

THE STRUCTURAL AND FUNCTIONAL CONSEQUENCES OF THE INTERACTION OF
THE VINCULIN TAIL DOMAIN WITH F-ACTIN AND PIP₂

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ABSTRACT

Peter Matthew Thompson: The Structural and Functional Consequences of the Interaction of the Vinculin Tail Domain with F-actin and PIP₂
(Under the direction of Sharon L. Campbell)

Vinculin is an essential, highly-conserved eukaryotic scaffolding protein. It localizes to focal adhesions and adherens junctions, where it assists in physically linking the actin cytoskeleton to the adhesive structure. Loss of vinculin causes embryonic lethality with observable cardiovascular and neural defects. Vinculin is comprised of three domains: a large helical head domain, an unstructured proline-rich linker, and a helical tail. Vinculin functions as a scaffold, alternating between an autoinhibited conformation in which it cannot bind ligands, and an open conformation in which it is free to bind ligands at all three domains.

The tail domain binds a variety of ligands, two of which are F-actin and phosphatidylinositol 4,5-bisphosphate (PIP₂). Both interactions lack good structural models, which has hindered the development of tools to understand the specific biological functions these interactions play. Using a variety of biochemical and biophysical techniques and tools, we have developed new structural models for these interactions. The PIP₂ model shows how the basic residues in helices 1 and 2 specifically recognize PIP₂, while basic residues on helix 3 are responsible for membrane association.

Actin-binding, conversely, is driven by a hydrophobic patch on helix 4. The application of cryo-electron microscopy has also revealed some of the conformational changes that take place in the tail domain when it binds F-actin: helix 4 straightens and helix 1 is discharged from the helix bundle.

Additionally, we characterize the backbone dynamics of Vt by nuclear magnetic resonance. These experiments reveal the presence of μ s-ms motions that reside where the C-terminal arm interacts with the helix bundle. These motions may be involved in the conformational change that takes place upon actin binding.

To Juliet. Thank you for being so understanding and generous
with your time while I have worked on this dissertation.

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In some ways, this is the most difficult part of the dissertation to compose. It is impossible to, in a few paragraphs, adequately recognize and thank the numerous individuals who have supported and encouraged me in my development as a scientist.

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In addition to Sharon, the other members of my thesis committee have each played a part in my development as a scientist. They encouraged me when I changed projects, offered both scientific and professional advice, and refused to hold me to anything but a high standard. Their support will definitely be missed.

A number of other scientists have played essential roles in my scientific growth. Dr. Charles Cobb and Ron Davis were the first teachers to directly encourage me to try biological research. Dr. Bruce Novak and Dr. Andrew Wallace stoked my passion for science while I was at North Carolina State University and were generous to allow me to work in their laboratories. I also want to thank Dr. Michael Caplow. He was, in many ways, an informal mentor taught me, more than anyone else, the importance in being critical in evaluating the conclusions drawn from experimental data. Had I followed his three rules for graduate students better: 1) never teach; 2) always have an experiment running; and 3) always look out for number one, I may have had an even better graduate career.

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LIST OF ABBREVIATIONS AND SYMBOLS

2D	two-dimensional
3D	three-dimensional
AIC	Akaike's information criterion
AJ	adherens junction
Akt	protein kinase B
AUC	analytical ultracentrifugation
BME	β -mercaptoethanol
CD	circular dichroism
CLEANEX	CLEAN chemical exchange spectroscopy
CPMG	Carr-Purcell-Meiboom-Gill
cryo-EM	cryo-electron microscopy
CSA	chemical shift anisotropy
DMD	discrete molecular dynamics
DTT	dithiothreitol
ECM	extracellular matrix
EM	electron microscopy
FA	focal adhesion
FAK	focal adhesion kinase
FN	fibronectin
FRAP	fluorescence recovery after photobleaching
FRET	Förster resonance energy transfer
HDX	hydrogen-deuterium exchange

HSQC	heteronuclear single quantum coherence
IPTG	β -D-1-thiogalactopyranoside
K_d	dissociation constant
kDa	kilodalton
k_{ex}	exchange rate
k_{rc}	exchange rate of a residue in an unfolded peptide
LB	lysogeny broth
MEF	mouse embryonic fibroblast
μ s-ms	microsecond/millisecond
NMR	nuclear magnetic resonance
NOE	Nuclear Overhauser Effect
PDB	Protein Data Bank
P_f	protection factor
PI ₃ K	phosphoinositide 3-kinase
PIP ₂	phosphatidylinositol 4,5-bisphosphate
PIPKI γ	phosphatidylinositol phosphate kinase type 1 gamma
PKC α	protein kinase C alpha
POPC	1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine
PS	phosphatidylserine
ps-ns	picosecond/nanosecond
R_1	longitudinal relaxation rate constant
R_2	transverse relaxation rate constant
$R_{2,eff}$	effective transverse relaxation rate
R_{ex}	transverse relaxation rate constant due to chemical exchange

RT	real-time
RTCA	real-time cell analyzer
S^2	Lipari-Szabo order parameter
SDS	sodium dodecyl sulfate
src	tyrosine-protein kinase CSK
SUVs	small unilamellar vesicles
τ_e	internal correlation time
TEV	tobacco etch virus
T_m	denaturation midpoint temperature
τ_m	rotational correlation time
UV	ultraviolet
VASP	vasodilator-stimulated phosphoprotein
Vh	vinculin head domain
Vin $-/-$ MEFs	vinculin-null mouse embryonic fibroblasts
Vt	vinculin tail domain
WT	wild-type

Chapter 1: Introduction and Background

Introduction

Vinculin is an essential scaffolding protein, ubiquitously expressed in higher eukaryotes, that localizes to focal adhesions (FAs) and adherens junctions (AJs). Initially discovered in chicken gizzard in 1979 (1), vinculin is large (117 kDa) and highly conserved (2, 3). Vinculin plays a key role in signaling at sites of adhesion, and has been implicated in regulating cell morphology, cell migration (4), cell stiffness (5, 6), adhesion strength (7), adhesion turnover, and adhesion morphology (8, 9). It regulates these processes by binding to multiple biomolecules, creating physical linkages between proteins at precise times and in precise subdomains of focal adhesions and adherens junctions. Vinculin's structure makes it uniquely suited for this role.

Vinculin contains two distinct domains: an N-terminal head domain (Vh) and a C-terminal tail domain (Vt), which are connected by a proline-rich linker (**Figure 1.1**). Vh and Vt are alpha-helical in nature, while the proline-rich linker lacks defined structure (2). Vh can be further subdivided into four subdomains (D1-D4), each of which is made up of one or two helix bundles. Vt is a five-helix bundle with an N-terminal strap and a C-terminal extension (10). While neither the strap or C-terminal extension have a defined secondary structure, they make interactions with the helix bundle and are thought to be somewhat ordered. Each vinculin domain is capable of binding to a variety of ligands, each with unique cellular consequences. Vh can bind talin (11), α -catenin (12, 13), α -actinin (14), and MAPK (15). The proline-rich linker houses binding sites for p130CAS (16), vasodilator-stimulated phosphoprotein (VASP) (17), ponsin/CAP (18), vinexin α/β (19), and the Arp2/3 complex (20). Vt interacts with filamentous actin (F-actin) (21), paxillin (22), raver1 (23),

phosphatidylinositol-4,5-bisphosphate (PIP₂) (3), Hic-5 (24), protein kinase C alpha (PKC α) (25), tyrosine-protein kinase CSK (src) (26), and α -synemin (27).

In addition to the ligands mentioned above, Vh and Vt interact at high affinity (11, 28) in the absence of ligands (2, 28). This conformation is referred to as the “inactive” conformation, as vinculin is unable to bind ligands in this state. However, vinculin becomes activated by binding multiple ligands, which compete with autoinhibitory contacts between Vh and Vt (29), forming the “active” conformation (**Figure 1.2**). This exposes even more binding sites on vinculin, allowing binding of more biomolecules. In addition to regulation of vinculin conformation by the presence or absence of ligands, phosphorylation and other post-translational modifications may also regulate the equilibrium between the active and inactive conformations (30, 31).

While much is known about what ligands bind to vinculin, less is known about how these ligands bind, especially to Vt. As of the writing of this manuscript, there are co-structures in the PDB for vinculin with talin (32), α -actinin (33), α -catenin (34), ponsin/CAP (35), vinexin (36), two bacterial ligands that exploit vinculin (from *Shigella flexneri* and *Rickettsia rickettsii*) (37, 38), raver1 (39), and PIP₂ (40). Because the Vt is able to bind to so many ligands and because these binding sites are poorly characterized, it has been difficult to study the cellular consequences of these specific interactions. Frequently, multiple mutations (41, 42) or large deletions (7, 42) are made to vinculin constructs in cells, which result in the unintended consequences of disrupting Vt structure and/or disrupting multiple Vt:ligand interactions (43). Part of the difficulty in defining ligand-binding sites on Vt at high resolution comes from the experimental difficulties presented by PIP₂ and F-actin, which do not lend themselves to crystallization or easy study by NMR. However, recent advances in negative-stain and cryo-electron microscopies, nanodisc preparation, and in computational modeling have provided new ways to identify and characterize these structures and binding surfaces.

Focal adhesion architecture and signaling

While vinculin is localized to both FAs and AJs and has essential roles in each structure, its roles at FAs have been better characterized, primarily due to the development of vinculin-null fibroblasts and their ease of use. For this reason, much of the work described in this thesis has been conducted in collaboration with cell biologists who study FAs, and a brief description of FAs is provided.

FAs are large (up to 200 nm in length (44, 45)), multi-protein substructures in cells that are responsible for mechanically linking the cytoskeleton to the extracellular matrix (ECM). Of the proteins that comprise FAs, the vast majority are cytoplasmic, save for the protein integrin, a transmembrane protein. Integrins are receptors, comprised of an α and β chain, that physically contact fibronectin, collagen, vitronectin, or other ligands present in the ECM (46-48). Syndecan 4 (49) is another transmembrane protein that has been identified in FAs, though its role seems to be less integral.

The cytoplasmic side of FAs is much larger and contains a greater variety of proteins. Integrins span the plasma membrane, and so part of the protein does reside in the cytoplasm. These tails change conformation upon binding (outside-in signaling), and can also receive internal signals that are passed to the integrin receptors (inside-out signaling) (50). The intracellular integrin tail domain can bind to talin, another protein localized to focal adhesions. Talin, like vinculin, is a scaffolding protein that exists in an autoinhibited state (51). It unfurls to bind actin, linking the actin cytoskeleton to integrin, though it does this somewhat poorly, as its actin-binding affinity is somewhat weak (52). The unfurling of talin exposes multiple vinculin binding sites, and it is thought that the recruitment of vinculin by talin stabilizes the actin linkage to integrins (53).

Like vinculin, paxillin is a scaffolding protein that localizes to FAs. Much less is known about paxillin, though it has a number of phosphorylation sites that mediate its interactions with a variety of proteins (54). However, unlike vinculin and talin, which physically span

from the plasma membrane to the actin cytoskeleton, paxillin is found very near the membrane (55). A variety of other proteins are localized to FAs. VASP, profilin, Arp2, and Arp3 are involved in promoting actin polymerization and branching, and have all been identified at FAs.

Many of these proteins are regulated by phosphorylation. Indeed, a common way to visualize FAs in cells is to use a phosphotyrosine antibody coupled to a fluorophore (56). Vinculin, paxillin, and focal adhesion kinase (FAK) are all targets of tyrosine kinases. FAK, src, csk, and fyn are the primary tyrosine kinases responsible for tyrosine phosphorylation at FAs (57). Additionally, PKC α , a serine kinase, is a key FA component and its ability to phosphorylate Vt is dependent on PIP₂ signaling (58). PKC α -mediated phosphorylation of vinculin has been implicated in vinculin activation and subsequent FA maturation (30), though direct validation of this mechanism has remained elusive.

All of this organization and signaling is designed to regulate attachment to the actin cytoskeleton, which resides approximately 80+ nm from the plasma membrane (55). While talin and vinculin are responsible for physically linking actin to the FA, other proteins can bind actin and crosslink individual filaments, such as α -actinin (59). Vinculin likely crosslinks filaments right as they reach the FA, while α -actinin continues to crosslink filaments further from the FA (55). These crosslinked filaments form stress fibers, through which the majority of the contractile forces are initiated. This contractility is generated by myosin II and is regulated by the Rho family of small GTPases (60).

Metavinculin is a tissue-specific splice variant of vinculin

Transcripts of *VCL* are alternatively spliced in muscle tissues, leading to expression of both vinculin and metavinculin (150 kDa) (61). Both isoforms are localized to the cell membrane, the I-band in the sarcomere, and to intercalated discs (62). Metavinculin transcripts contain an extra exon (exon 19) that codes for a 68-residue insert in the tail domain. While vinculin is highly conserved across species, exon 19 is much less conserved

(63). This extra exon codes for residues between helices 1 and 2 in the Vt primary sequence and confers unique functions to metavinculin (64). Structurally, these new residues replace the residues 879-915, which code for the N-terminal strap and helix 1 in Vt (65) (**Figure 1.3**). Functionally, this helix swap confers higher affinity for raver1 (23) and weaker affinity for PIP₂ (66) in metavinculin compared to vinculin. Metavinculin interactions with F-actin appear more complex. The tail domain of metavinculin can sever actin filaments, though this function appears to be regulated by the proline-rich linker in the head domain and such activity has not been observed *in vivo* (67). Metavinculin tail also appears to be less a less-capable actin crosslinking protein (68), though these results require further study, as the potential actin-severing properties may have skewed crosslinking analyses.

While the immediate cellular consequences of these structural and biochemical differences requires further study, it is currently believed that metavinculin is specialized for mechanotransduction, as its expression levels positively correlate with the force exerted on cells (44, 66, 69). These levels are high in the smooth muscle of the aorta and uterus (66), tissues that experience substantial amounts of force. However, when smooth muscle cells from human aorta (44, 62, 70) and chicken gizzard (71) are subcultured, they exhibit a marked decrease in metavinculin expression levels. This occurs as they are cultured, during which they experience weaker forces and tension than *in vivo*. This suggests that there is a feedback mechanism between the forces a cell experiences and the amount of metavinculin it expresses.

Vinculin in health and disease

Because of its roles in regulating adhesions, vinculin is required for embryogenesis and cardiovascular development, processes that require tight regulation of cell movement and cellular forces. Vinculin null mice fail to develop properly, ceasing to grow after embryonic day 10, exhibiting neural and cardiac defects (8). Mice with conditional vinculin expression fail to survive more than a few weeks without vinculin, dying from ventricular tachycardia or

dilated cardiomyopathy (72). Mice with hemizygous expression of vinculin fare better under normal conditions but will expire weeks after undergoing cardiac pressure loading (73). Additionally, mutations in metavinculin are associated with the development of cardiomyopathies in humans (65, 68, 74-77). These mutations are believed to disrupt the interactions with of metavinculin with F-actin (68), though other possible mechanisms for these mutants have not been thoroughly explored.

With a role in cell migration, many have hypothesized that vinculin plays a significant role in cancer, specifically in metastasis (78). However, a clear link has been difficult to establish. The first clues came from vinculin-null murine embryonic fibroblasts, which are resistant to apoptosis and anoikis (15) and exhibit increased motility, weakened adherence, and a rounded morphology (4, 9). These results suggest that vinculin is a tumor suppressor. Indeed, studies have correlated lower expression levels in both transformed cell lines (79) and in tumors (80) with a more tumorigenic phenotype. Activation of vinculin in M21 melanoma cells conferred greater susceptibility to chemotherapies (81). However, greater vinculin expression was found in castration-resistant prostate tumors, while decreased expression was observed in benign hyperplasias (82). Additionally, a transcriptome study of colon cancer showed that, for primary tumors and metastases, vinculin expression is increased, while metavinculin expression is decreased (83). Another study showed an increase in vinculin expression in lung cancer metastases (84). Increased vinculin localization to FAs in stiffer substrates correlates with increased phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) signaling in mammary tissue, suggesting a similar trend for breast cancer metastases (85). These conflicting reports speak both to the diversity of mechanisms that drive different cancers and to the complexity of vinculin signaling.

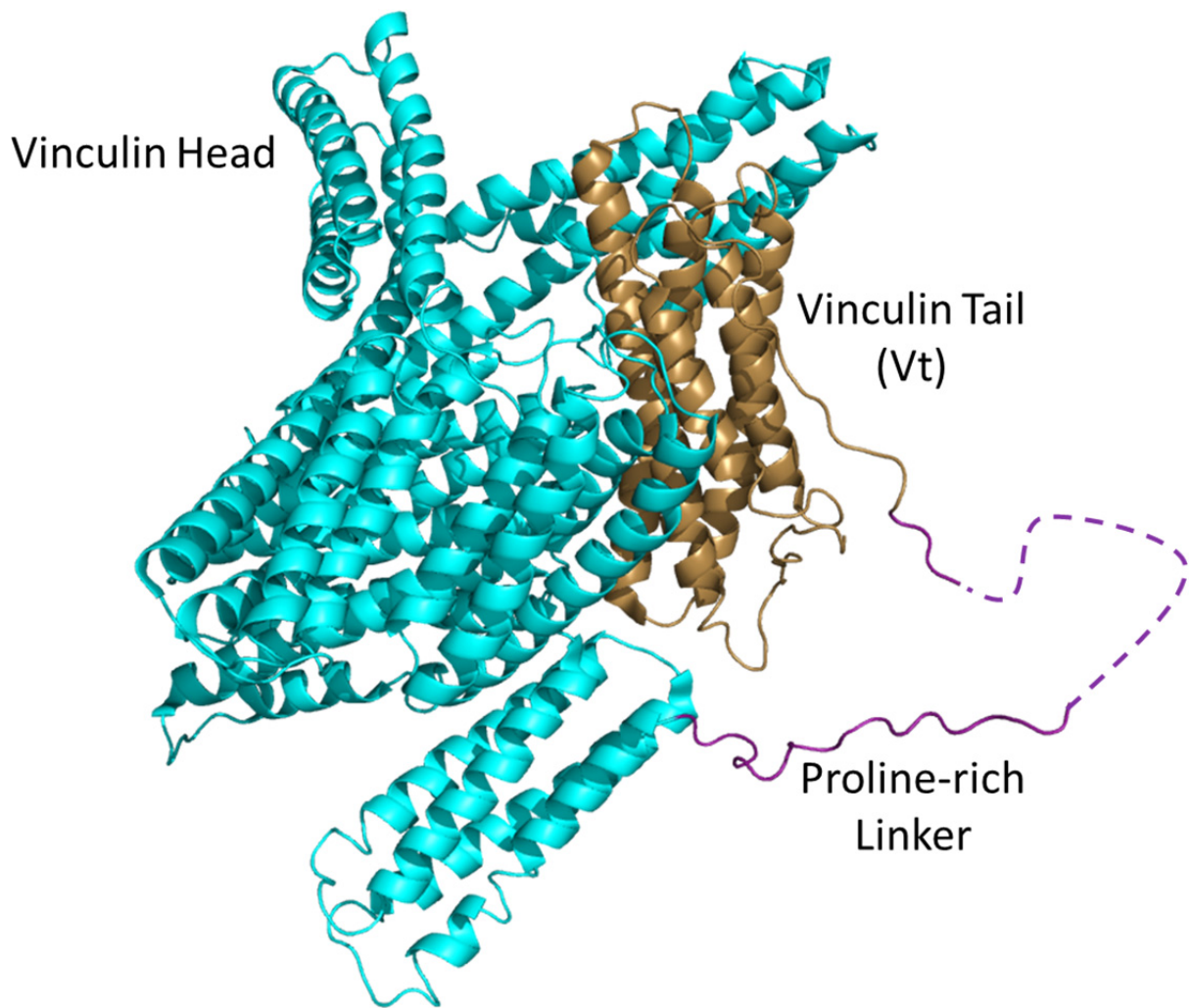


Figure 1.1. The structure of vinculin.

The crystal structure of chicken vinculin (PDB code 1ST6) is shown in a cartoon representation. The vinculin head domain is shown in cyan, the proline-rich linker in purple, and the vinculin tail domain (Vt) in sand.

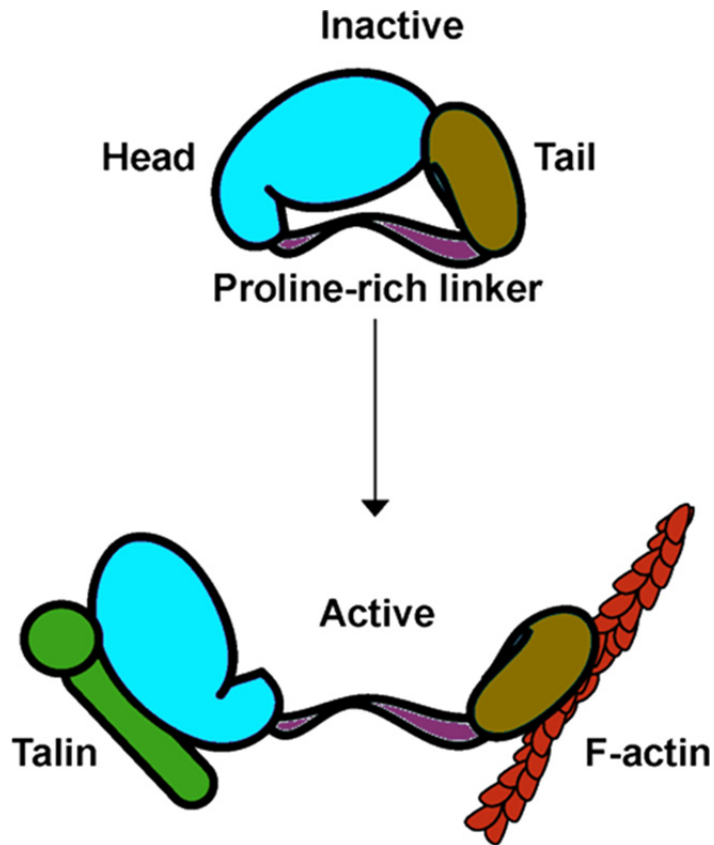


Figure 1.2. Diagram of vinculin activation

In the absence of ligands, vinculin exists in an autoinhibited conformation, mediated by a strong interaction between the head and tail domains. Activation of vinculin requires the presence of both a head ligand, such as talin, and a tail ligand, such as F-actin. When both ligands are available, the head domain and tail domain release their autoinhibitory interaction and bind to their respective ligands. This conformational change allows for binding of additional ligands on vinculin surfaces protected by the autoinhibited conformation.

