603. "Catalytic competence of human alpha- and gamma-thrombin in the activation of fibrinogen and factor XIII"
152. Bezeaud, A., Denninger, M. H., and Guillin, M. C. (1985) *Eur J Biochem* **153**, 491-496. "Interaction of human alpha-thrombin and gamma-thrombin with antithrombin III, protein C and thrombomodulin"
153. Berliner, L. J., Birktoft, J. J., Miller, T. L., Musci, G., Scheffler, J. E., Shen, Y. Y., and Sugawara, Y. (1986) *Ann N Y Acad Sci* **485**, 80-95. "Thrombin: active-site topography"
154. Chandra, T., Stackhouse, R., Kidd, V. J., and Woo, S. L. (1983) *Proc Natl Acad Sci U S A* **80**, 1845-1848. "Isolation and sequence characterization of a cDNA clone of human antithrombin III"
155. Fuchs, H. E., and Pizzo, S. V. (1983) *J Clin Invest* **72**, 2041-2049. "Regulation of factor Xa in vitro in human and mouse plasma and in vivo in mouse. Role of the endothelium and plasma proteinase inhibitors"
156. Fuchs, H. E., Trapp, H. G., Griffith, M. J., Roberts, H. R., and Pizzo, S. V. (1984) *J Clin Invest* **73**, 1696-1703. "Regulation of factor IXa in vitro in human and

- mouse plasma and in vivo in the mouse. Role of the endothelium and the plasma proteinase inhibitors"
157. Gitel, S. N., Medina, V. M., and Wessler, S. (1984) *J Biol Chem* **259**, 6890-6895. "Inhibition of human activated Factor X by antithrombin III and alpha 1-proteinase inhibitor in human plasma"
 158. Jesty, J. (1986) *J Biol Chem* **261**, 10313-10318. "The kinetics of inhibition of alpha-thrombin in human plasma"
 159. Hunt, L. T., and Dayhoff, M. O. (1980) *Biochem Biophys Res Commun* **95**, 864-871. "A surprising new protein superfamily containing ovalbumin, antithrombin-III, and alpha 1-proteinase inhibitor"
 160. Schreuder, H. A., de Boer, B., Dijkema, R., Mulders, J., Theunissen, H. J., Grootenhuys, P. D., and Hol, W. G. (1994) *Nat Struct Biol* **1**, 48-54. "The intact and cleaved human antithrombin III complex as a model for serpin-proteinase interactions"
 161. Carrell, R. W., Stein, P. E., Fermi, G., and Wardell, M. R. (1994) *Structure* **2**, 257-270. "Biological implications of a 3 A structure of dimeric antithrombin"
 162. Olson, S. T., and Shore, J. D. (1982) *J Biol Chem* **257**, 14891-14895. "Demonstration of a two-step reaction mechanism for inhibition of alpha-thrombin by antithrombin III and identification of the step affected by heparin"
 163. Danielsson, A., and Bjork, I. (1982) *Biochem J* **207**, 21-28. "Mechanism of inactivation of trypsin by antithrombin"
 164. Latallo, Z. S., and Jackson, C. M. (1986) *Thromb Res* **43**, 523-537. "Reaction of thrombins with human antithrombin III: II. Dependence of rate of inhibition on molecular form and origin of thrombin"
 165. Craig, P. A., Olson, S. T., and Shore, J. D. (1989) *J Biol Chem* **264**, 5452-5461. "Transient kinetics of heparin-catalyzed protease inactivation by antithrombin III. Characterization of assembly, product formation, and heparin dissociation steps in the factor Xa reaction"
 166. Wong, R. F., Windwer, S. R., and Feinman, R. D. (1983) *Biochemistry* **22**, 3994-3999. "Interaction of thrombin and antithrombin. Reaction observed by intrinsic fluorescence measurements"
 167. Stone, S. R., and Hermans, J. M. (1995) *Biochemistry* **34**, 5164-5172. "Inhibitory mechanism of serpins. Interaction of thrombin with antithrombin and protease nexin 1"
 168. Jornvall, H., Fish, W. W., and Bjork, I. (1979) *FEBS Lett* **106**, 358-362. "The thrombin cleavage site in bovine antithrombin"
 169. Bjork, I., Danielsson, A., Fenton, J. W., and Jornvall, H. (1981) *FEBS Lett* **126**, 257-260. "The site in human antithrombin for functional proteolytic cleavage by human thrombin"
 170. Bjork, I., Jackson, C. M., Jornvall, H., Lavine, K. K., Nordling, K., and Salsgiver, W. J. (1982) *J Biol Chem* **257**, 2406-2411. "The active site of antithrombin. Release of the same proteolytically cleaved form of the inhibitor from complexes with factor IXa, factor Xa, and thrombin"
 171. Erdjument, H., Lane, D. A., Panico, M., Di Marzo, V., and Morris, H. R. (1988) *J Biol Chem* **263**, 5589-5593. "Single amino acid substitutions in the reactive site of