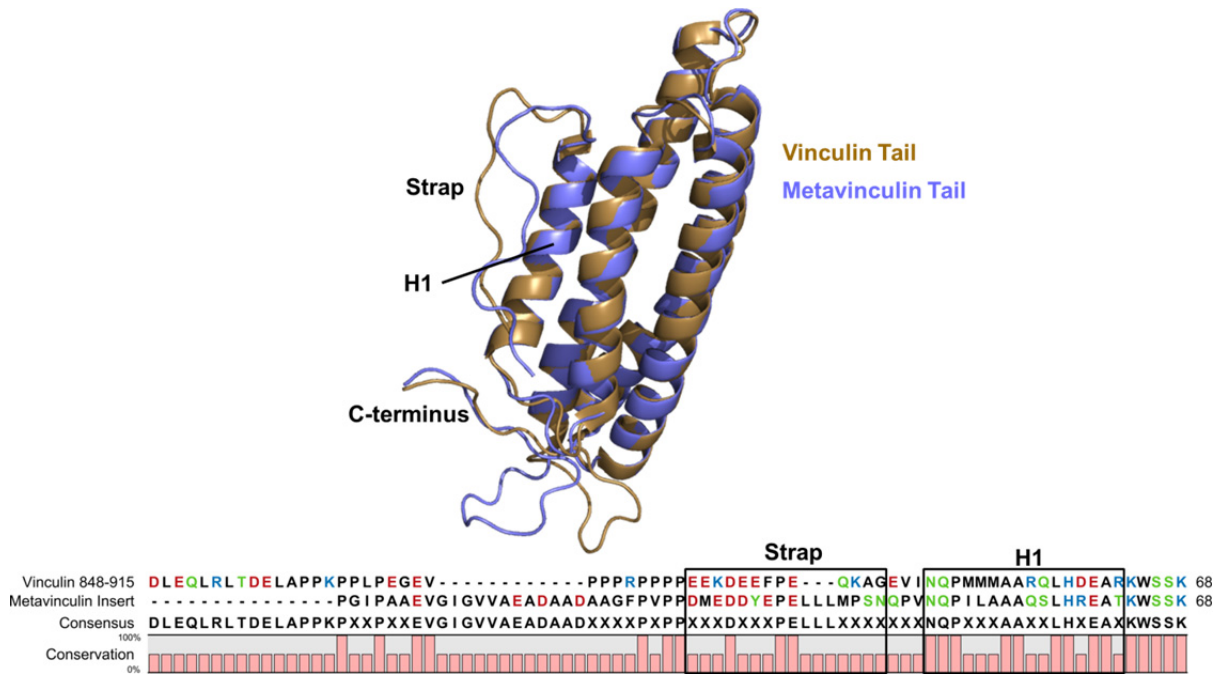


Figure 1.3. Comparison of vinculin and metavinculin tails.

The overlaid structures of human Vt and metavinculin tail domains (structures used are from PDB code 1RKE and Rangarajan *et al.* (65), respectively). Vinculin is shown in sand and metavinculin in lavender. The structures are very similar, especially in the helix bundle. Below is an alignment of the metavinculin 68-residue insert with vinculin residues 848 to 915. The second half of the insert shows high sequence similarity and homology to the vinculin sequence that forms the N-terminal strap and helix 1.

Chapter 2: Characterization of the Interaction Between the Vinculin Tail Domain and PIP₂-containing Membranes

Introduction

Vinculin tail (Vt) can bind to many different ligands, including acidic phospholipids (86), specifically phosphatidylinositol 4,5-bisphosphate (PIP₂) (43). This interaction may activate vinculin by disrupting the vinculin head:tail interaction (10, 32, 87). PIP₂ is an important signaling lipid for numerous cellular processes, including membrane ruffle formation (88), exocytosis (89), and phagocytosis (90). In the case of focal adhesions (FAs), PIP₂ is generated by phosphatidylinositol phosphate kinase type 1 gamma (PIP1 γ) (91), which is recruited to focal adhesions by talin (92, 93). Talin recruits vinculin, meaning that vinculin colocalizes with and PIP₂, which regulates the interaction of vinculin with talin (11, 94). PIP1 γ regulates FA dynamics (95, 96), is required for FA formation (97), and is thought to be involved in recruitment and activation of vinculin at FAs (96, 98). While much is known about the general role of PIP₂ at FAs, the consequences of the vinculin:PIP₂ interaction are much less understood, in part because of the lack of understanding for how Vt binds PIP₂. The interaction has been implicated in cell spreading, cell migration, FA turnover, and force transduction (41, 42, 99), though the vinculin constructs used in these studies may not be selective in their disruption of PIP₂-binding.

When the structure of Vt was first solved by X-ray crystallography, two surfaces on Vt were identified that contain a high concentration of basic residues: the basic ladder and the basic collar (**Figure 2.1**) (10). These regions were predicted to be potential binding sites for acidic phospholipids, but defining which site drives lipid binding has been quite difficult. Different labs have provided evidence for a loss in PIP₂ binding by mutating the basic collar

(2, 58, 99), the basic ladder (41), or deleting the C-terminus (41, 42, 58, 99, 100). Other groups have expressed peptide fragments of Vt and found that the basic ladder (3) or the C-terminus (101) are sufficient for PIP₂ binding.

However, the use of multiple mutations, deletions, and fragments of Vt likely alter its structure and perturb other Vt functions (43). Hence, their use in studying the vinculin:PIP₂ interaction in cells provides potentially misleading results. For example, the use of two separate vinculin variants with reported PIP₂ defects of comparable severity, vinculinK952Q/K956Q/R963Q/R966Q and vinculin R1060Q/K1061Q, resulted in two distinct cellular phenotypes; the double mutant disrupted the ability of the cells to exert force on their substrate by 50%, the quadruple mutant did not (42). These disparate and confusing results result largely from the lack of a good structural model for the interaction of Vt with PIP₂ and the use of poorly characterized PIP₂-defective vinculin variants. This problem is not unique to the interaction of vinculin with PIP₂, as similar approaches have been used to study the interaction of vinculin with actin (Chapter 3).

Recently, a crystal structure of a Vt mutant bound to a soluble, short-chain PIP₂ was solved by Chinthalapudi *et al.*, providing the first structural model for how PIP₂ binds to Vt (PDB file 4PR9) (40). In the crystal structure, Vt R1060A forms trimers upon associating with PIP₂. Two of the Vt molecules recognize the PIP₂ headgroup via residues in the basic collar whereas the third Vt molecule interacts with the PIP₂ headgroup through residues K944 and R945. To test their model, mutations were made to residues in the basic collar (Vt K1061Q) or the basic ladder (K944Q/R945Q), and were found to disrupt PIP₂-binding by lipid co-sedimentation. The authors posit that these mutations support their model for how Vt binds PIP₂ and that their model accurately depicts the oligomerization state of Vt upon binding to PIP₂. Vt has been shown, by crosslinking, to dimerize and trimerize in the presence of PIP₂- or phosphatidylserine (PS)-containing liposomes (10, 43, 102). However, while the crystal structure does provide possible models for Vt dimers and trimers linked by

PIP₂, these models do not explain how Vt would insert into a lipid bilayer (3). Additionally, vinculin-null mouse embryonic fibroblasts expressing vinculin showed phenotypes effects for two different vinculin constructs with weak PIP₂ binding. The K944Q/R945Q double mutation, as evaluated by fluorescent recovery after photobleaching (FRAP), prevents exchange of vinculin at FAs, while the K1061Q mutant only mildly disrupts exchange of vinculin at FAs. We applied biochemical and computation methods to this system to address these discrepancies.

Materials and Methods

Expression and purification of Vt

Vt constructs expression constructs were a gift from Susan Craig. The pET15b Vt expression vector encoded a His-tag followed by a thrombin cleavage site and then chicken Vt residues 884-1066. Vt was expressed by transforming BL21 *E. coli* with the vector, growing to an OD at 600 nm of 0.6, dropping the temperature to 18 C°, and inducing expression with 0.25 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). Cells were grown overnight, centrifuged, resuspended in lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 5 mM imidazole, 5 mM β-mercaptoethanol), and frozen until sonication. Vinculin tail protein was purified as previously described (43).

Lipid co-sedimentation assays

Vt binding to PIP₂ was evaluated by lipid co-sedimentation assays using small, unilamellar vesicles (SUVs) as reported (43, 103). SUVs were composed of 250 μg lipid mixture, with the reported PIP₂% and a 3:1:1 ratio of phosphatidylethanolamine to phosphatidylcholine to PS and/or PIP₂. The lipids (Avanti Polar Lipids, Alabaster, AL, USA) were resuspended by vortexing in the lipid co-sedimentation buffer (40 mM MES pH 6.0, 150 mM NaCl, and 2 mM dithiothreitol (DTT)) and subsequently extruded in a mini-extruder

(Avanti Polar Lipids) to produce the SUVs. Relative protein amounts were quantified using ImageJ (104).

Binding to PS was evaluated similarly, with the only difference being the ratio of lipids used. When assembling SUVs, the reported percentage of PS was used, and the remaining lipids were a 3:1 mixture of phosphatidylethanolamine to phosphatidylcholine.

Actin co-sedimentation assays

Actin co-sedimentation assays for both actin binding and actin crosslinking (bundling) properties of Vt were performed as previously reported (6, 103).

Mutagenesis of DNA constructs

Vt variants were generated using QuikChange site-directed mutagenesis (Stratagene) and sequences were verified by DNA sequencing (Genewiz).

Computational modeling

Modeling of the interaction of the headgroup of PIP₂ with Vt (residues 896-1055) was performed with MedusaDock (105, 106). MedusaDock utilizes a library of rotamers for the sidechains of Vt and a separate library for rotamers of the PIP₂ headgroup. Docking simulations are performed using these libraries, while the secondary structure remains fixed, and the results are ranked by MedusaScore. The lowest energy pose is reported.

Modeling of the interaction of the full PIP₂ molecule, 1-stearoyl-2-arachidonoyl-*sn*-glycero-3-phospho-(1'-myo-inositol-4',5'-bisphosphate), with Vt in the absence of a lipid bilayer was performed with discrete molecular dynamics (DMD) simulations (107-109). The initial docking pose was taken from the MedusaDock simulations. In these all-atom DMD simulations, Gō constraints were used to maintain intramolecular Cβ/Cα contacts between Vt residues during the simulation. These constraints were applied for all residues in Vt or only residues in alpha helices in different simulations. The Gō constraints limit the potential for Vt unfolding while the PIP₂ is allowed to sample the protein surface. This combination

allows for greater sampling of potential Vt:PIP₂ interactions while maintaining the structural integrity of Vt. The assumption that Vt retains its fold upon binding to PIP₂ is supported by the lack of large conformational changes in the helix bundle in the crystal structure of Vt bound to a short-chain PIP₂ (40).

Modeling of the interaction of PIP₂ within a lipid bilayer was performed using all-atom, explicit solvent molecular dynamics simulations. A lipid bilayer of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) with a single PIP₂ molecule was generated, and an initial pose of Vt (residues 896-1055) bound to PIP₂ using the model generated from MedusaDock. Simulations were done with GROMACS (110) using a CHARMM27 force field (111) and performed for 150 nanoseconds. A control experiment with a bilayer consisting entirely of POPC was also conducted.

Circular dichroism spectroscopy

Circular dichroism (CD) spectra were collected as reported (43). Spectra were collected on a Jasco J-815 CD Spectrometer at 25°C in 10 mM potassium phosphate, pH 7.5, 50 mM Na₂SO₄, and 1 mM DTT. For spectral scans, Vt was concentrated to 5 µM for 260-190 or 450 µM for 350-250 nm. For thermal melts, 5 µM Vt was used and ellipticity at 222 nm was monitored as the temperature was increased from 25 C° to 95 C° at a rate of 2 C°min⁻¹.

Results

Computational modeling predicts distinct roles for the basic collar and basic ladder

To develop a structural understanding for how PIP₂ is bound by Vt, modeling of the interaction of PIP₂ with Vt was performed in collaboration with the Dokholyan Lab at UNC Chapel Hill. The headgroup of PIP₂, D-inositol 4,5-bisphosphate, was docked to Vt residues 895-1065 using MedusaDock (105, 106). The N-terminal strap was omitted from Vt for docking. It is likely that the N-terminal strap is displaced when Vt binds to PIP₂ as removal of the N-terminal strap increases binding to PIP₂ (43), suggesting that this deletion is not

unreasonable. MedusaDock identified the basic collar, specifically residues R910, K915, K924, and R925, as the site on Vt with the highest affinity for the PIP₂ headgroup. R910 interacted primarily with the hydroxyl of C4, K915 with the hydroxyl of C6 (which would place it near the phosphoryl group on C1 in the full PIP₂ molecule), K924 with the hydroxyl of C5, and R925 with the phosphoryl group on C4 (**Figure 2.2**). This finding is in agreement with the general geometry of the basic collar compared to the basic ladder, in which the positive charges within the basic collar are clustered in a smaller surface area more compatible with headgroup coordination relative to the basic ladder.

Next, DMD simulations (107-109) were performed with a complete PIP₂ molecule, 1-stearoyl-2-arachidonoyl-*sn*-glycero-3-phospho-(1'-myo-inositol-4',5'-bisphosphate). Simulating the interaction with the entire PIP₂ molecule was the logical next step from the initial simulations with just the inositol headgroup. Simulations were performed using Gō constraints on Cb/Ca contacts (with the interaction square-well depth 0.5 ϵ , where ϵ is the DMD energy unit (108, 112)) for all residues in Vt (residues 896-1065) or Gō constraints only for residues in alpha-helices (using Vt constructs 896-1065 or 896-1059). After selecting 2000 snapshots with a binding energy less than -10 kcal/mol (105), the residues in contact with PIP₂ were tallied (**Figure 2.2**). In all cases, the contacts at the basic collar remained high. When using Gō constraints only for helical residues, some contacts were observed at the basic ladder, though contacts at the basic collar were more frequent.

While both MedusaDock and DMD simulations suggested that the basic collar was the primary determinant for PIP₂ headgroup recognition, these simulations were done only with the PIP₂ molecule or headgroup, not in the context of a lipid bilayer. To better mimic the physiological interaction at the membrane, all-atom, explicit solvent molecular dynamics simulations of Vt bound to PIP₂ in a 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) bilayer were conducted. The initial pose had Vt (residues 896-1055) bound to PIP₂ in an orientation that best matched the docking pose. The Vt helix bundle was oriented

roughly parallel to the lipid bilayer at the start of the simulation. However, by the end of the simulation, the side chains of K944, R945, K952, and K956, all in helix 3, show lipid interactions resulting in insertion in the lipid bilayer (**Figure 2.2**). Throughout the simulation, the basic collar maintained contacts with PIP₂. A control simulation was performed without PIP₂, and no specific interaction with the POPC membrane was observed. Results from these simulations suggest that, while the basic collar is responsible for PIP₂ recognition, the basic ladder is required for insertion into and full association with lipid bilayers.

A common Vt variant deficient in PIP₂-binding is a poor actin crosslinker

The vinculin variant, LD-CT (vinculin R1060Q/K1061Q), has been used in a number of studies as a lipid-binding deficient vinculin construct (41, 42, 58). While Ziegler *et al.* (58) observed no defects in actin binding or bundling for Vt LD-CT, Diez *et al.* reported that this construct may have “impaired actin-vinculin binding” that would explain the lower strain energy cells generate when expressing vinculin Vt LD-CT (42). To address this controversy, we performed actin co-sedimentation assays with Vt LD-CT. High-speed co-sedimentation assays revealed that Vt LD-CT has no observable actin-binding defect (**Figure 2.3**).

However, low-speed actin co-sedimentation assays show that Vt LD-CT is deficient in F-actin crosslinking (**Figure 2.3**). These results further highlight the importance of the C-terminus in actin crosslinking and the ability of vinculin to regulate mechanical force through actin crosslinking (6). This can explain the decreased strain energy seen by Diez *et al.* (42) as a consequence of the lost vinculin crosslinking function in vinculin LD-CT.

Mutation of residues in the basic collar impairs binding to PIP₂-containing liposomes and Vt dimerization

While previous work has shown that mutations at the basic collar or the basic ladder of Vt decrease lipid binding, our molecular dynamics simulations suggested that the key

residues for PIP₂ headgroup recognition are R910, K915, K924, and R925. The T6 construct (Vt T6) construct, reported by Cohen *et al.* (28), consists of a K924A/R925A double mutation. We measured the PIP₂-binding activity of Vt T6 and the quadruple mutant R910A/K915A/K924A/R925A (Vt BC-4A) using a PIP₂ co-sedimentation assay (43). While Vt T6 exhibited a modest decrease in binding to PIP₂, with roughly half as much Vt binding at 5% PIP₂ (**Figure 2.4**), Vt BC-4A showed an even greater drop in PIP₂ binding, with a 6-fold decrease in binding at 5% PIP₂. Fitting the curves to a one-site binding model resulted in an approximate K_D of 43 ± 12 μM for Vt, 191 ± 29 μM for Vt T6, and 551 ± 66 μM for Vt BC-4A. These findings suggest that the basic collar, specifically residues R910, K915, K924, and R925, plays a key role in PIP₂ binding.

To assess the viability of these variants as tools for study of a PIP₂-specific defect in cells, the basic collar Vt variants were tested for their ability to bind F-actin and to dimerize to bundle F-actin. Using an actin co-sedimentation assay, no significant change in binding to F-actin for any basic collar Vt variant was observed (**Figure 2.4**). These results are not surprising, given that the basic collar is separate from the actin-binding surface of Vt. However, mutation of the basic collar did result in a significant decrease in the ability of Vt to bundle F-actin filaments, as measured by a low-speed co-sedimentation assay (**Figure 2.4**). This decrease was not rescued by using the more conservative Vt variant, Vt BC-4Q (R910Q/K915Q/K924Q/R925Q) (**Figure 2.5**).

Mutation of residues in the basic ladder impairs binding to PIP₂-containing liposomes but not Vt dimerization

Since mutations to the basic collar disrupt the ability of Vt to bundle F-actin, vinculin variants at this site would be poor tools to study lipid-binding by vinculin in cells. Observed phenotypes could not be solely attributed to the loss of PIP₂ binding. Previous work has shown that mutation of basic residues within helix 3 of Vt can also disrupt binding to PIP₂-containing liposomes (40, 41), suggesting that these residues may be good targets for

disrupting PIP₂-binding. Three constructs were generated to test the importance of helix 3 in binding to PIP₂-containing liposomes: Vt K944A/R945A (BL-2A), Vt K944A/R945A/K952A (BL-3A), and Vt K944A/R945A/K952A/K956A (BL-4A). These constructs all show decreased binding to PIP₂-containing liposomes (**Figure 2.6**). Vt BL-4A exhibited the most severe defect, with an effective K_d 13-fold weaker than Vt WT. Vt BL-2A and Vt BL-3A have K_d values roughly 6.5-fold weaker than Vt WT.

Actin binding and crosslinking activities for these variants were evaluated to test the specificity of the PIP₂-binding defect. Vt BL-2A, Vt BL-3A, and Vt BL-4A do not have significantly different effective K_d values for binding actin (**Figure 2.6**). Additionally, slow speed actin co-sedimentation assays show that there is no significant difference in the ability of these variants to crosslink F-actin (**Figure 2.6**). These data, in conjunction with the lipid co-sedimentation data, support the use of these basic ladder variants in studying the biological function of the vinculin:PIP₂ interaction.

Mutations to the basic collar decrease Vt stability

To evaluate potential alterations in Vt structure by mutations to the basic collar and basic ladder, CD data were collected. Far-UV data show a slight increase in helical secondary structure for Vt BL-4A (**Figure 2.7**). This increase in helical character may result from the K944A and R945A mutations, which may extend helix 3 by incorporating some of the helix 2-helix 3 loop residues into helix 3. Near-UV CD data, which report on the packing of W912 with W1058 and act as a proxy for tertiary structure, show that Vt BC-4Q and Vt BL-4A retain the packing interaction, as both peaks at 283 and 290 nm are maintained. Their signatures are slightly different, especially for Vt BL-4A, though, suggesting that the packing of W912 and W1058 has been somewhat altered. Thermal stability of Vt BC-4A, Vt BC-4Q, and Vt BL-4A was measured by monitoring CD at 222 nm as temperature increased (**Figure 2.7**). After normalizing the data, subtracting buffer contributions, and fitting to a sigmoidal curve (**Figure 2.7**), the denaturation midpoint (T_m) was calculated (**Table 2.1**). Vt BC-4A

has a 10 C° decrease in T_m that is only partially rescued with the more conservative Vt BC-4Q. Vt BL-4A is slightly more stable than Vt WT, but only by 2.2 C°. These results suggest that the basic collar, in addition to mediating Vt dimerization and actin crosslinking activity, is important for Vt stability.