- antithrombin leading to thrombosis. Congenital substitution of arginine 393 to cysteine in antithrombin Northwick Park and to histidine in antithrombin Glasgow"
172. Lane, D. A., Erdjument, H., Thompson, E., Panico, M., Di Marzo, V., Morris, H. R., Leone, G., De Stefano, V., and Thein, S. L. (1989) *J Biol Chem* **264**, 10200-10204. "A novel amino acid substitution in the reactive site of a congenital variant antithrombin. Antithrombin pescara, ARG393 to pro, caused by a CGT to CCT mutation"
 173. Owen, M. C., Beresford, C. H., and Carrell, R. W. (1988) *FEBS Lett* **231**, 317-320. "Antithrombin Glasgow, 393 Arg to His: a P1 reactive site variant with increased heparin affinity but no thrombin inhibitory activity"
 174. Olson, S. T., Bock, P. E., Kvassman, J., Shore, J. D., Lawrence, D. A., Ginsburg, D., and Bjork, I. (1995) *J Biol Chem* **270**, 30007-30017. "Role of the catalytic serine in the interactions of serine proteinases with protein inhibitors of the serpin family. Contribution of a covalent interaction to the binding energy of serpin-proteinase complexes"
 175. Le Bonniec, B. F., Guinto, E. R., and Stone, S. R. (1995) *Biochemistry* **34**, 12241-12248. "Identification of thrombin residues that modulate its interactions with antithrombin III and alpha 1-antitrypsin"
 176. Rezaie, A. R. (1996) *Biochemistry* **35**, 1918-1924. "Tryptophan 60-D in the B-insertion loop of thrombin modulates the thrombin-antithrombin reaction"
 177. Olson, S. T. (1985) *J Biol Chem* **260**, 10153-10160. "Heparin and ionic strength-dependent conversion of antithrombin III from an inhibitor to a substrate of alpha-thrombin"
 178. Patston, P. A., Gettins, P., Beechem, J., and Schapira, M. (1991) *Biochemistry* **30**, 8876-8882. "Mechanism of serpin action: evidence that C1 inhibitor functions as a suicide substrate"
 179. Fish, W. W., and Bjork, I. (1979) *Eur J Biochem* **101**, 31-38. "Release of a two-chain form of antithrombin from the antithrombin-thrombin complex"
 180. Cooperman, B. S., Stavridi, E., Nickbarg, E., Rescorla, E., Schechter, N. M., and Rubin, H. (1993) *J Biol Chem* **268**, 23616-23625. "Antichymotrypsin interaction with chymotrypsin. Partitioning of the complex"
 181. Griffith, M. J. (1982) *J Biol Chem* **257**, 7360-7365. "Kinetics of the heparin-enhanced antithrombin III/thrombin reaction. Evidence for a template model for the mechanism of action of heparin"
 182. Jordan, R. E., Oosta, G. M., Gardner, W. T., and Rosenberg, R. D. (1980) *J Biol Chem* **255**, 10081-10090. "The kinetics of hemostatic enzyme-antithrombin interactions in the presence of low molecular weight heparin"
 183. Olson, S. T., and Bjork, I. (1991) *J Biol Chem* **266**, 6353-6364. "Predominant contribution of surface approximation to the mechanism of heparin acceleration of the antithrombin-thrombin reaction. Elucidation from salt concentration effects"
 184. Olson, S. T., Bjork, I., Sheffer, R., Craig, P. A., Shore, J. D., and Choay, J. (1992) *J Biol Chem* **267**, 12528-12538. "Role of the antithrombin-binding pentasaccharide in heparin acceleration of antithrombin-proteinase reactions."

- Resolution of the antithrombin conformational change contribution to heparin rate enhancement"
185. Olson, S. T., and Shore, J. D. (1986) *J Biol Chem* **261**, 13151-13159. "Transient kinetics of heparin-catalyzed protease inactivation by antithrombin III. The reaction step limiting heparin turnover in thrombin neutralization"
 186. Koide, T., Odani, S., Takahashi, K., Ono, T., and Sakuragawa, N. (1984) *Proc Natl Acad Sci U S A* **81**, 289-293. "Antithrombin III Toyama: replacement of arginine-47 by cysteine in hereditary abnormal antithrombin III that lacks heparin-binding ability"
 187. Borg, J. Y., Owen, M. C., Soria, C., Soria, J., Caen, J., and Carrell, R. W. (1988) *J Clin Invest* **81**, 1292-1296. "Proposed heparin binding site in antithrombin based on arginine 47. A new variant Rouen-II, 47 Arg to Ser"
 188. Borg, J. Y., Brennan, S. O., Carrell, R. W., George, P., Perry, D. J., and Shaw, J. (1990) *FEBS Lett* **266**, 163-166. "Antithrombin Rouen-IV 24 Arg----Cys. The amino-terminal contribution to heparin binding"
 189. Gandrille, S., Aiach, M., Lane, D. A., Vidaud, D., Molho-Sabatier, P., Caso, R., de Moerloose, P., Fiessinger, J. N., and Clauser, E. (1990) *J Biol Chem* **265**, 18997-19001. "Important role of arginine 129 in heparin-binding site of antithrombin III. Identification of a novel mutation arginine 129 to glutamine"
 190. Najjam, S., Chadeuf, G., Gandrille, S., and Aiach, M. (1994) *Biochim Biophys Acta* **1225**, 135-143. "Arg-129 plays a specific role in the conformation of antithrombin and in the enhancement of factor Xa inhibition by the pentasaccharide sequence of heparin"
 191. Olson, S. T., Halvorson, H. R., and Bjork, I. (1991) *J Biol Chem* **266**, 6342-6352. "Quantitative characterization of the thrombin-heparin interaction. Discrimination between specific and nonspecific binding models"
 192. Carlstrom, A. S., Lieden, K., and Bjork, I. (1977) *Thromb Res* **11**, 785-797. "Decreased binding of heparin to antithrombin following the interaction between antithrombin and thrombin"
 193. Preissner, K. T., Anders, E., Grulich-Henn, J., and Muller-Berghaus, G. (1988) *Blood* **71**, 1581-1589. "Attachment of cultured human endothelial cells is promoted by specific association with S protein (vitronectin) as well as with the ternary S protein-thrombin-antithrombin III complex"
 194. de Boer, H. C., Preissner, K. T., Bouma, B. N., and de Groot, P. G. (1995) *J Biol Chem* **270**, 30733-30740. "Internalization of vitronectin-thrombin-antithrombin complex by endothelial cells leads to deposition of the complex into the subendothelial matrix"
 195. Wells, M. J., and Blajchman, M. A. (1998) *J Biol Chem* **273**, 23440-23447. "In vivo clearance of ternary complexes of vitronectin-thrombin-antithrombin is mediated by hepatic heparan sulfate proteoglycans"
 196. Essex, D. W., Miller, A., Swiatkowska, M., and Feinman, R. D. (1999) *Biochemistry* **38**, 10398-10405. "Protein disulfide isomerase catalyzes the formation of disulfide-linked complexes of vitronectin with thrombin-antithrombin"