Mutation of residues in the basic ladder result in a larger defect in PS binding relative to mutation of residues in the basic collar

As mutations at both the basic collar and basic ladder impair Vt binding to PIP₂-containing liposomes, it is difficult to determine which site is responsible for PIP₂ specificity by lipid co-sedimentation. Co-sedimentation experiments conducted with increasing amounts of PIP₂ did not show a large difference in the ability of Vt BC-4Q or Vt BL-4A to bind PIP₂-containing liposomes (**Figure 2.8**). However, since our computational modeling and biochemical data suggest that the basic ladder is responsible for insertion into the lipid membrane and the basic collar provides PIP₂ headgroup specificity, but that both functions are important for lipid binding in our co-sedimentation assays, we tested the hypothesis that the basic ladder is critical for insertion by using lipid co-sedimentation assays with increasing concentrations of PS and no PIP₂. While both Vt BC-4Q and Vt BL-4A bound PS-containing liposomes weaker than Vt WT, Vt BC-4Q retained binding, with 15% of Vt BC-4Q bound when liposomes were 60% PS (**Figure 2.8**). Vt BL-4A, however, did not show significant binding, even at high levels of PS. These data suggest that the basic ladder residues K944, R948, K952, and K956 drive the non-specific interactions with negatively-charged lipid membranes. Because mutations at the top of the basic ladder impair lipid association but do not impair actin interactions, these variants are reasonable tools to study the effect of lipid binding by vinculin in cells.

Discussion

While it is known that vinculin binds PIP₂ with high specificity (43) and facilitates membrane association (3), the biological role of this interaction remains unclear. The study of the vinculin:PIP₂ interaction has been frustrated by a number of factors. The lack of a good structural model and seemingly contradictory biochemical data resulted in the use of vinculin variants with multiple ligand-binding defects, not solely PIP₂ (41, 42, 99, 113, 114). The use of these tools has resulted in publication of seemingly contradictory data, even within the same manuscript (40, 42)! The recent structure from Chinthalapudi *et al.* (PDB file 4PR9) provides a good start to developing a structural model of the interaction of vinculin with PIP₂, but it falls short of explaining how this interaction takes place at a biological membrane (40).

The biochemical data presented here are in agreement with those published by Chinthalapudi *et al.*, who observed that mutating K944 and R945 or mutating K1061 weaken PIP₂-binding activity by Vt (40). Both the basic collar and the basic ladder play important roles in binding to PIP₂-containing membranes. However, while Chinthalapudi *et al.* attribute the effects of mutating K944 and R945 to the presence of a second PIP₂-binding site for the PIP₂ headgroup on Vt that also assists in oligomerization, our results suggest that these residues interact with the lipid bilayer in a PIP₂-independent manner; the larger defect in binding to PS-containing liposomes and our computational model point to the role of these residues as critical for insertion into the plasma membrane. This interpretation of the biochemical data is further supported by previous work from Palmer *et al.* who found that, in solution, short-chain PIP₂ binds non-specifically to Vt (43), supporting the notion that the native interaction of Vt with PIP₂ requires the presence of a membrane. Because the structure generated by Chinthalapudi *et al.* was done in the absence of a membrane and with a R1060A mutation, some of the PIP₂ contacts observed may be non-specific or non-native.

The structural and biochemical data also explain the lower affinity of metavinculin tail for PIP₂ (66). Metavinculin has a threonine at position 978, which replaces R910 in Vt. The loss of one of the key residues for binding of PIP₂ at the basic collar explains why metavinculin still binds PIP₂, though weaker than Vt.

The data presented here also support our recent structural model of the Vt-F-actin interaction, which highlights the role of helices 4 and 5, not helix 3. Further, actin co-sedimentation assays reveal that mutation of the basic collar and basic ladder do not significantly affect actin binding. However, mutations at the basic collar do disrupt the ability of Vt to crosslink actin filaments into bundles (**Figures 2.3, 2.4, 2.5**). Even the use of conservative mutations to glutamine did not preserve Vt's crosslinking activity. The failure of Vt LD-CT to crosslink F-actin further highlights the importance of the C-terminus in F-actin bundling (6, 115). The weaker crosslinking activity of Vt BC-2A and the nearly absent crosslinking activity Vt BC-4A suggest that residues R910, K915, K924, and R925 are integral in forming the Vt dimer. The failure of Vt BC-4Q to improve crosslinking activity over Vt BC-4A indicates that the positive nature of the helix 1-helix 2 loop is likely required for Vt to dimerize and crosslink actin filaments. It also provides new data to help develop a model for the actin-induced Vt dimer.

These data provide new insights with which to develop better structural models for the vinculin:PIP₂ interaction and, ultimately, better tools to study the biological function of this interaction. There remain further challenges to an accurate structural model, including understanding the conformational changes that take place when Vt binds to PIP₂ (10) and the formation of higher order oligomers at membranes (10, 43, 66, 102). The Vt:PIP₂ structure by Chinthalapudi *et al.* identifies an oligomeric species that coordinates PIP₂, but this model is unclear regarding how vinculin inserts into the membrane (40). Similarly, it does not show any significant conformation change in Vt upon binding this short-chain PIP₂. While we favor our experimentally-derived computational model (**Figure 2.2**), additional

structural information derived from crystallography, NMR, or even cryo-electron microscopy of vinculin associated with PIP₂ in lipid bilayers will aid in further understanding vinculin's insertion into the membrane. Of the mutants described here, Vt BL-3A and Vt BL-4A are likely the best mutants to use in cells to study the biological role of the vinculin:PIP₂ interaction. They exhibit a strong PIP₂-binding defect, but, unlike the basic collar mutants, retain the ability to crosslink actin filaments. Cellular studies with these vinculin variants will be conducted to study the effects of the vinculin:PIP₂ interaction of vinculin activation, vinculin localization, FA development, and force transduction.

	T_m (C°)
WT	76.8 ± 0.1
BC-4A	66.5 ± 0.1
BC-4Q	73.8 ± 0.1
BL-4A	79.0 ± 0.2

Table 2.1. Denaturation midpoints of Vt variants as assessed by CD thermal melts.

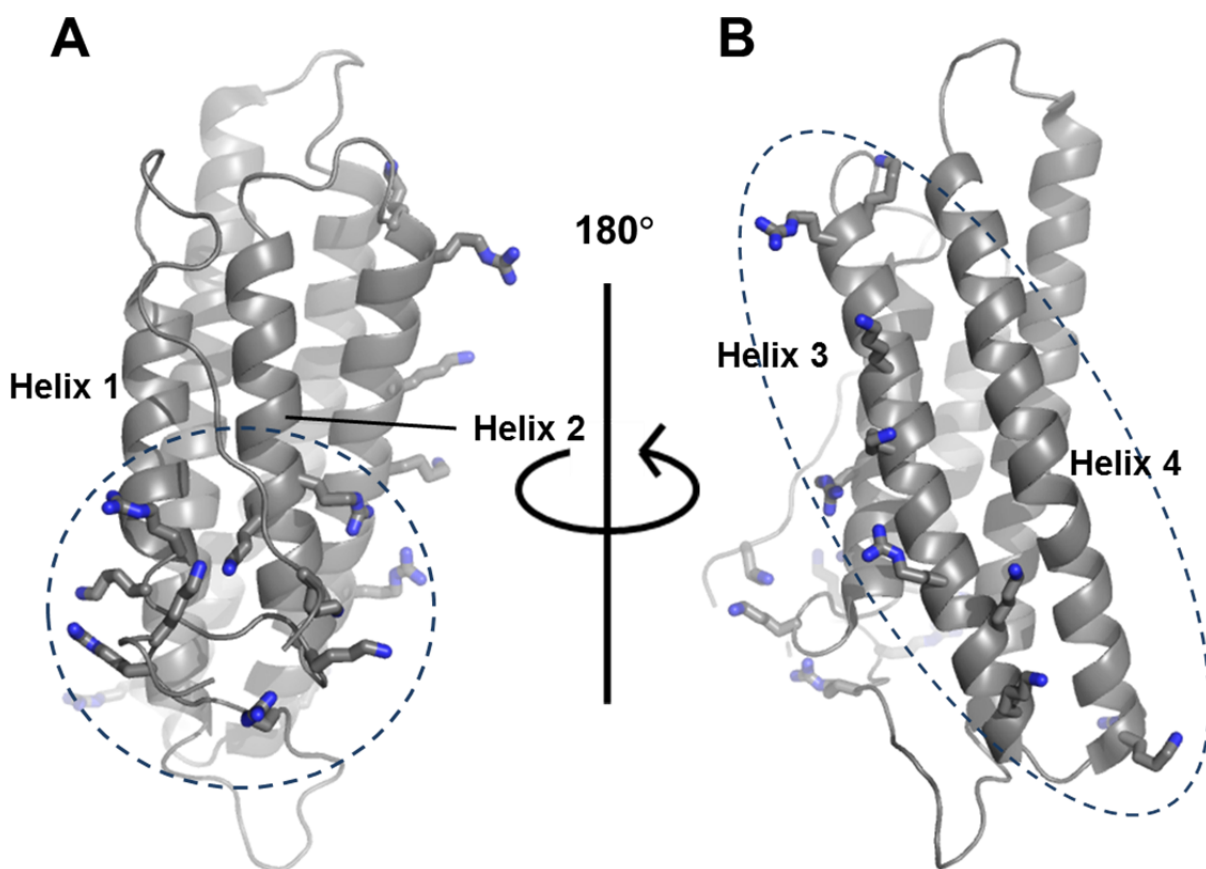


Figure 2.1. The basic collar and basic ladder of Vt.

Shown on Vt (PDB file 1ST6) are the basic collar (A) and basic ladder (B) residues. The basic collar is comprised of K881, R910, K911, K915, K924, R925, R1057, R1060, and K1061, which are found in the N-terminal strap, helix 1, the helix 1-helix 2 loop, helix 2, and the C-terminal hairpin. The basic ladder is comprised of K944, R945, K952, K956, R963, K966, K970, K975, and R976, which are found in helices 3 and 4. Various studies have claimed that the basic ladder (3), the basic collar (2, 99, 101), or both (40-42) are responsible for PIP₂-binding by vinculin.

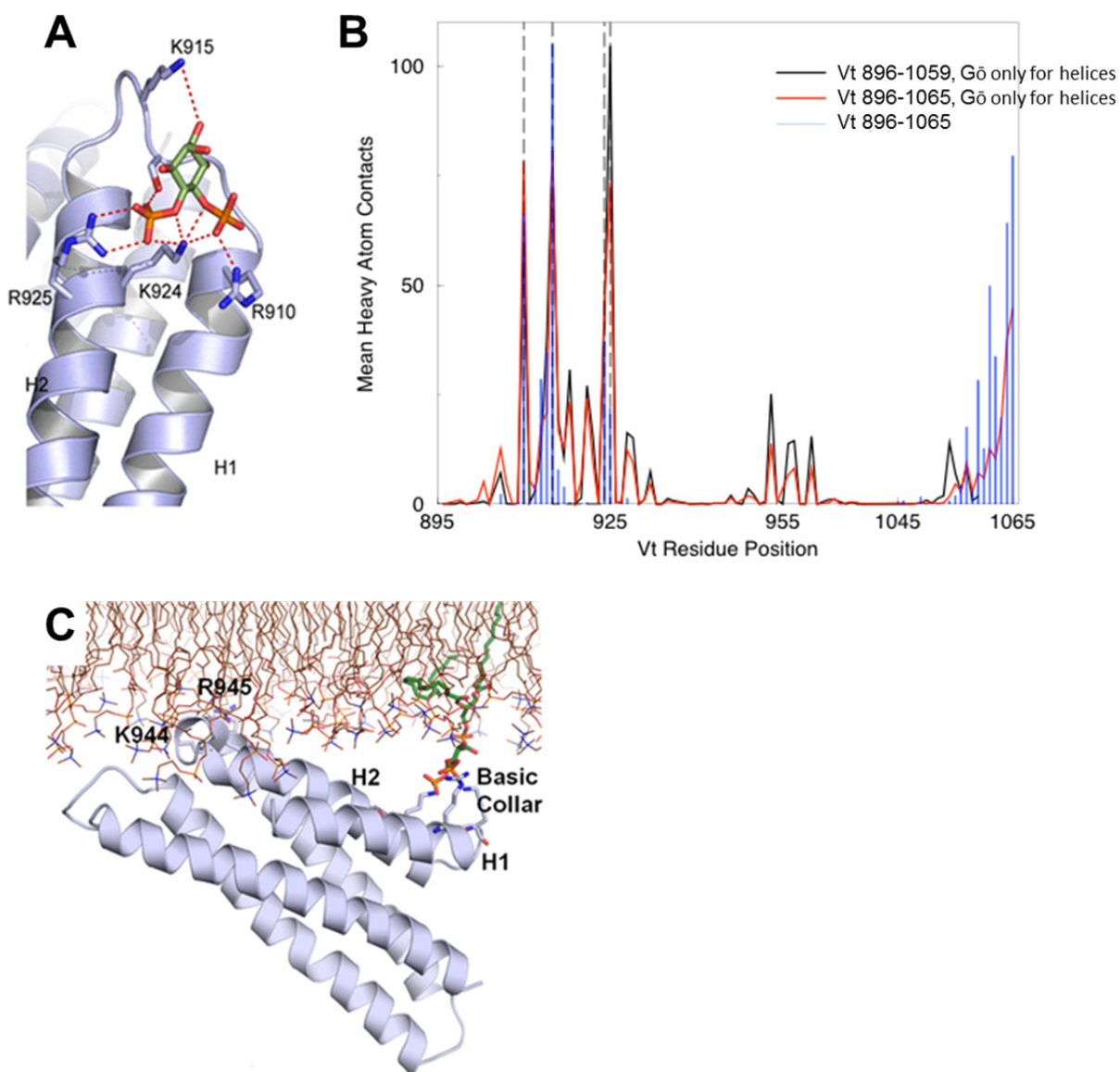


Figure 2.2. Modeling of the PIP₂:Vt interaction.

The results of MedusaDock (105, 106) (A), DMD (B), and GROMACS (C) simulations are shown. The MedusaDock simulations, using the PIP₂ headgroup, identified residues R910, K915, K924, and R925 as residues that mediate PIP₂ binding. These results were followed by DMD simulations with the full PIP₂ molecule (B). The average number of heavy atom contacts with PIP₂ is reported for each Vt residue in the three simulations. R910, K915, K924, and R925 are identified with vertical dashed lines. The basic collar shows the highest

amount of contact. The final pose of the GROMACS molecular dynamics simulation (C) shows PIP₂ (green) still bound to the basic collar and residues K944 and R945 inserting into the lipid bilayer.

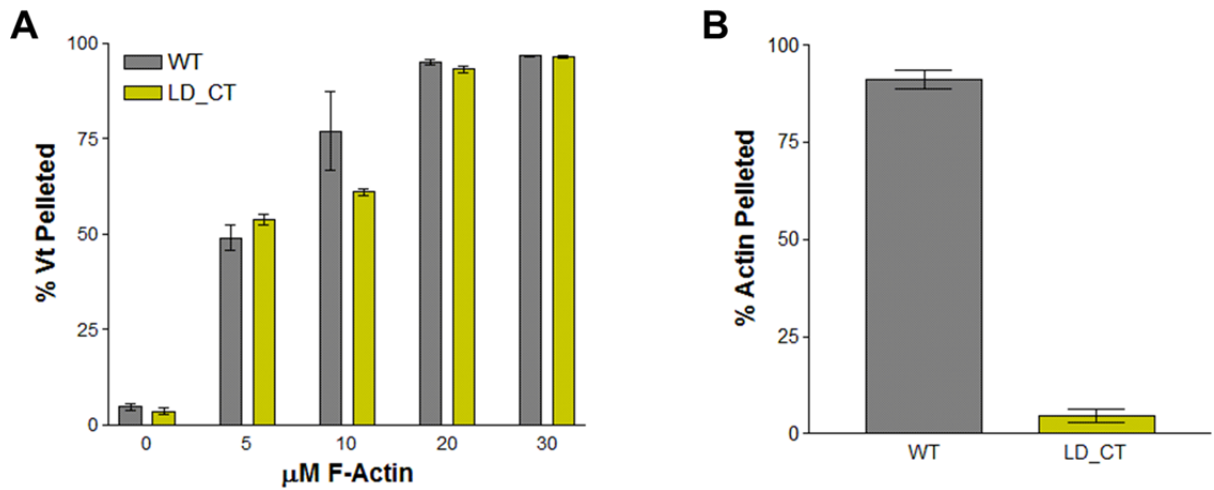


Figure 2.3. Actin-binding and crosslinking activity of Vt LD-CT (R1060Q/K1061Q).

Shown are the results from actin co-sedimentation assays evaluating the ability of Vt LD-CT to bind (A) and crosslink (B) F-actin. Vt LD-CT binds to F-actin well, but is significantly impaired in its ability to crosslink F-actin.

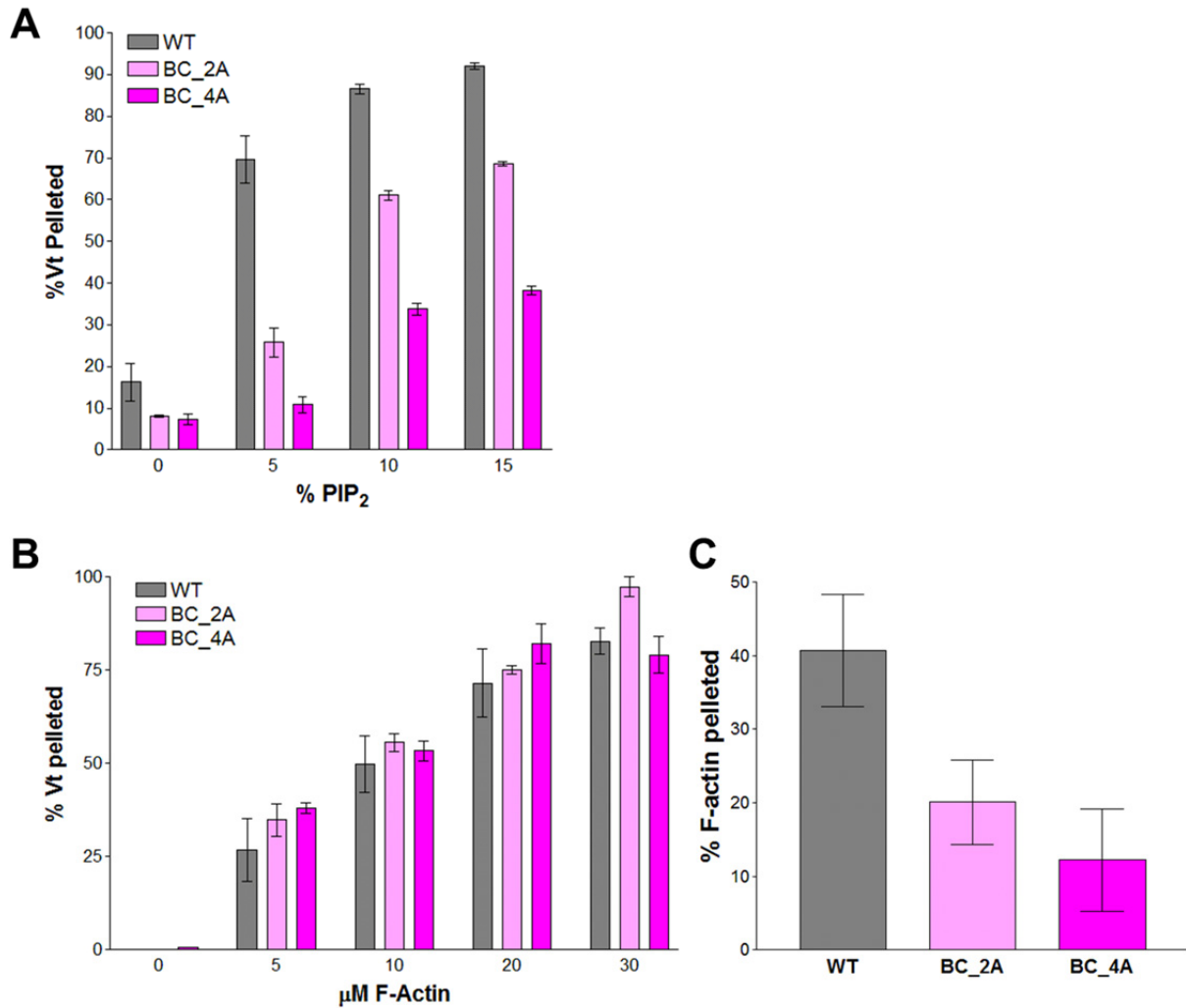


Figure 2.4. Mutations at the basic collar disrupt binding to PIP₂ and actin crosslinking functions.