197. Podack, E. R., Dahlback, B., and Griffin, J. H. (1986) *Journal of Biological Chemistry* **261**, 7387-7392. "Interaction of S-protein of Complement with Thrombin and Antithrombin III during Coagulation"
198. Izumi, M., Shimo-Oka, T., Morishita, N., Ii, I., and Hayashi, M. (1988) *Cell Struct Funct* **13**, 217-225. "Identification of the collagen-binding domain of vitronectin using monoclonal antibodies"

Appendices

APPENDIX A: Polypeptide Information

POLYPEPTIDE 1-100

Peptide Sequence

DQESCKGRCT EGFNVDKKCQ CDELCSTTQS CCTDYTAECK PQVTRGDVFT
NPEDEYTVYD DGEEKNNATV HEQVGGPSLT SDLQAQSKGN PEQTPVLKPE

Amino Acid Composition

residue	number	mole percent
A	3	2.439
B	0	0.0
C	8	6.504
D	9	7.317
E	12	9.756
F	2	1.626
G	10	8.130
H	8	6.504
I	0	0.0
K	7	5.691
L	6	4.878
M	3	2.439
N	4	3.252
P	7	5.691
Q	8	6.504
R	3	2.439
S	11	8.943
T	9	7.317
V	8	6.504
W	0	0.0
Y	5	4.065
Z	0	0.0

Molecular Weight (Daltons)

13,679.85

Isoelectric Point

4.88

POLYPEPTIDE 51-150

Peptide Sequence

NPEDEYTVYD DGEEKNNATV HEQVGGPSLT SDLQAQSKGN PEQTPVLKPE
EEAPAPEVGA SKPEGIDSRP ETLHPGAPQP PAEEELCSGK PSTLHRLKNG

Amino Acid Composition

residue	number	mole percent
A	7	5.645
B	0	0.0
C	1	0.806
D	8	6.452
E	16	12.903
F	2	1.613
G	11	8.871
H	9	7.258
I	1	0.806
K	6	4.839
L	8	6.452
M	3	2.419
N	4	3.226
P	15	12.097
Q	5	4.032
R	3	2.419
S	11	8.871
T	6	4.839
V	6	4.839
W	0	0.0
Y	2	1.613
Z	0	0.0

Molecular Weight (Daltons)

13,450.48

Isoelectric Point

4.62

POLYPEPTIDE 101-200

Peptide Sequence

EEAPAEVGA SKPEGIDSRP ETLHPGAPQP PAEEELCSGK PSTLHRLKNG
SLFAFRGQYC YELDEKAVRP GYPKLIRDVW GIEGPIDAAF TRINCQGKTY

Amino Acid Composition

residue	number	mole percent
A	9	7.317
B	0	0.0
C	3	2.439
D	6	4.878
E	12	9.756
F	5	4.065
G	13	10.569
H	8	6.504
I	5	4.065
K	6	4.878
L	8	6.504
M	2	1.626
N	2	1.626
P	13	10.569
Q	3	2.439
R	7	5.691
S	9	7.317
T	4	3.252
V	4	3.252
W	1	0.813
Y	3	2.439
Z	0	0.0

Molecular Weight (Daltons)

13,512.97

Isoelectric Point

6.30

POLYPEPTIDE 151-250

Peptide Sequence

SLFAFRGQYC YELDEKAVRP GYPKLIRDVW GIEGPIDAAF TRINCQGKTY
LFKGSQYWRF EDGVLDPDYP RNISDGFDDI PDNVDAALAL PAHSYSGRER

Amino Acid Composition

residue	number	mole percent
A	8	6.452
B	0	0.0
C	2	1.613
D	11	8.871
E	6	4.839
F	6	4.839
G	14	11.290
H	8	6.452
I	6	4.839
K	4	3.226
L	9	7.258
M	2	1.613
N	4	3.226
P	8	6.452
Q	3	2.419
R	8	6.452
S	9	7.258
T	2	1.613
V	5	4.032
W	2	1.613
Y	7	5.645
Z	0	0.0