Shown are results from PIP₂ co-sedimentation (A) and actin co-sedimentation (B, C) assays. Vt BC-2A and Vt BC-4A exhibit decreased binding to PIP₂-containing liposomes (A). Vt BC-4A has a more severe PIP₂-binding defect than Vt BC-2A, yet both retain F-actin binding function (B). Vt BC-2A and Vt BC-4A are impaired F-actin bundling (C), with Vt BC-4A having the largest defect.

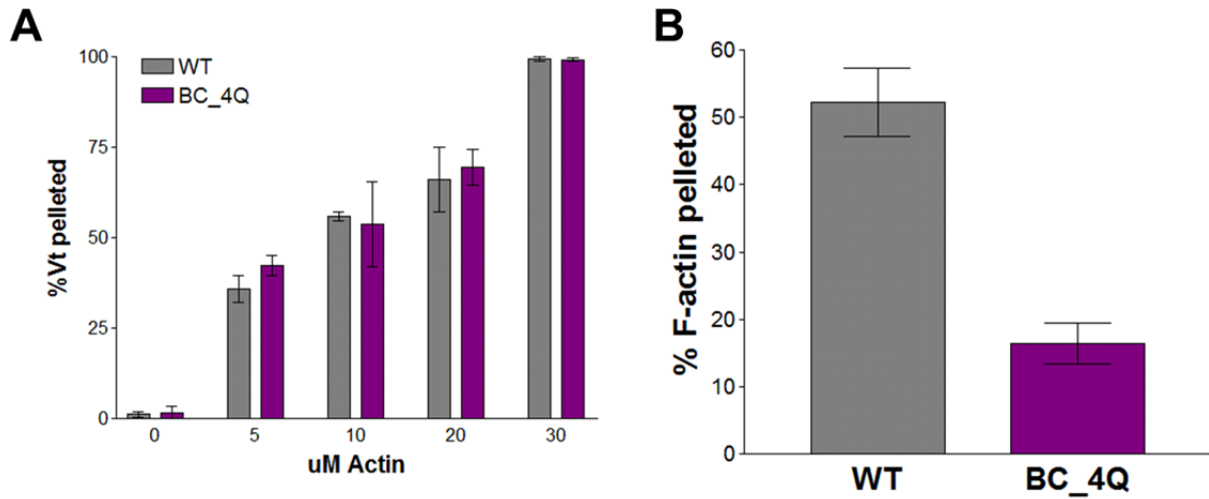


Figure 2.5. Mutation of basic collar residues to glutamine does not rescue F-actin crosslinking activity.

Shown are the results of actin co-sedimentation assays measuring the ability of Vt BC-4Q to bind (A) and bundle (B) F-actin. Vt BC-4Q contains more conservative mutations (basic to polar) than Vt BC-4A. Vt BC-4Q binds F-actin with a similar affinity to Vt WT. However, it, like Vt BC-4A, has a significant deficiency in actin crosslinking activity (C).

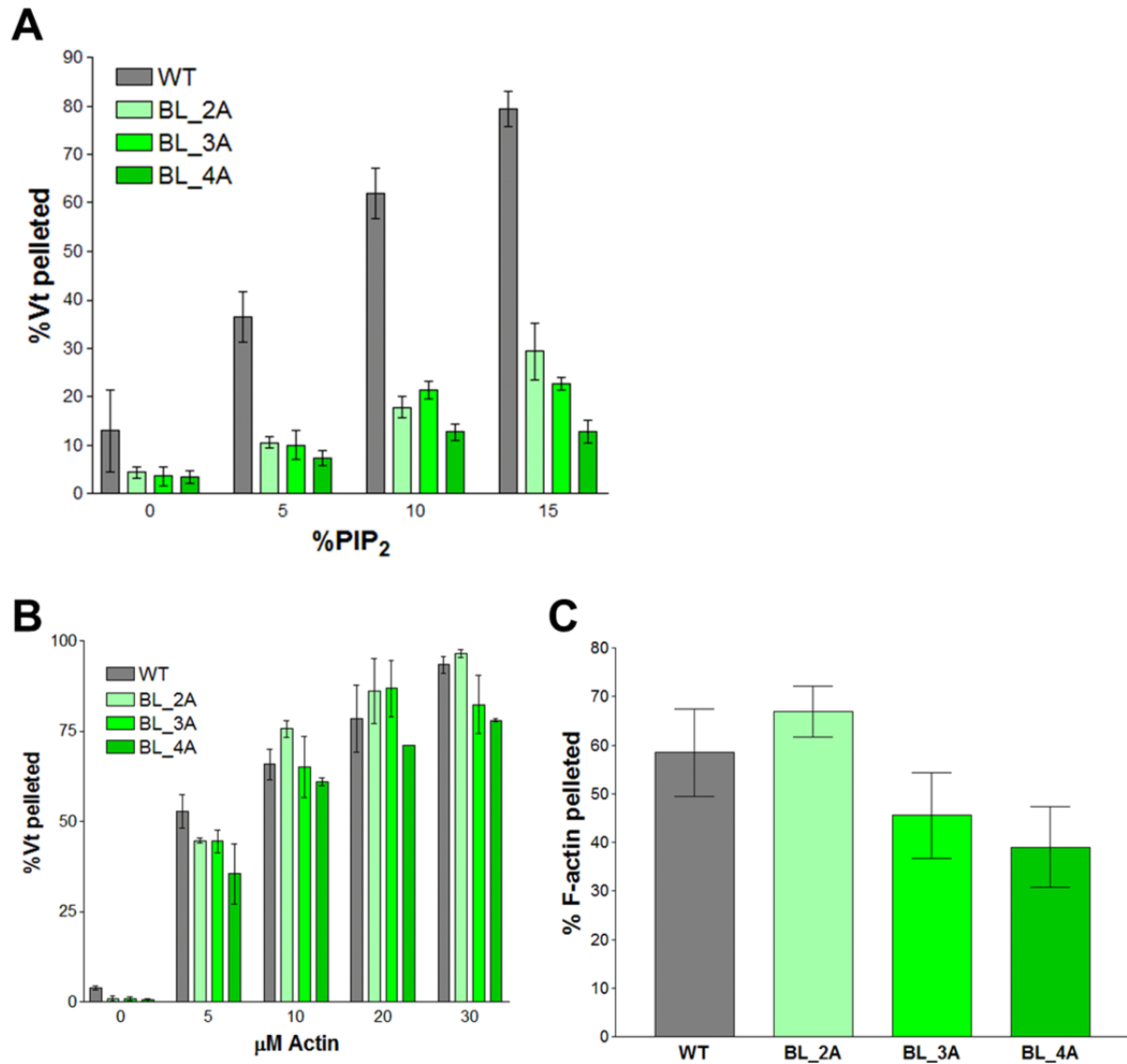


Figure 2.6. Mutations to the basic ladder disrupt PIP₂ binding, but do not significantly impair F-actin binding or crosslinking.

Shown are results from PIP₂ co-sedimentation (A) and actin co-sedimentation (B, C) assays. Vt BL-2A, Vt BL-3A, and Vt BL-4A exhibit decreased binding to PIP₂-containing liposomes (A), but retain F-actin binding (B) and crosslinking activity (C) in comparison to Vt WT. As with the basic collar mutations, mutating four residues in the basic ladder results in a stronger PIP₂-binding defect.

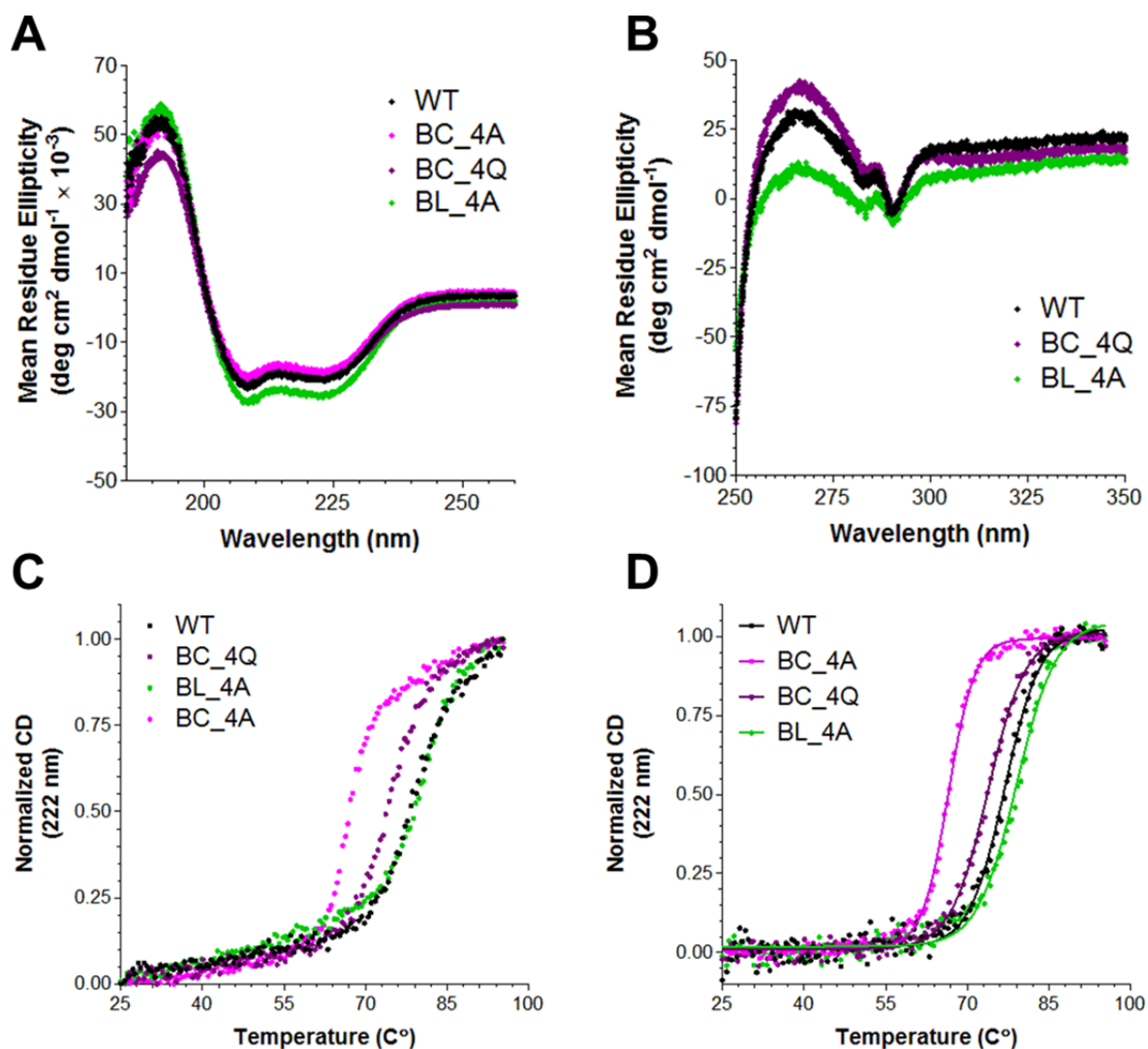


Figure 2.7. CD characterization of basic collar and basic ladder mutants.

Far-UV (A) and near-UV (B) spectra of Vt WT and Vt mutants. All constructs exhibit the characteristic signature of an α -helical protein (A). The near-UV spectra for Vt BL-4A suggests the packing interaction between W912 and W1058 has been altered, but not abolished, as characteristic peaks are present at 283 and 290 nm. The normalized thermal denaturation profiles for the Vt proteins (C) were adjusted by subtracting the buffer contributions and fit with a sigmoidal curve (D, Table 2.1). Mutations to the basic collar destabilize Vt, as evidenced by the decreased melting temperatures, though Vt BC-4Q, the more conservative variant, is more stable than Vt BC-4A.

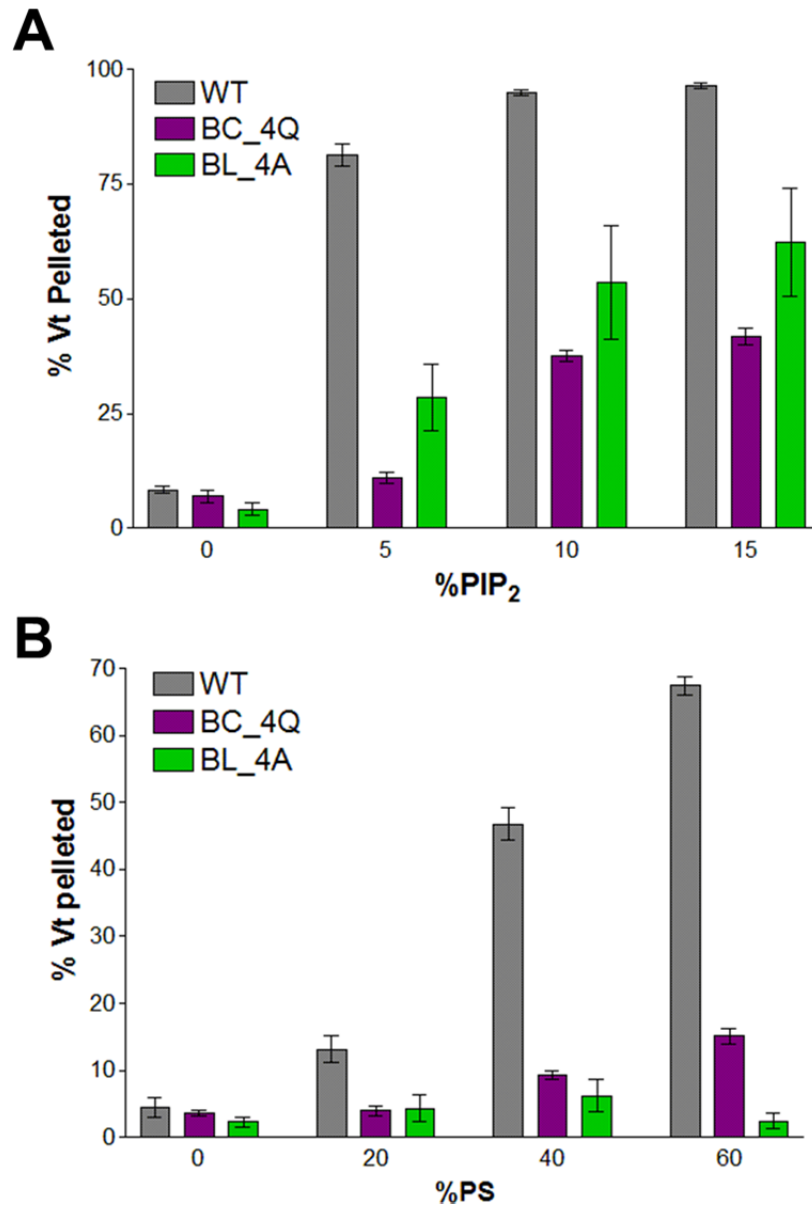


Figure 2.8. Comparative lipid co-sedimentation assays for Vt BC-4Q and Vt BL-4A.

Lipid-cosedimentation assays with PIP₂-containing (A) or PS-containing liposomes. Both Vt BC-4Q and Vt BL-4A exhibit a PIP₂-binding defect relative to Vt WT, as expected (A). Both variants also are defective in associating with PS-containing liposomes (B), though Vt BL-4A fares worse than Vt BC-4Q. In liposomes with 60% PS, only 2% of Vt BL-4A binds the liposomes, while 15% of Vt BC-4Q does.

Chapter 3: Structural, Biochemical, and Functional Characterization of the Actin-Binding Site on Vinculin Tail¹

Introduction

Focal adhesions (FAs) and adherens junctions (AJs) are essential structures in eukaryotic cells that link cells to their environment: the extracellular matrix (ECM) and other cells, respectively. These structures serve both structural and signaling purposes, both of which are mediated through interactions with the actin cytoskeleton. Proper signaling between these adhesions and the actin cytoskeleton is essential for cell migration, cell survival, and the biological generation of force as reviewed by Gardel *et al.* (116). To ensure efficient signaling, a large number of proteins present at FAs and AJs can bind to and/or remodel the actin cytoskeleton.

Vinculin is one such protein, though defining its interaction with actin has remained a difficult and somewhat contentious endeavor for scientists. The ability of vinculin bind F-actin was debated for years after vinculin's discovery until it was determined that the Vh:Vt interaction masks actin binding and must be disrupted for vinculin to bind actin (117). Subsequently, it has been shown that vinculin can cap actin filaments at the barbed end (inhibiting polymerization) (118), nucleate actin polymerization in low ionic strength buffer (119), and crosslink with other vinculin molecules to bundle F-actin filaments (120). While

¹A significant portion of this chapter previously appeared in an article in *Structure*. The original citation is as follows:

Thompson, P. M., Tolbert, C. E., Shen, K., Kota, P., Palmer, S. M., Plevock, K. M., Orlova, A., Galkin, V. E., Burridge, K., Egelman, E. H., Dokholyan, N. V., Superfine, R., and Campbell, S. L. (2014) "Identification of an actin binding surface on vinculin that mediates mechanical cell and focal adhesion properties," *Structure* 22, 697-706.

these interactions have been studied biochemically, the lack of structural models for the interactions has hindered our understanding of their roles in a cellular context.

Within the past eight years, a number of studies have been published attempting to describe, at greater resolution and with techniques other than random mutagenesis and deletions, the interaction of Vt with F-actin. The first such study was published by Janssen *et al.* and used mutagenesis data from Cohen *et al.* (28) and negative-stain electron microscopy (EM) to propose two patches on Vt that bind two longitudinally adjacent F-actin protomers within the filament (121). These two patches, termed the “upper” and “lower” sites, were evaluated by Golji and Mofrad with molecular dynamics simulations (122). They identified interactions between Vt helix 4 not identified by Janssen *et al.*, slightly shifting the perceived upper site. Additionally, they simulated Vt binding to the barbed end of the actin filament. Not long after, Thompson *et al.* used negative stain EM and new mutagenesis data to further support the role of helix 4, specifically its hydrophobic patch, in binding to F-actin (103). Their mutagenesis data provided two new vinculin mutants with the most significant actin-binding defects reported (I997A and V1001A). Another point mutant, R1049E, was identified as being a weak actin binder, especially in low ionic strength solutions (123). This mutant disrupts binding at the lower site. These mutants, when used in cells, show that vinculin binding to actin is required for cell reinforcement, cell-spreading (103), cell migration, and generation of traction forces (123). Vinculin does this by binding to F-actin, slowing its retrograde flow, and altering the size, density, and growth rate of focal adhesions (124).

No published structural model, however, perfectly accounts for all the data present. A significant part of this is that Vt undergoes a conformational change when it binds F-actin, a change that is not well-understood (120). The conformational changes are not large enough to see with negative stain EM due to the resolution limitations. Recent unpublished work out

of the Campbell and Alushin labs is beginning to identify the conformational changes using cryo-EM. Some of that work, as well as work published previously (103), is described below.

Materials and Methods

Protein expression and purification

The plasmids for expression of vinculin and Vt were generated by the Liddington lab (2, 10). The gene for full-length chicken vinculin (residues 1-1066) was placed in a pET15b vector. The gene for chicken Vt (residues 879-1066) was also placed into a pET15b vector for protein expression. All mutagenesis was performed using QuikChange site-directed mutagenesis (Stratagene, La Jolla, CA, USA) and the DNA sequence was verified by sequencing (Genewiz, RTP, NC, USA).

To express either protein, chemically competent BL21 DE3 *E. coli* with the Rosetta2 plasmid (EMD Millipore, Billerica, MA, USA) were transformed with the vector and plated on lysogeny broth (LB) plates containing ampicillin and chloramphenicol. A single colony was selected and grown overnight. The overnight growth was used to inoculate flasks with 1 L LB. Cells were grown at 37C° until OD reached 0.6. For full-length vinculin, expression was induced with 1 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) and expression proceeded for three hours. For Vt, expression was induced with 250 μ M IPTG and the incubator temperature was cut to 16-18 C°, and cells were harvested after 16-20 hours. To harvest, cells were pelleted via centrifugation and then resuspended in lysis buffer (20 mM Tris, 150 mM NaCl, 5 mM imidazole, and 2 mM β -mercaptoethanol (BME)). Cells were lysed either with sonication or homogenization and the lysate was subsequently clarified by centrifugation at 25,000 $\times$ g for 45 minutes. Both full-length vinculin and Vt were purified using Ni-NTA beads (Qiagen, Germantown, MD, USA). The lysate was applied to the beads, which were subsequently washed with lysis buffer. For full-length vinculin, the beads were then washed with lysis buffer with 50 mM imidazole, while with Vt, the beads were washed

with lysis buffer with 60 mM imidazole. The protein was eluted with lysis buffer with 500 mM imidazole.

After purification by nickel column, proteins were dialyzed into thrombin cleavage buffer (20 mM Tris pH 7.5, 500 mM NaCl, 2 mM CaCl_2 , and 5 mM BME) and their histidine tags cleaved by addition of thrombin (Sigma-Aldrich, St. Louis, MO, USA). Proteins were then further purified by ion exchange chromatography (Q-column for vinculin, S-column for Vt) and size exclusion chromatography (S-200 superdex for both vinculin and Vt).