Molecular Weight (Daltons)

13,948.35

Isoelectric Point

6.30

POLYPEPTIDE 201-300

Peptide Sequence

LFKGSQYWRF EDGVLDPDYP RNISDGFDDGI PDNVDAALAL PAHSYSGRER
VYFFKGKQYW EYQFQHQPSQ EECEGSSLSA VFEHFAMMQR DSWEDIFELL

Amino Acid Composition

residue	number	mole percent
A	6	4.839
B	0	0.0
C	1	0.806
D	10	8.065
E	10	8.065
F	9	7.258
G	10	8.065
H	10	8.065
I	3	2.419
K	3	2.419
L	8	6.452
M	4	3.226
N	3	2.419
P	6	4.839
Q	7	5.645
R	6	4.839
S	13	10.484
T	0	0.0
V	5	4.032
W	3	2.419
Y	7	5.645
Z	0	0.0

Molecular Weight (Daltons)

14,497.77

Isoelectric Point

5.21

POLYPEPTIDE 251-350

Peptide Sequence

VYFFKGKQYW EYQFQHQPSSQ EECEGSSLSA VFEHFAMMQR DSWEDIFELL
FWGRTSAGTR QPQFISRDPWH GVPQGQVDAAM AGRIYISGMA PRPSLAKKQR

Amino Acid Composition

residue	number	mole percent
A	7	5.654
B	0	0.0
C	1	0.806
D	5	4.032
E	8	6.452
F	8	6.452
G	11	8.871
H	10	8.065
I	4	3.226
K	4	3.226
L	6	4.839
M	6	4.839
N	0	0.0
P	6	4.839
Q	10	8.065
R	8	6.452
S	14	11.290
T	3	2.419
V	5	4.032
W	4	3.226
Y	4	3.226
Z	0	0.0

Molecular Weight (Daltons)

14,364.02

Isoelectric Point

7.38

POLYPEPTIDE 301-400

Peptide Sequence

FWGRTSAGTR QPQFISRDWH GVPGQVDAAM AGRIYISGMA PRPSLAKKQR
FRHRNRLGYR SQRGHSRGRN QNSRRPSRAT WLSLFSSEES NLGANNYDDY

Amino Acid Composition

residue	number	mole percent
A	7	5.645
B	0	0.0
C	0	0.0
D	5	4.032
E	3	2.419
F	4	3.226
G	13	10.484
H	10	8.065
I	3	2.419
K	3	2.419
L	7	5.645
M	5	4.032
N	6	4.839
P	6	4.839
Q	6	4.839
R	17	13.710
S	17	13.710
T	3	2.419
V	3	2.419
W	3	2.419
Y	3	2.419
Z	0	0.0

Molecular Weight (Daltons)

14,172.67

Isoelectric Point

12.16

POLYPEPTIDE 351-459

Peptide Sequence

FRHRNRLGYR SQRGHSRGRN QNSRRPSRAT WLSLFSSEES NLGANNYDDY
RMDWLVPATC EPIQSVFFFS GELYRVNLR TRRVDTVDPP YPRSIAQYW
LGCPAPGHL

Amino Acid Composition

residue	number	mole percent
A	5	3.759
B	0	0.0
C	2	1.504
D	7	5.263
E	4	3.008
F	5	3.759
G	10	7.519
H	10	7.519
I	2	1.504
K	2	1.504
L	9	6.767
M	4	3.008
N	7	5.263
P	9	6.767
Q	4	3.008
R	18	13.534
S	16	12.030
T	3	2.256
V	6	4.511
W	3	2.256
Y	7	5.263
Z	0	0.0

Molecular Weight (Daltons)