IpaA peptide (Ac-NNIYKAAKDVTTSLSKVLKNIN-NH₂) was provided by both the UNC High-Throughput Synthesis and Array Facility (Chapel Hill, NC, USA) and LifeTein (Hillsborough, NJ, USA). The lyophilized peptide was resuspended in actin co-sedimentation buffer (10 mM Tris pH 7.5, 100 mM KCl, 2.5 mM MgCl_2 , and 2 mM dithiothreitol (DTT)) and centrifuged to remove aggregates and insoluble contaminants. Peptide concentration was determined by absorbance at 280 nm using an extinction coefficient of 1200 M⁻¹ cm⁻¹.

Actin co-sedimentation

Actin co-sedimentation assays were performed as previously described (6, 103). G-actin was polymerized by addition of 2X and 1X actin co-sedimentation buffer so that the final concentration of actin is 45.6 μM and the buffer is at 1X. Polymerization was carried out for 30 minutes. Ten μL of 100 μM Vt or vinculin was then mixed with enough actin and buffer to give the reported actin concentration in a total volume of 100 μL . When using the IpaA peptide, 10 μL of a 1 mM stock was added to the reaction for a final peptide concentration of 100 μM . The volume for these reactions was also 100 μL .

The actin and vinculin or Vt were incubated together at room temperature for 1 hour. The samples were then spun for 30 minutes at 150,000 $\times$ g. The supernatant was collected by pipetting and the pellet resuspended in an equal volume of actin co-sedimentation buffer. The supernatant and pellet fractions were run on a gel using SDS-PAGE, and band

intensities were compared using ImageJ (104). The percentage of protein pelleted was calculated by dividing the intensity of the pellet band by the sum of the intensities of the pellet and supernatant bands.

Circular dichroism

Circular dichroism (CD) spectra were collected on a Jasco J-815 CD Spectrometer (JASCO, Easton, MD, USA). CD data were collected in 10 mM potassium phosphate, pH 5.5 or pH 7.5, 50 mM Na₂SO₄, and 1 mM DTT at 25°C unless otherwise specified. For near-ultraviolet (UV) measurements, protein was concentrated to 450 µM, while far-UV measurements were collected on protein at 5 µM.

NMR spectroscopy

To make uniformly ¹⁵N-enriched Vt for NMR, expression was done in M9 media with ¹⁵NH₄Cl as the sole nitrogen source. ¹⁵N-Vt was purified in the same manner as Vt from LB, exchanged into NMR buffer (10 mM potassium phosphate, pH 5.5, 50 mM NaCl, 0.1% NaN₃, 2 mM DTT, and 10% D₂O), and concentrated to 50 µM. Two-dimensional heteronuclear single quantum coherence (HSQC) spectra were collected on a Varian INOVA 700 MHz spectrometer at 37°C. Spectra were processed with NMRPipe (125) and analyzed with NMRViewJ (126).

Generation of negative stain EM images, manual fit model, and discrete molecular dynamics (DMD) model

Collection of the EM images and generation of both structural models have been described by Thompson *et al.* (103).

Cell culture, cell-spreading assays, and microscopy

Maintenance of vinculin-null mouse embryonic fibroblasts (Vin ^{-/-} MEFs), real-time cell analyzer (RTCA) assays, and microscopy were performed as previously reported (103, 115).

Results

Mutation of the hydrophobic patch on Vt disrupts binding to F-actin

For eight years after the publication of the first structural model for the interaction of Vt with F-actin (121), there were no specific mutations identified in vinculin that disrupted the interaction. This hampered the ability of cell biologists to tease out the role of vinculin's interaction with F-actin without disrupting other interactions mediated by vinculin's tail domain. The best mutation identified for disrupting actin binding, at that point, was a triple mutant, D959A/E960A/R963A, that disrupted binding to actin by 20% relative to wild type Vt (28).

A former student in the Campbell lab, Sean Palmer, was trying to make mutations in Vt to disrupt its propensity to self-associate (Chapter 4) (127). This self-association is extremely weak (~330 μ M) and is mediated through a hydrophobic patch on Vt that resides on helices 4 and 5 (10, 127). Surprisingly, mutations at this interface disrupted binding to F-actin, even though these mutations were not near the actin-binding surfaces identified in the Janssen model. Specifically, Vt variants I997A and V1001A both significantly disrupted binding to F-actin (**Figure 3.1**) (103, 124), though I997A disrupts binding more. As expected, these mutations also impair Vt's ability to bundle actin filaments (**Figure 3.1**). Mutations to L928 and I948, residues in the upper site defined by Janssen *et al.*, fail to significantly disrupt F-actin binding (**Figure 3.1**).

While Vt separates from the vinculin head domain upon vinculin activation, it was important to test the efficacy of these mutations in the context of the full-length vinculin

protein. Without the addition of IpaA peptide, neither wild type vinculin nor the actin-binding deficient variants I997A and V1001A were able to bind, F-actin (**Figure 3.1**). This was expected, as vinculin must be activated by the presence of a head ligand to bind F-actin (117). The addition of an activating IpaA peptide, however, rescued actin binding in vinculin (103, 124). In I997A and V1001A vinculin, the addition of the IpaA peptide only partially rescued binding to F-actin (103, 124), showing that the actin-binding mutations have a similar effect in the context of the full-length protein.

To test the structural integrity of these Vt variants, CD spectra were collected from 185 nm to 350 nm. The far-UV spectra report on secondary structure, while the near-UV spectra report on packing of W912 and W1058. These spectra were collected at both pH 5.5 and pH 7.5, as the charge on the sidechain of H906 has been reported to have a small effect on Vt structure (127, 128). The CD spectra show a small change in the helicity of Vt I997A at pH 7.5, though the change is quite modest, suggesting that these mutations do not significantly impact the structure of Vt (**Figure 3.2**) (103). To further test the structural integrity, ¹H-¹⁵N HSQC NMR spectra were collected. For both Vt I997A and V1001A, only a few residues show changes in their chemical shift values, and these residues are near to the sites of mutation, suggesting that any change in Vt structure caused by these mutations is small and localized (**Figure 3.3**) (103). The NMR and CD data suggest that both I997A and V1001A mutations do not significantly affect Vt structure and that the actin-binding defect of these Vt variants are due to disruption of the actin-binding site.

Development of a new structural model for the interaction of Vt with F-actin

Using the information from these mutations, new EM images of Vt-decorated F-actin, and computational modeling, two possible structural models for the interaction of F-actin with Vt were posited, a manual fit model and a discrete molecular dynamics (DMD) model (**Figure 3.4**) (103). These models fit both the EM data and the new mutation results. Additionally, they support the role of the previously identified lower site in binding, but

reassign the upper site to helix 4, not helix 2. These models place the hydrophobic patch on helix 4 of Vt in a hydrophobic groove in the actin filament (103). This groove is a canonical binding site for actin-binding proteins (129).

While both the manual fit and DMD models place helix 4 in the hydrophobic groove in the actin filament, the orientation of Vt is quite different. In the manual fit model, the C-terminal end of helix 4 faces the barbed end of the actin filament, while in the DMD model, the C-terminal end of helix 4 faces the pointed end (**Figure 3.4**). In this manner, the DMD model has Vt rotated 180° relative to the manual fit model. Because of the resolution limitations from the negative stain EM images, both models fit well into the three-dimensional reconstruction and explain the effective disruption of actin binding by the I997A and V1001A mutations.

Two additional mutations, T993A and E1015A, were made to further test these models. In both models, T993 faces F-actin, though only in the DMD model does the sidechain of E1015 face F-actin (**Figure 3.4**). Both of these mutations decrease actin binding, though the effects are weaker than the I997A and V1001A mutations (**Figure 3.1**). These additional data support choosing the DMD model over the manual fit model, though they are not sufficient to eliminate consideration of the manual fit model.

Disruption of F-actin binding increases F-actin flow at focal adhesions, altering adhesion size and function

With the development of point mutations in vinculin with specific defects in F-actin binding, the tools were in place to evaluate the roles of actin-binding by vinculin in cell and FA biology without disrupting many of vinculin's other functions. Using vinculin-null mouse embryonic fibroblasts (Vin^{-/-} MEFs) expressing vinculin I997A, Thievensen *et al.* showed that vinculin acts as a clutch to mechanically link F-actin flow to FAs. This regulation of actin flow is coupled to FA growth, suggesting that, by regulating actin flow, vinculin helps regulate the growth of FAs.

To further examine the role of actin-binding, Thompson *et al.* expressed vinculin I997A and vinculin V1001A in Vin^{-/-} MEFs. MEFs expressing vinculin I997A or vinculin V1001A had slightly fewer, but larger FAs, similar to what Thievessen *et al.* observed (**Figure 3.5**) (103, 124). To test the ability of these cells to respond appropriately to external forces, three dimensional force microscopy (130) was used. MEFs expressing the various vinculin constructs were plated and then magnetic beads coated in fibronectin (FN) were added. After time passed to allow for FAs to develop between the MEFs and the beads, successive pulses of magnetic force were applied. MEFs expressing wild type vinculin underwent a strengthening of their focal adhesions, reinforcement, which decreased the movement of beads upon successive pulses. However, MEFs expressing vinculin I997A or vinculin V1001A failed to reinforce (**Figure 3.6**) (103).

Vinculin has also been implicated as having a role in cell spreading, though the role of actin-binding in this function is unknown (4). Vin^{-/-} MEFs and Vin^{-/-} MEFs expressing vinculin, vinculin I997A, and vinculin V1001A were allowed to spread on FN and monitored with the xCELLigence real-time cell analyzer system (131). Vin^{-/-} MEFs exhibited a spreading defect, as evident by the lower cell index readout, which was only partially rescued by vinculin I997A or vinculin V1001A (**Figure 3.7**) (103). This suggests that, while actin-binding by vinculin plays a role in cell-spreading, it is not the only vinculin function involved in regulation of cell spreading.

Cryo-EM modeling reveals details of Vt conformational change

Vt undergoes a conformational change when it binds to F-actin, though the structural details of the conformational change are relatively unknown (10, 120). Bakolitsa *et al.* showed that actin-binding by Vt leads to an increase in susceptibility to proteolytic cleavage at A901 and S913, residues that start and end helix 1. Because of the lack of structural information, interpreting structural and biochemical data regarding the Vt:F-actin interaction has been difficult.

Collaboration with the Alushin Lab at the National Heart, Lung, and Blood Institute was initiated to address some of these concerns. Using cryo-EM and Vt $\Delta C5$, a Vt variant defective in actin bundling (6), an 8.5 Å structural model for Vt bound to F-actin was developed (**Figure 3.8**) (132). This model shows two key conformational changes that take place upon actin binding: the straightening of helix 4, which is kinked by the presence of P989, and the displacement of helix 1 (**Figure 3.9**). Whether helix 1 unfolds or remains a helix and whether it has any interactions with F-actin is unclear, though there is some evidence that it may bind to the barbed-end actin protomer (unpublished). However, it is clear that binding to F-actin converts Vt structure from a five-helix bundle to a four-helix bundle.

While the 8.5 Å resolution of the cryo-EM model limits the detail with which conformational changes in Vt can be described, it does provide insight to the mode of actin binding. In comparing the cryo-EM model to the published models (103, 121, 122), it is clear that, while none of the previous models could predict the Vt conformational changes, the DMD model published by Thompson *et al.* comes the closest to predicting the actin-binding surface on Vt (**Figure 3.8**) (103). Though the resolution limitation precludes the identification of specific contacts made by sidechains, those closest to F-actin are more likely to interact. Thus, to further test the validity of the cryo-EM model, two Vt residues that had not been mutated, R987 and M1022, previously were mutated to alanine. Actin co-sedimentation assays show that these mutants disrupt binding to F-actin (**Figure 3.10**). These mutations are not as severe in their binding defect as I997A or V1001A, but R987 and M1022 are located further from the center of the upper actin-binding site on Vt.

Discussion

For a protein like vinculin with many different functions, determining the biological role of any one particular function presents a significant challenge, as special care must be taken to target only the desired function. The lack of a good structural model for vinculin

bound to F-actin has long hampered a better understanding of the biological role of this interaction. This has been further complicated by the conformational change that vinculin undergoes upon binding to F-actin and by the intrinsic difficulties F-actin poses to structural biologists.

Using some fortuitous mutagenesis results, a combination of EM, modeling, and biochemical techniques, the highest-resolution model for the interaction of vinculin with F-actin has been developed. This model confirms that the upper F-actin binding site on Vt is localized to the hydrophobic patch on helices 4 and 5 (103). Additionally, the Vt helix bundle loses helix 1, becoming a four-helix bundle upon binding to F-actin, explaining the limited proteolysis data suggesting an increase in flexibility around helix 1 (10). The predictive power of this structural model in disrupting actin binding further supports its validity.

The development of these structural models has allowed for rational use of specific point mutations in cells to determine the biological role of actin binding by vinculin. Actin-binding by vinculin regulates FA growth and maturation, affecting both cell spreading and mechanotransduction in mouse fibroblasts. Further experiments using vinculin mutants selectively deficient in F-actin binding showed that, by binding to actin, vinculin serves as a mechanical clutch at FAs to regulate retrograde actin flow, which is an important factor regulating FA development (124).

With the tools now in place, future studies should be able to determine the role of actin binding by vinculin at adherens junctions and in cellular migration. Additionally, the role of actin binding by metavinculin can now also be evaluated using the tools reported here. As vinculin's role in cancer becomes more defined, the role of actin binding by vinculin in both tumor growth and in metastasis will be of interest. If actin binding by vinculin is important for cancer progression, perhaps the Vt conformational change can be targeted as a therapeutic.

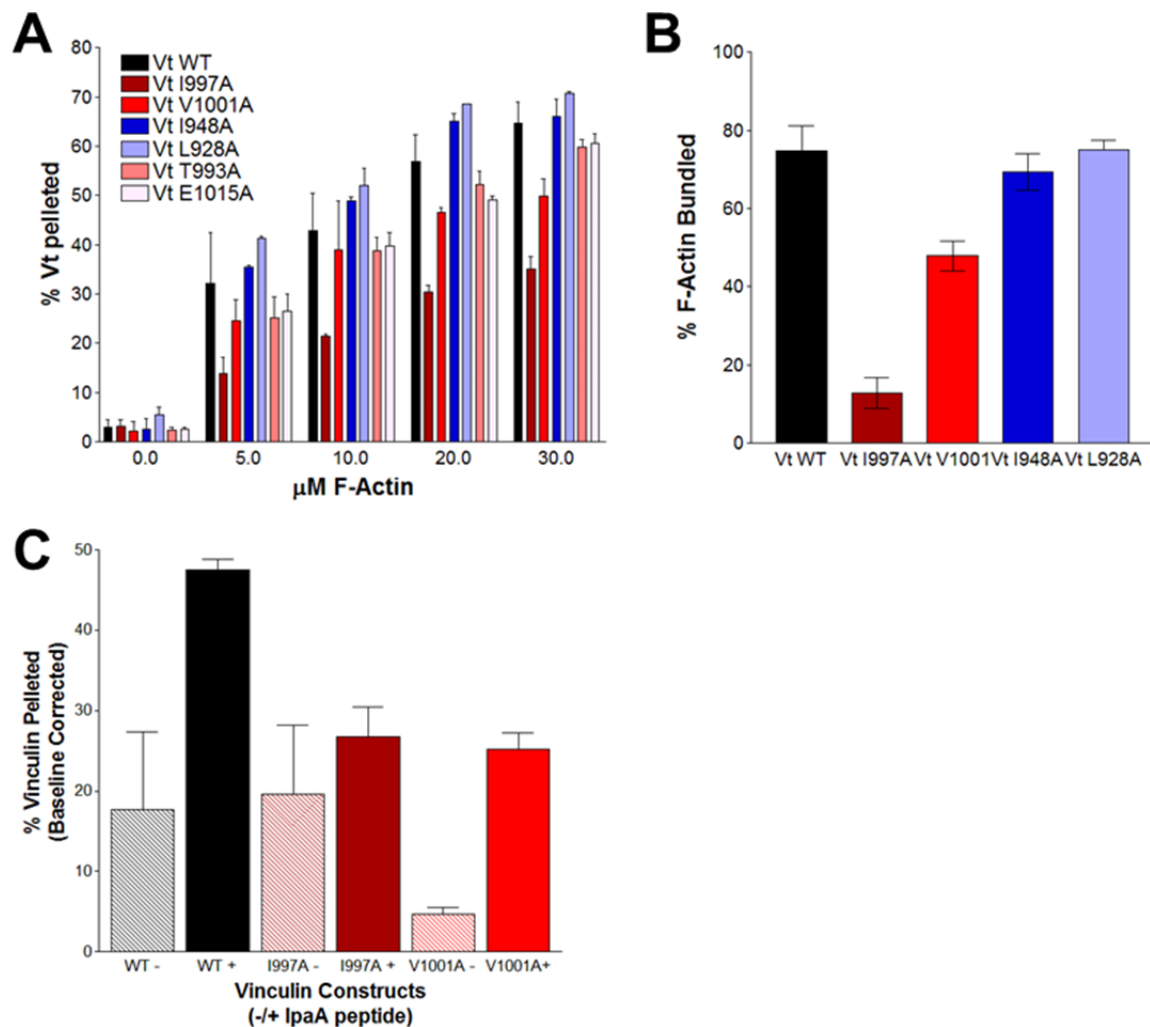


Figure 3.1. Mutations at and near I997 and V1001 disrupt F-actin binding.

Shown are actin results from actin co-sedimentation assays. Panel A is an actin binding co-sedimentation, showing that I997 and V1001 are important residues for Vt binding to F-actin. I948 and L928, residues at the binding surface for a different model (121), are not involved in binding to F-actin. Panel B shows that actin-binding defects result in actin-bundling defects. Panel C shows that the defects are not a consequence of working with just Vt, but that full-length vinculin with these mutations has an actin-binding defect.

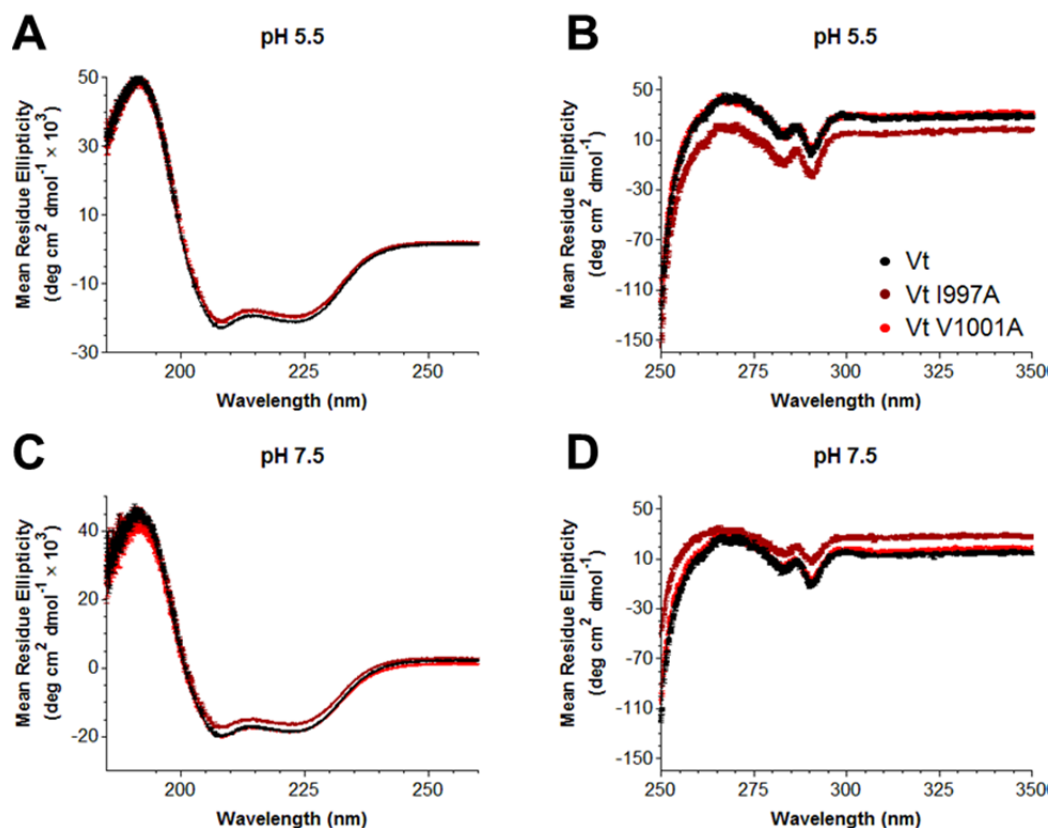


Figure 3.2. CD spectra of Vt variants.

The CD spectra for Vt, Vt I997A, and Vt V1001A are shown for far-UV wavelengths (A, C) and near-UV wavelengths (B, D) at pH 5.5 (A, B) and 7.5 (C, D). The spectra are very similar, except for Vt I997A. The near-UV spectra for Vt I997A appear to be shifted, even though they retain the peaks at 283 and 290 nm associated with the packing of W912 and W1058 in Vt.