15,594.26

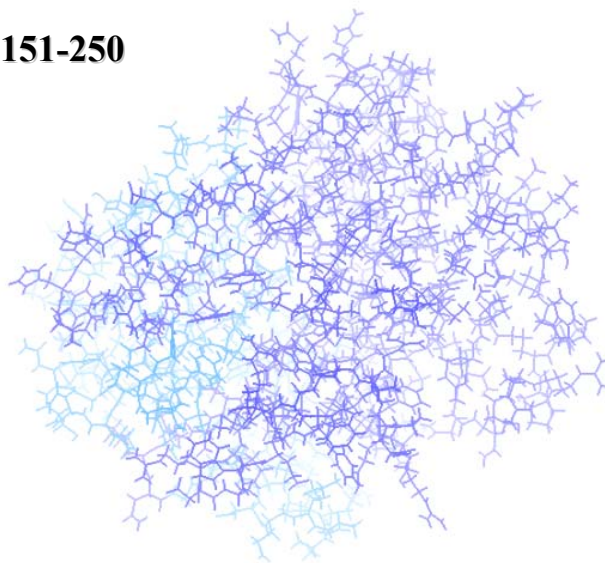
Isoelectric Point

11.08

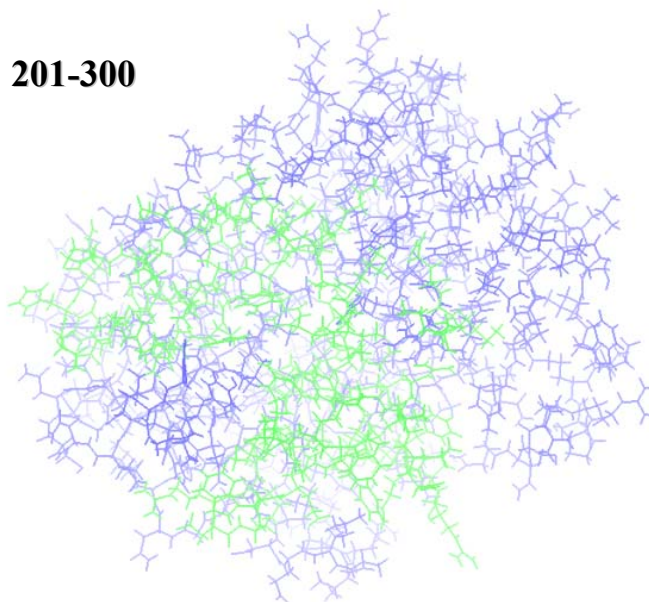
APPENDIX B: Polypeptide Modeling

Using INSIGHT© software, the following polypeptides were highlighted in the predicted three-dimensional structure of vitronectin determined by Xu et al (2001).