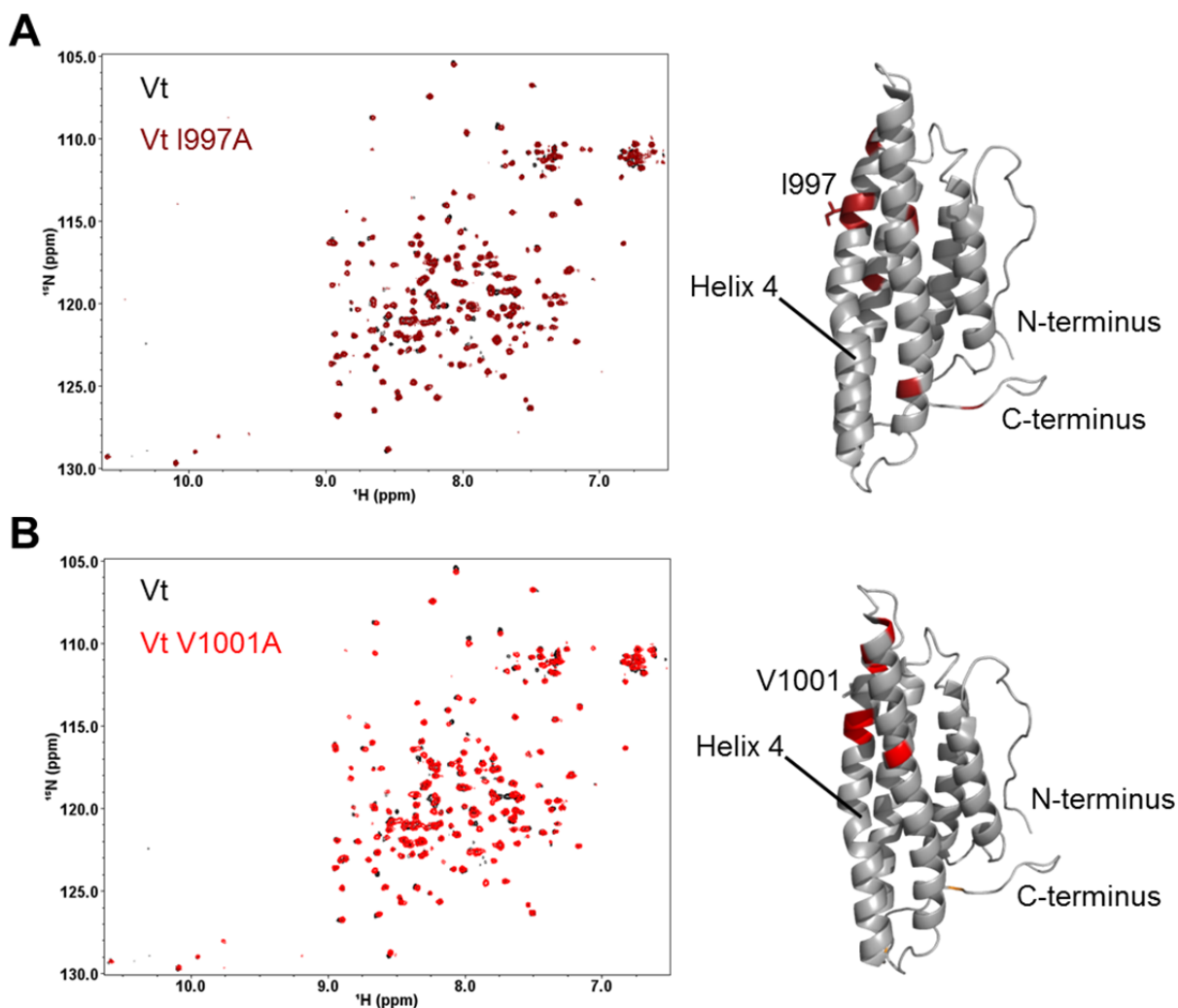


Figure 3.3. ^1H - ^{15}N HSQC spectra of Vt, Vt I997A, and Vt V1001A.

Shown are NMR spectral overlays of Vt I997A (A) and Vt V1001A (B) over Vt. The majority of peaks do not shift, and those that do are mostly near the site of mutation. The mutated residues are labeled and shown as sticks on Vt (PDB file 1ST6). Residues whose peaks shift are colored red, and those that broaden are colored orange.

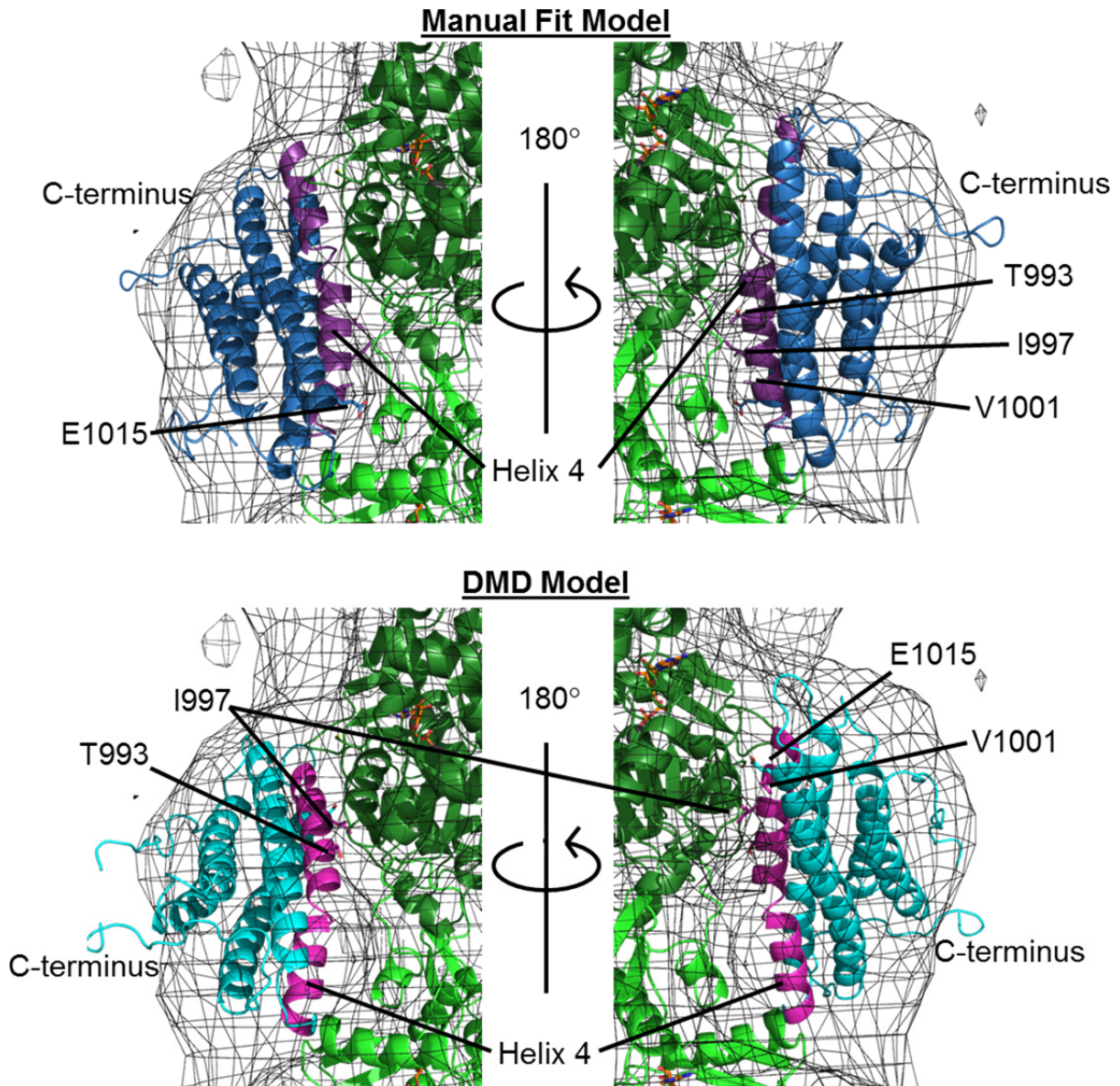


Figure 3.4. The manual fit and DMD models for the binding of Vt to F-actin.

Shown are the manual fit (above) and DMD (below) models for Vt bound to F-actin. F-actin is in green, with the pointed end protomer in dark green and the barbed end protomer is in light green. Vt is in a blue shade, with helix 4 in purple (manual fit) or pink (DMD). Residues T993, I997, V1001, and E1015 are shown as sticks. These residues, when mutated to alanine, weaken the binding affinity of Vt for F-actin. The two models both have helix 4 facing the actin filament; however, the manual fit model has the C-terminus closer to the pointed end and the DMD model has the C-terminus closer to the barbed end.

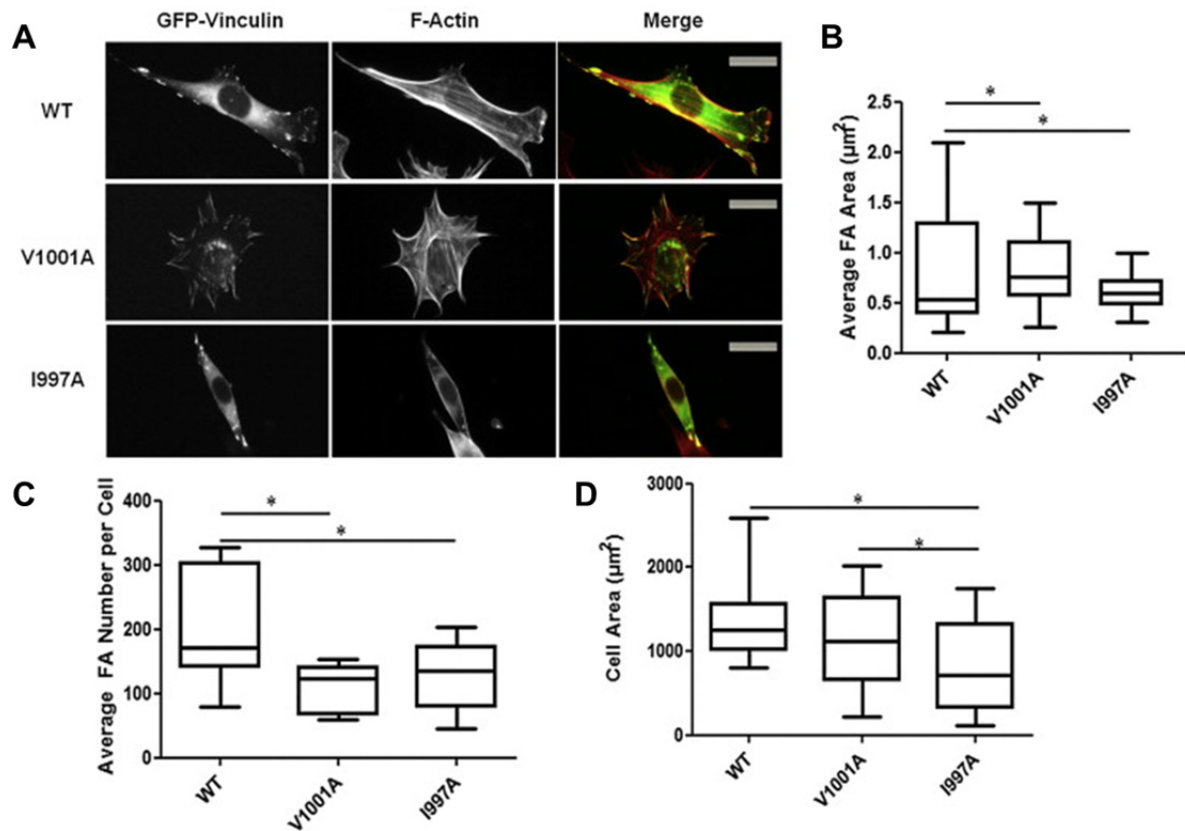


Figure 3.5. Loss of actin-binding function in vinculin results in fewer, but larger, FAs.

Panel A shows representative images of Vin $-/-$ MEFs expressing wild type (WT), V1001A, or I997A GFP-tagged vinculin. The vinculin mutants localize normally to focal adhesions. The scale bar is 25 μm . Panels B, C, and D are box plots that show the average size of FAs, average number of FAs, and the cell area, respectively, for Vin $-/-$ MEFs expressing these vinculin constructs. Cells expressing vinculin V1001A or vinculin I997A have significantly fewer, but larger FAs. Cells expressing vinculin I997A are significantly smaller. This may be due to a mild PIP₂-binding defect or the increased severity of the actin-binding defect relative to vinculin V1001A. * represents $p < 0.05$. Figure adapted with permission from Thompson *et al.* (103).

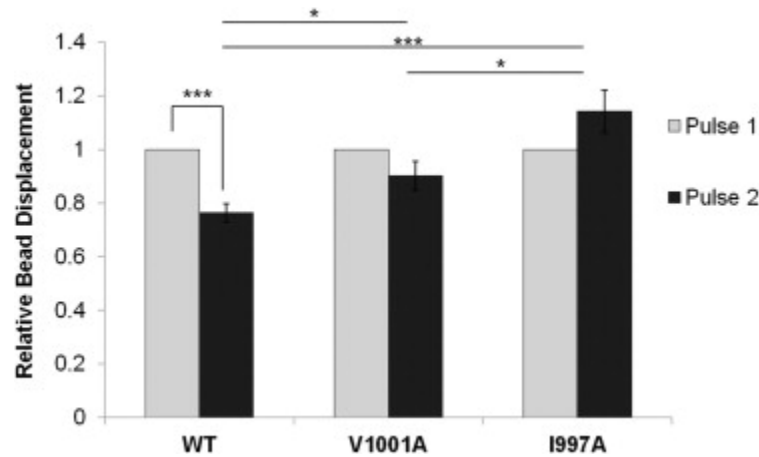


Figure 3.6. Actin-binding by vinculin is required for reinforcement at FAs.

Vin ^{-/-} MEFs expressing wild type vinculin (WT), vinculin V1001A, or vinculin I997A were incubated with magnetic beads coated in fibronectin. Pulses of force were then applied to the beads and the bead displacement measured. The normal cellular response is a stiffening, resulting in a smaller displacement of the bead upon the second pulse. MEFs expressing vinculin constructs with an actin-binding defect are not able to reinforce. The greater defect for MEFs expressing vinculin I997A could be from the stronger actin-binding defect or the slight PIP₂-binding defect. * represents $p < 0.05$ and *** represents $p < 0.001$. Figure adapted with permission from Thompson *et al.* (103).

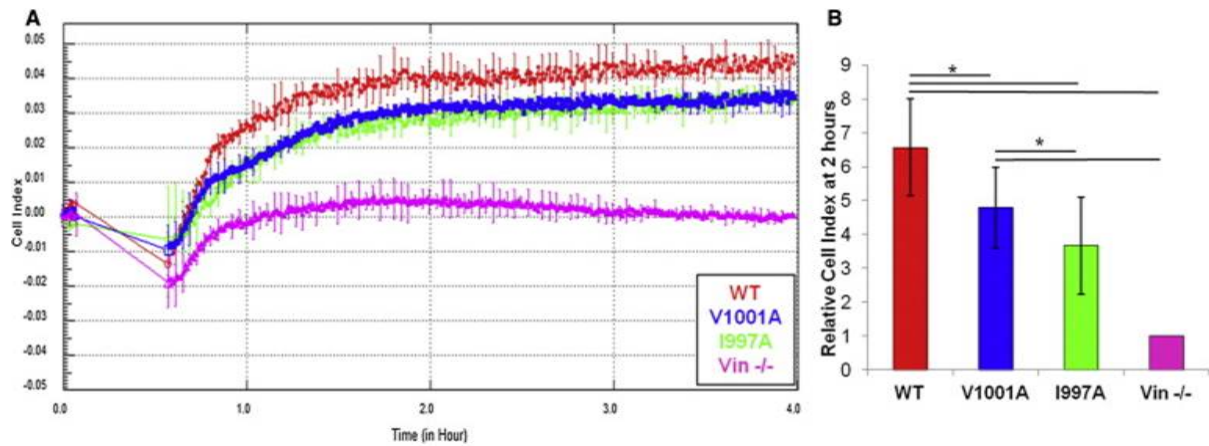


Figure 3.7. Cell spreading of Vin $-/-$ MEFs expressing various vinculin constructs.

Panel A is the raw data from the RTCA experiment. Data were collected for Vin $-/-$ MEFs expressing wild type vinculin (WT, red), vinculin V1001A (blue), vinculin I997A (green), or no vinculin (magenta). Vinculin mutants with actin-binding defects only partially rescue the poor spreading phenotype in the vinculin null cells. Panel B shows the relative cell index at the 2 hour timepoint. * represents $p < 0.05$. Figure adapted with permission from Thompson *et al.* (103).

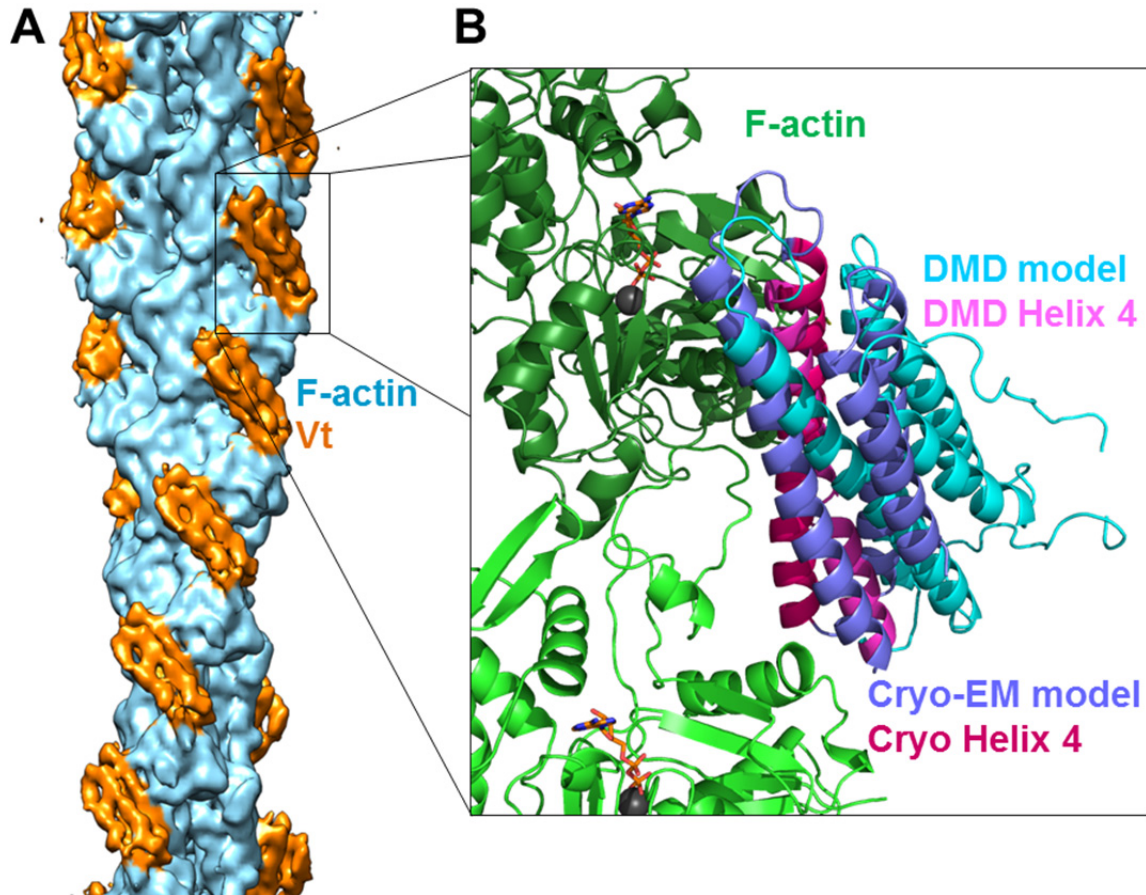


Figure 3.8. The cryo-EM model of Vt bound to F-actin.

Shown is the model of Vt bound to F-actin derived from cryo-EM data (A). Vt is in orange and F-actin in cyan. Panel B compares the DMD model with the cryo-EM model. The color scheme is maintained from Figure 3.4. The cryo-EM model is similar in that helices 4 and 5 are involved in actin binding. Discrepancies between the two models are, in large part, due to the conformational changes in Vt that are observed in the cryo-EM model.