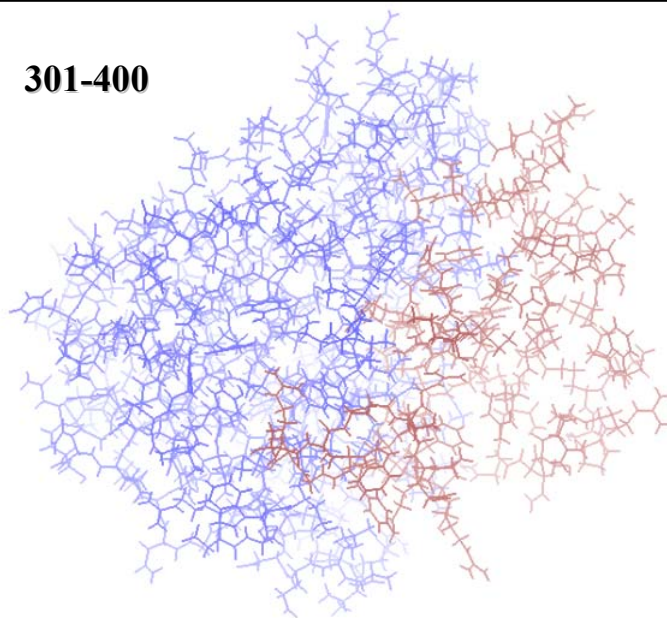
151-250



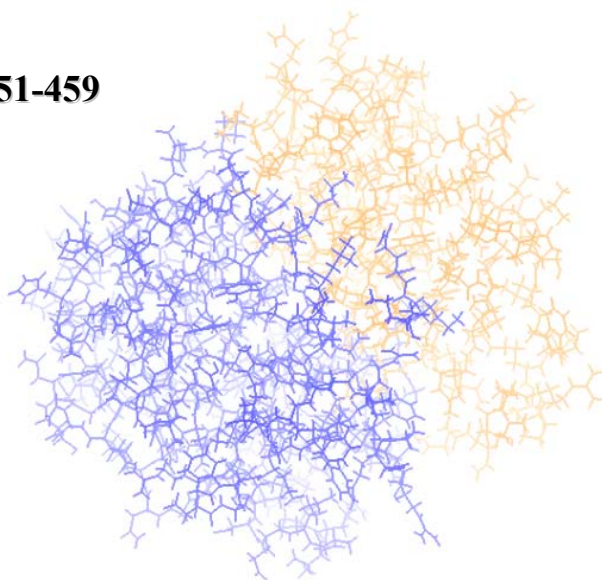
201-300



301-400



351-459



APPENDIX C: Domain Organization of Vitronectin

N-terminal Domain

**DQ▼ESCKGRCTEGFNVDKKCQCDELCSYYQSCCTDY
TAECKPQ▼VT**

Connecting Region

**RGDVFTMPED EYTVYDDGEEKNNATVHERVGGPSLTSDLQAQS
KGNPEQTPALKPEEEAPAPEVGASKP▼EGIDSRPET**

Hemopexin Homology Domain I

**LHPGRPQPPAEEELCSGKPFDAFTDLKNGSLFAFR▼
GQYCYELDEKAVRPGYPKLIRDVW**

**GIEGPIDAAFRINCQGKTYLKF▼GSQYWRFEDGVLDPDYPRNI
SDGFDGIPDNADAAL**

**ALPAHSYSGRERVYFFK▼GKQYWEYQFQHQPSEEECEGSSLSA
VFEHFAMMQRDS**

EDIFELLFWGRTS▼AGTRQPQFISRDWHGV

Hemopexin Homology Domain II

PGQVDAAMAGRIYISGMAPRPSLAKKQRF

**HRNRKGYRSQRGHSRGRNQNSRRPSRATWLSLFSSEESNLGANN
YDDYRMDWLVPATCE**

**PIQSVFFFSG▼DKYYRVNLRTRRVDTVDPPYPRSIAQYWLGC
PA
PGHL**

This is the mature vitronectin peptide sequence organized into four domains. **Green** indicates the N-terminal domain, **gray** is the connecting region, **orange** represents hemopexin homology domain (HPD) I, and **blue** is hemopexin homology domain II. An ▼ indicates an exon junction. HPD I and II are further organized into four and three parts, respectively, to indicate individual “blades” of the predicted β -propeller structure (Xu et al, 2001).

VITA

Jodi Lyn Watson was born in Knoxville, Tennessee on August 18, 1978, the daughter of Don and Judi Watson. She attended school in the Monroe and McMinn County public school systems. In May 1996, she graduated from Sequoyah High School in Madisonville, Tennessee. She entered King College in Bristol, Tennessee in August of 1996 and was awarded a Bachelor of Science degree in May 2000, with a major in biology and a minor in chemistry. During college, she served as a laboratory instructor for the Biology Department and worked during summers as a medical office assistant in a physician's office in her hometown of Madisonville. She entered the Masters program in Biochemistry at the University of Tennessee, Knoxville in August 2000 where she joined the laboratory of Cynthia B. Peterson to conduct research on vitronectin. She received a Master of Science Degree in Biochemistry in May 2004.