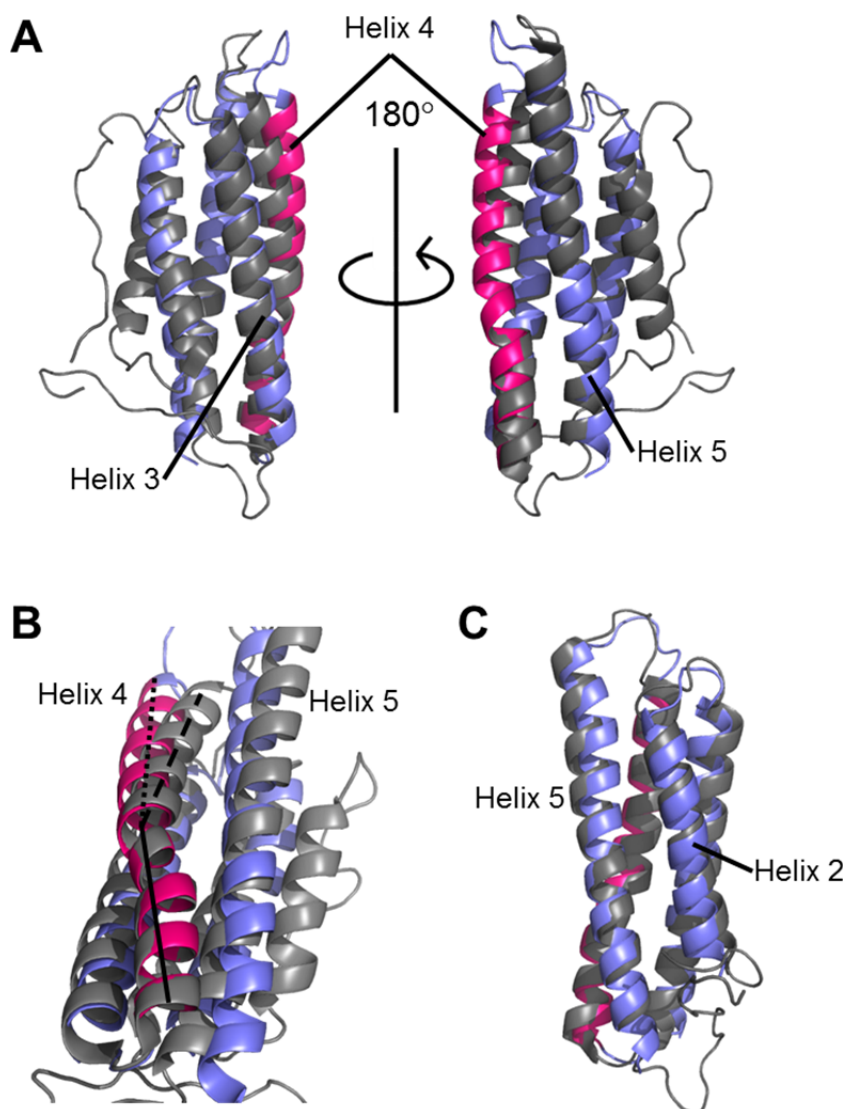


Figure 3.9. The Vt helix bundle changes conformation upon binding F-actin.

Shown in Panel A is a comparison of the structure of unbound Vt (gray, PDB file 1ST6) with Vt from the cryo-EM model of Vt bound to F-actin (blue and pink, Figure 3.8). The color scheme is maintained from Figure 3.8. Panel B shows how helix 4 straightens considerably upon binding F-actin. Lines have been drawn along helix 4 to highlight this change, with long dashes for unbound Vt and small dashes for the cryo-EM model of bound Vt. To help compensate for this straightening, helices 3 and 5 shift slightly, with helix 3 moving out from the bundle at its C-terminus and helix 5 moving back to the bundle at its C-terminus. Note that helix 1 is not present in the cryo-EM model, suggesting it leaves the helix

bundle. Panel B removes helix 1 from the unbound Vt to show how helices 2 and 5 shift towards each other. This shift suggests that helix 1 is excluded and blocked from accessing the hydrophobic core of the bundle.

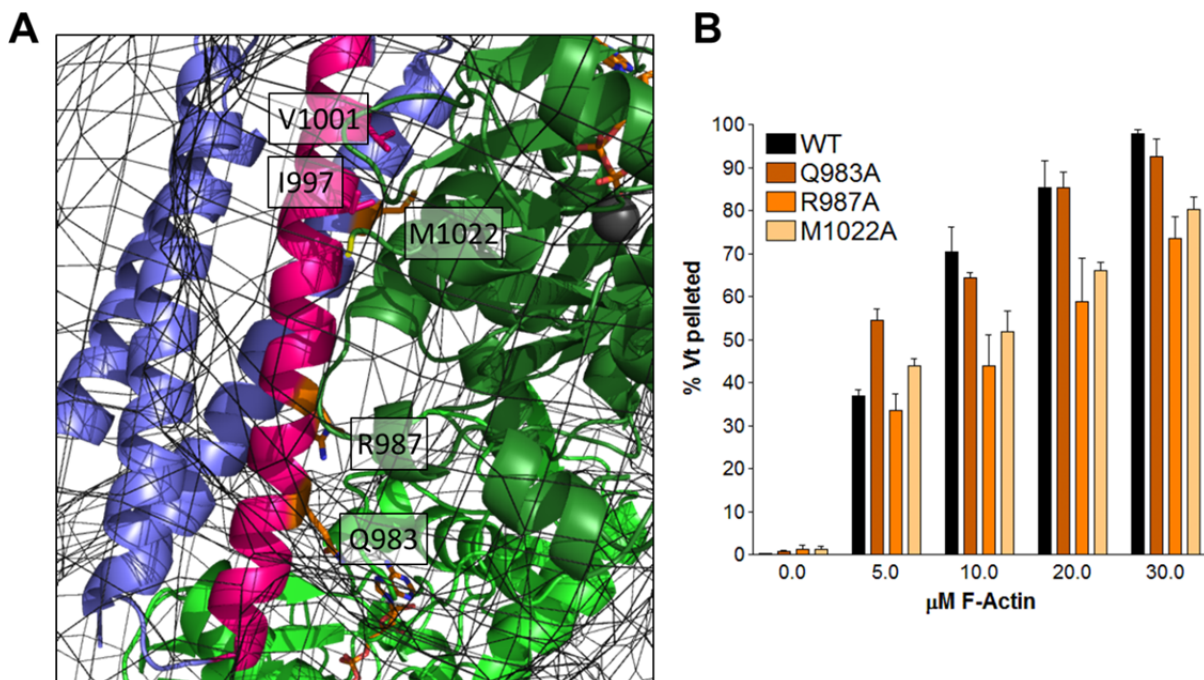


Figure 3.10. Two model-predicted mutants disrupt F-actin binding.

Panel A shows the cryo-EM model. The color scheme is the same from Figure 3.8. Helix 4 is colored in pink, and the labeled residues have their sidechains shown with sticks. Q983, R987, and M1022 are in orange, and were identified as Vt residues that contact F-actin. Panel B shows the results of actin co-sedimentation assays done to assess the contribution of these residues. Mutation to alanine of R987 and M1022 result in a slight actin-binding defect. Mutation of Q983 has no significant effect on actin binding.

Chapter 4: Characterization of Vt Q1018K, a Vt variant with a Decreased Propensity for Self-Association at High Concentrations

Introduction

Vinculin and Vt have been studied extensively by crystallography, providing a number of static crystal structures of Vt (2, 10, 39, 133). These structures have shown that Vt has the same general structure, a five-helix bundle, independent of binding to the vinculin head domain. Additionally, the binding sites for F-actin and PIP₂ are localized to Vt (86, 134), suggesting that study of the isolated Vt reasonably mimics native structure and interactions. It has become apparent that, on its own or in the presence of ligands, Vt has a propensity to oligomerize. Upon binding of Vt to F-actin (120, 121) or PIP₂ (10, 66, 102) and subsequent addition of a crosslinking agent, dimers and higher-order oligomers are observed. The F-actin-induced dimer is responsible for F-actin crosslinking by Vt (120), which is essential for proper focal adhesion development and force transduction, though the structure of this oligomer is unknown. The structural and functional natures of the PIP₂-induced oligomers have not been well defined. Recently a crystal structure of Vt with a short-chain PIP₂ molecule has been solved (40), though the physiological relevance of this oligomer is somewhat questionable, as its formation in the presence of a lipid bilayer is unlikely. The oligomerization of Vt in the absence of ligands has also been reported, though at a much lower K_d than in the presence of ligands (127, 128).

Not surprisingly, when the structure of Vt was first solved, it was solved as a dimer (10). The dimer in the crystallographic space group has the C-terminal arm of one Vt pack against the N-terminal strap and helix 1 of the second Vt (**Figure 4.1**) (10). In addition to this dimer, a second dimer is formed across the repeating units (**Figure 4.1**), which is the

dimer that forms when Vt is found at higher concentrations ($> 50 \mu\text{M}$) in solution (10, 127). This dimer is mediated by a hydrophobic patch on helices 4 and 5 (**Figure 4.1**). The K_d for this dimer is reported to be roughly $300 \mu\text{M}$. Given the physiological concentrations of vinculin in vivo, this dimer is unlikely to be physiologically relevant and does not contribute significantly in most biochemical assays (127). However, nuclear magnetic resonance (NMR) experiments often require concentrations greater than $200 \mu\text{M}$, at which roughly 43% or more of Vt would exist in this weak, self-associative dimer. As NMR is highly sensitive to the rotational correlation time of molecules in solution, with sensitivity and detection more difficult as the molecular size increases, dimerization of Vt limits NMR structural and dynamic analyses.

NMR spectroscopy is a biophysical technique that uses the property of nuclear spin to obtain specific signals for each nucleus in a molecule. The frequency of the signals originates from the precession of the magnetic moment of the nuclear spin, which is dependent on the electrochemical environment of the nucleus. Additionally, the relaxation of the nuclear magnetic moment provides information on the motion of the nucleus. The transverse relaxation (R_2) is quite sensitive to this, and also scales with the size of the molecule. A Vt dimer, at 43 kDa, has unfavorable relaxation properties, further making NMR an unsuitable technique for the study of Vt. To overcome these limitations, a Vt variant was designed to weaken the affinity of this dimer and allow for the study of Vt by NMR as well as other biophysical/structural approaches.

Materials and Methods

Protein expression and purification

The plasmid for expression of Vt was generated by the Liddington lab (10). The gene for chicken Vt (residues 879-1066) was placed into a pET15b vector for protein expression. All mutagenesis was performed using QuikChange site-directed mutagenesis (Stratagene, La

Jolla, CA, USA) and the DNA sequence was verified by sequencing (Genewiz, RTP, NC, USA). Additionally, the Vt gene was cloned into the pQLinkH vector using BamHI and NotI as the 5' and 3' restriction endonucleases, respectively. pQLinkH, like pET15b, codes for an N-terminal histidine tag to the protein. However, it codes for a TEV protease cleavage site instead of a thrombin cleavage site.

To express Vt, chemically competent BL21 DE3 *E. coli* with the Rosetta2 plasmid (EMD Millipore, Billerica, MA, USA) were transformed with either vector and plated on LB plates containing ampicillin and chloramphenicol. A single colony was selected and grown overnight. The overnight growth was used to inoculate flasks with 1 L Luria Broth. Cells were grown at 37°C until OD reached 0.6. Expression was induced with 250 µM IPTG, the incubator temperature was cut to 16-18 °C, and cells were harvested after 16-20 hours. To harvest, cells were pelleted via centrifugation and then resuspended in lysis buffer (20 mM Tris, 150 mM NaCl, 5 mM imidazole, and 2 mM β-mercaptoethanol (BME)). Cells were lysed either with sonication or homogenization and the lysate was subsequently clarified by centrifugation at 25,000 × g for 45 minutes. The first purification step was over Ni-NTA beads (Qiagen, Germantown, MD, USA). The lysate was applied to the beads, which were subsequently washed with lysis buffer. The beads were then washed with lysis buffer with 60 mM imidazole. The protein was eluted with lysis buffer containing 500 mM imidazole.

After purification using nickel-agarose beads, vinculin protein expressed in the pET15b system was dialyzed into thrombin cleavage buffer (20 mM Tris pH 7.5, 500 mM NaCl, 2 mM CaCl₂, and 5 mM BME) and the histidine tag cleaved by addition of thrombin (Sigma-Aldrich, St. Louis, MO, USA). Proteins were then further purified by ion exchange chromatography (S-column) and size exclusion chromatography (S-200 superdex).

For expression using the pQLinkH vector, vinculin proteins were purified by nickel affinity chromatography and the N-terminal histidine tag was cleaved by the addition of TEV protease. The mixture was dialyzed overnight at room temperature into TEV cleavage buffer

(20 mM Tris pH 7.5, 50 mM NaCl, 5 mM BME). The protein mixture was then passed over Ni-NTA beads a second time to remove the histidine tag, TEV protease, and any contaminating proteins that bound to the Ni-NTA beads. After this step, ion exchange and size exclusion chromatography were used to further purify the protein sample.

Analytical ultracentrifugation

Sedimentation equilibrium data were collected as previously described (127). Experiments were run on a Beckman Optima XL-I analytical ultracentrifuge with a Ti50 8-hole rotor and six-sectored centerpieces. Samples were dialyzed into analytical ultracentrifugation (AUC) buffer (10 mM potassium phosphate pH 7.5, 50 mM NaCl, 2 mM dithiothreitol) and concentrated. Samples were run first at 3000 rpm to obtain a flat line and to estimate the extinction coefficient at 305 nm. Absorbance was measured at 305 nm because the concentrations of Vt used were too large to get reliable readings at 280 nm. Samples were then spun at 19000 rpm for 20 hours, with measurements collected every two hours. Finally, samples were spun at 40000 rpm for eight hours with measurement collected at every two hours to determine the meniscus depletion and provide an offset for the absorbance of the buffer.

Data were analyzed using WinNonlin V1.06 (D. Yphantis, University of Connecticut, Storrs, CT; M. Johnson, University of Virginia, Charlottesville, VA; J. Lary, National Analytical Ultracentrifugation Facility Center) as reported previously (127).

Circular dichroism

Circular dichroism (CD) spectra were collected on a Jasco J-815 CD Spectrometer (JASCO, Easton, MD, USA). CD data were collected in 10 mM potassium phosphate, pH 5.5 or pH 7.5, 50 mM Na₂SO₄, and 1 mM DTT at 25°C unless otherwise specified. For near-ultraviolet (UV) measurements, protein was concentrated to 450 µM, while far-UV measurements were collected on protein at 5 µM.

NMR spectroscopy

To make uniformly ^{15}N -enriched Vt for NMR studies, Vt was expressed in M9 media with $^{15}\text{NH}_4\text{Cl}$ as the sole nitrogen source. ^{15}N -Vt was purified in the same manner as Vt from lysogeny broth (LB), exchanged into NMR buffer (10 mM potassium phosphate, pH 5.5, 50 mM NaCl, 0.1% NaN_3 , 2 mM DTT, and 10% D_2O), and concentrated to 30 μM . For uniformly ^{15}N - and ^{13}C -enriched Vt, M9 media was used with $^{15}\text{NH}_4\text{Cl}$ (1 g/L) and ^{13}C -D-glucose (2 g/L) as the nitrogen and carbon sources (Cambridge Isotope Laboratories, Tewksbury, MA, USA). Two-dimensional heteronuclear single quantum coherence (HSQC) spectra were collected on a Varian INOVA 600 MHz spectrometer at 37°C. HNCA and HNCO spectra for backbone assignments were collected on a Varian INOVA 700 MHz spectrometer at 37°C. The HNCO was collected using 16 transients, 32 real increments in the ^{15}N dimension, and 38 real increments in the ^{13}C dimension. The HNCA was collected using 32 transients, 30 real increments in the ^{15}N dimension, and 52 real increments in the ^{13}C dimension. Spectra were processed with NMRPipe (125) and analyzed with NMRViewJ (126).

Size exclusion chromatography

For evaluation of self-association, samples were run over Superdex 75 10/300 GL column run by an AktaFPLC (GE Healthcare Life Sciences, Pittsburgh, PA, USA). Sample volume was 500 μL at the reported concentration. AUC buffer was flowed over at a rate of 0.5 mL/minute and absorbance at 280 nm was recorded to track protein elution.

Actin co-sedimentation

Actin co-sedimentation assays were performed as previously described (6, 103). G-actin was polymerized by addition of 2X and 1X actin co-sedimentation buffer so that the final concentration of actin is 45.6 μM and the buffer is at 1X. Polymerization was carried out for 30 minutes. Ten μL of 100 μM Vt was mixed with enough actin and buffer to give the

reported actin concentration (0, 5, 10, 20, and 30 μM) in a total volume of 100 μL to give Vt:F-actin molar ratios of 1:0, 1:0.5, 1:1, 1:2, and 1:3.

Actin and Vt were incubated together at room temperature for 1 hour. The samples were then spun for 30 minutes at $150,000 \times g$. The supernatant was collected by pipetting and the pellet resuspended in an equal volume of actin co-sedimentation buffer. The supernatant and pellet fractions were run on a gel using SDS-PAGE, and band intensities were compared using ImageJ (104). The percentage of protein pelleted was calculated by dividing the intensity of the pellet band by the sum of the intensities of the pellet and supernatant bands. Values were adjusted to fit the percentages published previously (6, 43) by fitting to a binding curve and scaling the respective K_d values.

Results

Mutation of Q1018 to lysine disrupts Vt self-association at high concentrations

To study Vt by NMR requires the overcoming of certain technical challenges imposed by its propensity to self-associate. With the reported K_d of 300 μM , (127), one must choose to either run experiments at a low concentration or to study Vt as a dimer. Low concentrations necessitate prohibitively long NMR collection times for three-dimensional experiments or those designed to record relaxation properties. Studying the self-associating Vt dimer is not an attractive option, as it is a non-physiological oligomer with a molecular weight of 43.2 kDa, placing it near the upper size limit for structures amenable to NMR. Therefore, mutations at the self-associative interface (PDB file 1QKR) (10) were made to weaken this interaction and make Vt more amenable to NMR. Two such mutations, I997A and V1001A, disrupt self association, but exhibit poor binding to F-actin (103). A different mutation, Q1018K, was also made, with the intention of it not disrupting binding to F-actin. This mutation introduces two positive charges into the self-associative interface to decrease the affinity of the interaction with electrostatic repulsion.

To evaluate the self-association properties of Vt Q1018K, Vt and Vt Q1018K were sedimented using an analytical ultracentrifuge. Vt was centrifuged at concentrations of 470, 450, 313, 300, 157, and 150 μ M, while Vt Q1018K was centrifuged at 600, 400, and 200 μ M. Data were fit to using the monomer/dimer self-association model, both with and individual fit for each concentration and a collective fit, with and without offset values determined by spinning at 40,000 rpm (**Figures 4.2 and 4.3**). The fits were not particularly good, as evidenced by the residuals and the reported errors. Additionally, the K_d for the fit of Vt is much larger than 300 μ M (**Table 4.1**). These results suggest that, at the concentrations used, Vt may be forming trimers or other higher-order oligomers. However, the self-association affinity for Vt Q1018K is slightly lower than that for Vt, suggesting that this mutation may weaken the association interface.

To follow up on these results, both Vt and Vt Q1018K were run on a size exclusion column at various concentrations. Vt Q1018K eluted at the size of a Vt monomer at 200 μ M, suggesting that the K_d for its self-association is greater than that reported for wild type Vt (**Figure 4.4**). An NMR titration was also collected with Vt Q1018K to evaluate its self-associative properties. The ^1H - ^{15}N chemical shifts for the backbone amide of T962 and S1016 are good probes for formation of the dimer, as they shift as Vt concentration increases (127). Unlike Vt, there was no evidence of Vt Q1018K dimerization at 200 μ M and only slight dimerization at 650 μ M by these resonances (**Figure 4.5**). These experiments, in conjunction, show that the K_d for Vt Q1018K self-association is higher than Vt, indicating that the Vt Q1018K variant is amenable to detailed NMR studies.

Vt Q1018K is structurally similar to wild type Vt

Though Vt Q1018K shows reduced concentration-dependent self-association, for it to be a suitable substitute for wild type Vt in NMR experiments, it must be structurally similar to Vt. Circular dichroism (CD) of Vt and Vt Q1018K was used to assess secondary structure and tryptophan packing. At both far- and near-UV wavelengths, Vt Q1018K exhibited the

characteristic spectra of a well-folded Vt protein (**Figure 4.6**). The far-UV spectrum revealed the secondary structure of Vt Q1018K to be dominated by α -helical and the near-UV spectrum revealed the characteristic peaks at 283 and 290 nm indicative of the correct packing of the tryptophan 912 and tryptophan 1058 sidechains. These results suggest that mutation of glutamine 1018 to lysine does not significantly alter the structure of Vt.

In addition to evaluating the structure of Vt Q1018K by CD, NMR was also used to examine any potential change in Vt structure. A ^1H - ^{15}N two-dimensional (2D) HSQC reveals that there are a substantial number of peaks that shift relative to Vt (**Figure 4.7**). These resonances all shifted by greater than twice the average linewidth of the NH peaks. This was not surprising, as the backbone resonances were assigned under conditions at which the Vt dimer was the prevalent species (135). The NH backbone resonances that shift correspond to V985, E986, R987, T993, K996, I997, L998, S999, E1014, S1016, L1023, V1024, and I1046. The majority of these residues are located near the site of mutation, residue 1018, as expected. These results suggest that, while there may be some small, local changes to the structure of Vt upon mutation of glutamine 1018 to lysine, the protein retains its structure (**Figure 4.7**). This makes Vt Q1018K a valuable tool for studying Vt by NMR.

Vt Q1018K has a slight actin-binding defect

Because Vt I997A and Vt V1001A greatly impair binding to F-actin (103), these variants were not ideal for study by NMR, as the biochemical function of Vt was significantly disrupted. We tested Vt Q1018K for its actin-binding properties by actin co-sedimentation to ensure that it could still bind and crosslink F-actin. Vt Q1018K does exhibit a slight binding defect, as it binds to 30 μM F-actin at 87% of the level that Vt binds F-actin (**Figure 4.8**). This is congruent with the most recent Vt:F-actin structural model, as Q1018 is in helix 5, near the edge of the actin-binding interface. However, this change in binding is modest compared to mutations at the hydrophobic core of this upper site (Chapter 3).

NMR backbone assignment of Vt Q1018K

The ^1H - ^{15}N HSQC of Vt Q1018K exhibited shifts for at least 8.4% of the NH peaks, necessitating the assignment of Vt Q1018K backbone residues. Both three-dimensional HNCA and HNCOC data were collected for ^{15}N - ^{13}C labeled Vt Q1018K at a concentration of 200 μM . This was done without perdeuteration, which was required for the assignment of Vt, and practically removes the potential for signal from the Vt self-associated dimer. Assignments were made for 81.52% of the non-proline ^{15}N and ^1H resonances, compared to 84.24% of the non-proline ^{15}N and ^1H resonances for Vt (**Figure 4.9**) (135). The residues that were assigned for Vt but not for Q1018K are R903, I920, A922, A927, and L936, which are found in helix 1, helix2, or the helix 2-helix 3 loop.

Discussion

The ability of Vt to self-associate at high concentrations in a non-physiological manner limits the techniques and analyses available for studying Vt. The generation of Vt Q1018K, a Vt variant with a weaker affinity for the self-associative dimer in the absence of Vt ligands, provides a way to overcome many of these limitations. Though the AUC, size exclusion, and NMR data, individually, could not be used to quantify the K_d of the associative interaction for Vt Q1018K, these data taken together indicate that Vt Q1018K has a weaker K_d for self-association. These data are further supported by the correlation time of Vt Q1018k, 11.86 ns/rad (Chapter 5). This correlation time is indicative of a protein of roughly 20 kDa , slightly less than the calculated weight of monomeric Vt (136).

Not surprisingly, mutations that weaken this self-associative interface also weaken F-actin binding (103, 124), as the self-associative interface overlaps significantly with the upper actin binding site (103). Mutation of Q1018 to lysine avoids a large disruption of actin-binding, as seen with I997A and V1001A, while still disrupting self-association, making it a suitable candidate for studying Vt at high concentrations without disrupting its biochemical

functions. Indeed, the development of this tool has allowed for the utilization of a wide range of NMR experiments to study Vt, some of which have been published previously (103) and as well as applications discussed in Chapter 5.

	K_d (mM)	95% confidence interval (mM)
Vt	1.26	0.81-2.06
Vt Q1018K	1.53	0.98-2.47

Table 4.1 K_d values for Vt and Vt Q1018K self-association as determined by AUC.

The K_d values and 95% confidence intervals for the fits of the AUC data for Vt and Vt Q1018K to a monomer/dimer model are reported.

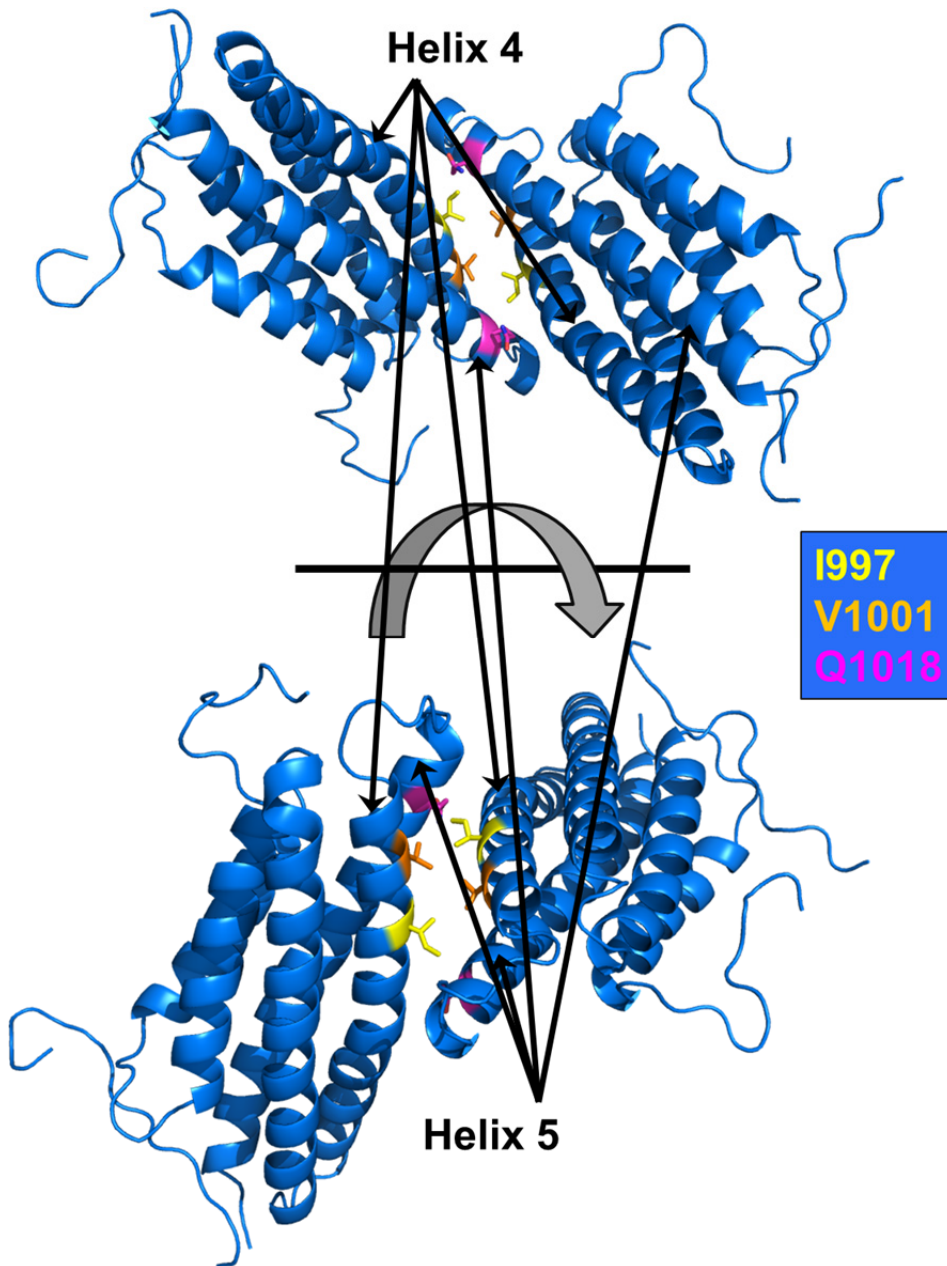


Figure 4.1. The structure of the Vt homodimer.

At high concentrations Vt self-associates to form a homodimer. This interaction is mediated primarily by the hydrophobic patch present on helices four and five. Isoleucine 997 and valine 1001 are shown in yellow and orange, respectively, and are the residues at the core of this interface. Glutamine 1018 is shown in magenta. The sidechains of these residues are shown as sticks.

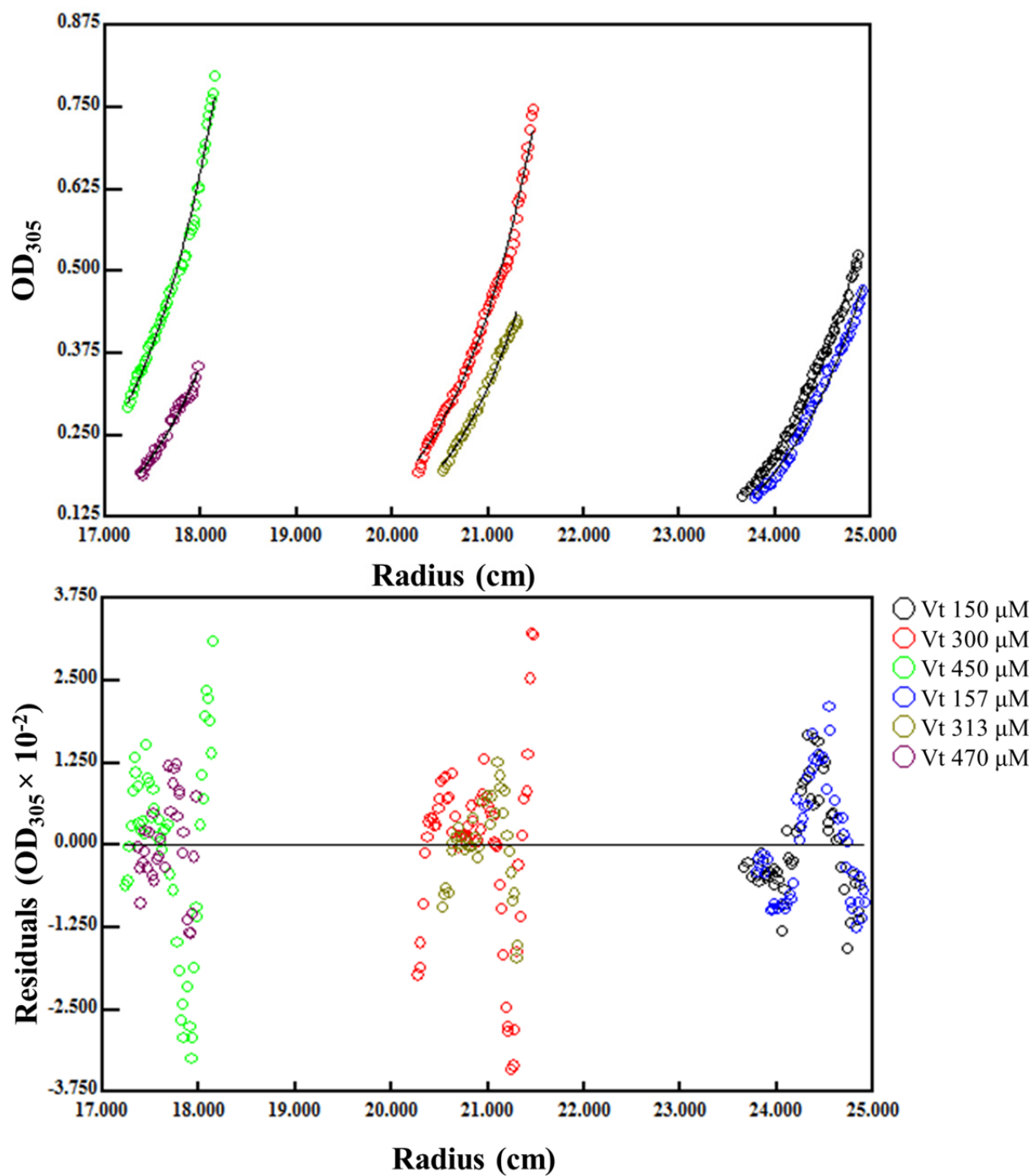


Figure 4.2. Sedimentation curves for Vt.

Sedimentation curves for Vt at six different concentrations are displayed with a fit to a monomer/dimer model. Residuals are also shown.

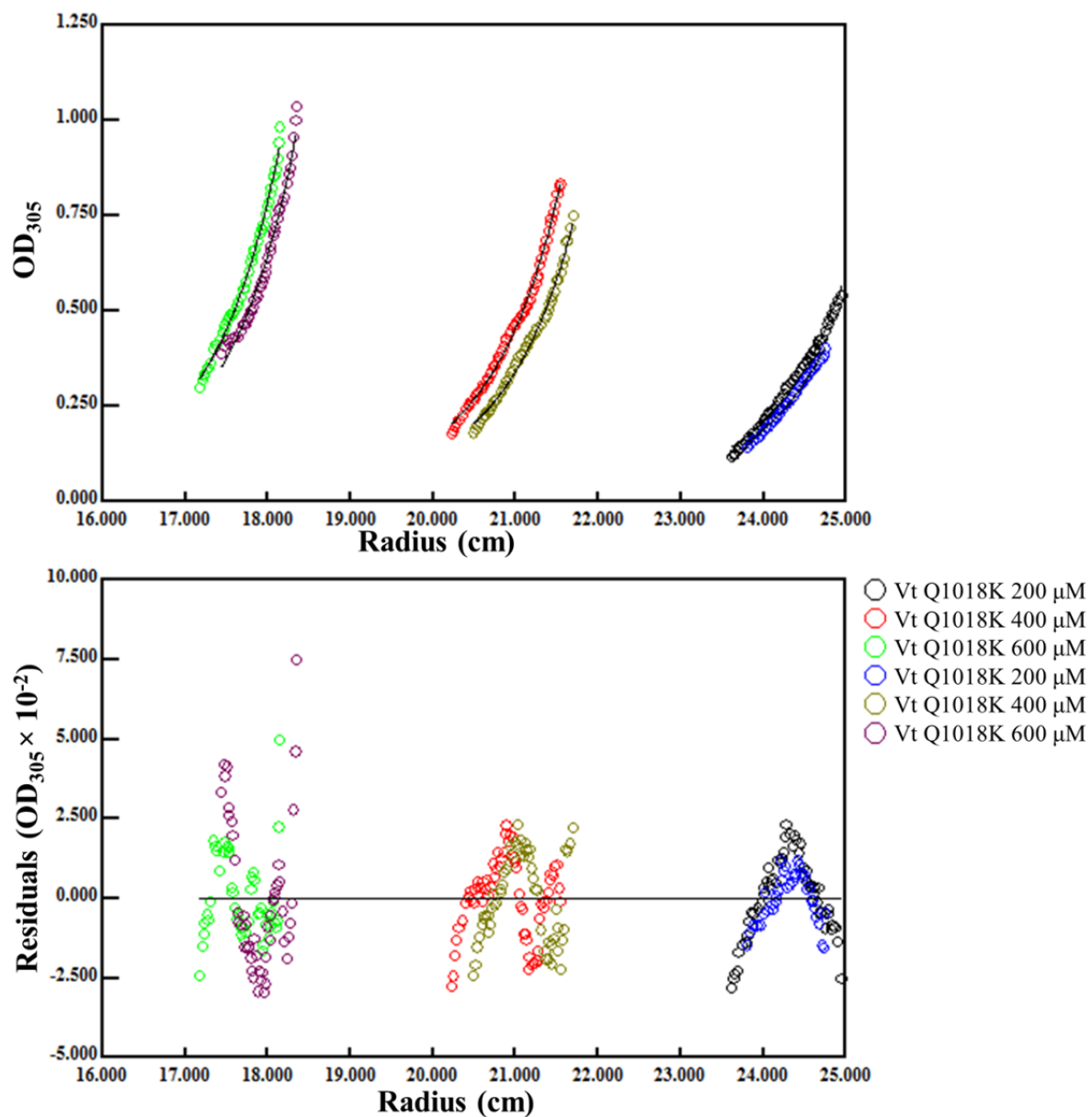


Figure 4.3. Sedimentation curves for Vt Q1018K.

Sedimentation curves for Vt Q1018K at three different concentrations are displayed with a fit to a monomer/dimer model. Residuals are also shown.

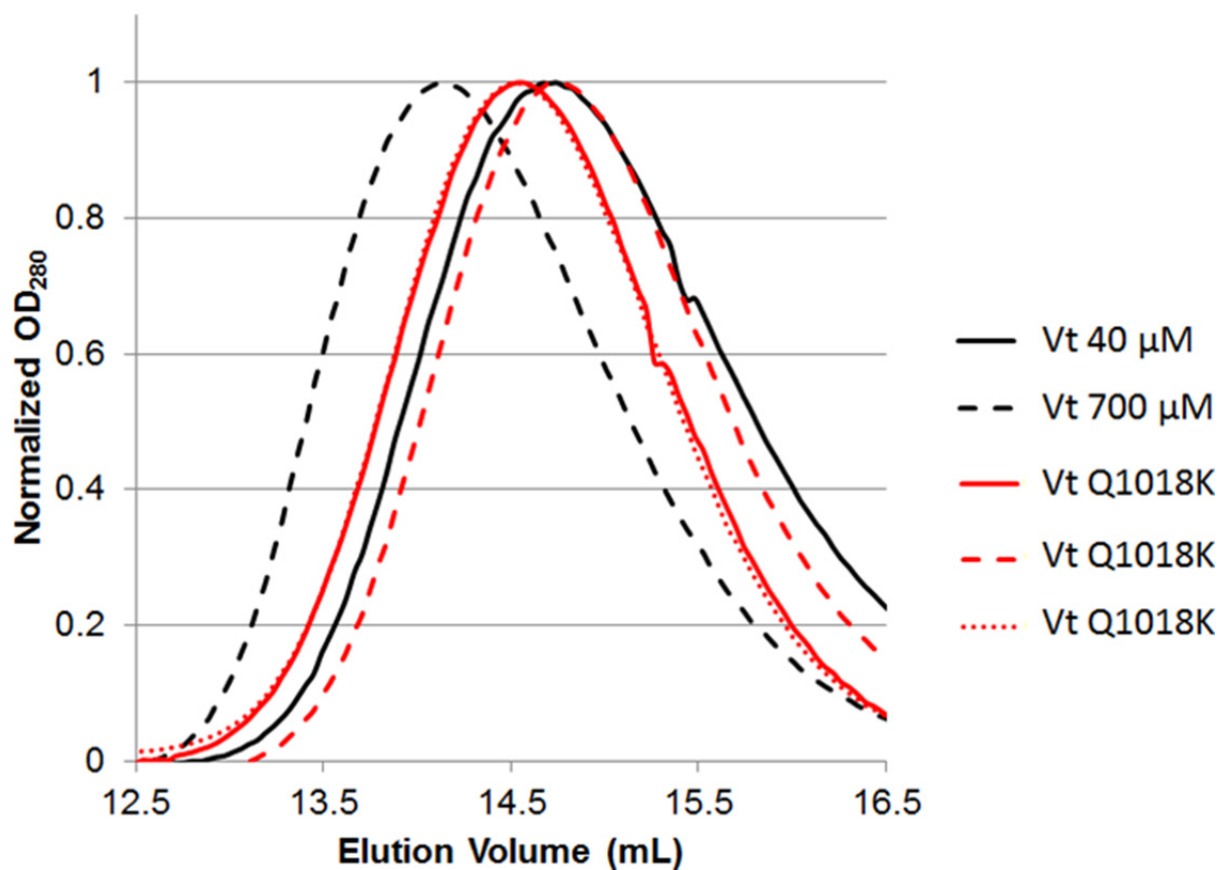


Figure 4.4. Size exclusion chromatography of Vt and Vt Q1018K.

The elution profiles for Vt and Vt Q1018K from a 25-mL Superdex-75 column. Vt runs are shown in black and Vt Q1018K elution profiles are shown in red, with the respective concentrations listed in the legend.

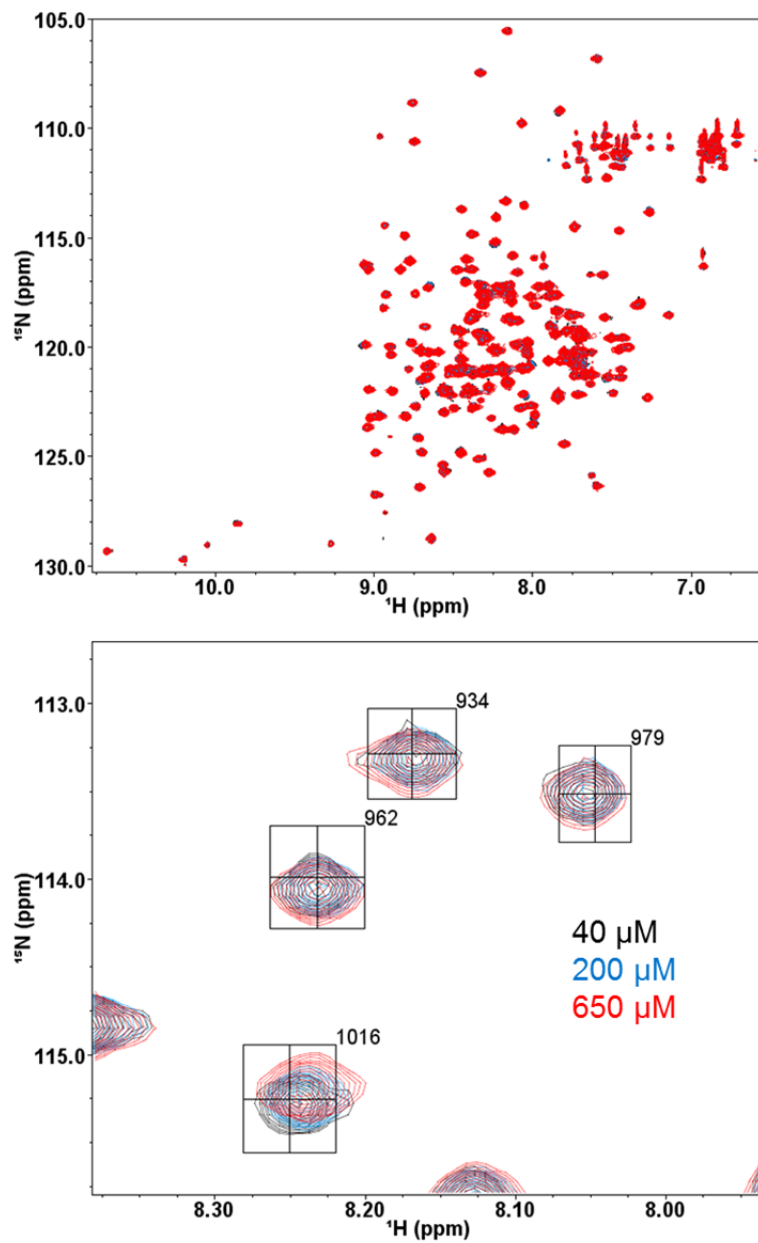


Figure 4.5. HSQC titration of Vt Q1018K.

Overlaid spectra of ^1H - ^{15}N HSQCs collected at various Vt Q1018K concentrations. The colors of the peaks (black, blue, red) correspond to the concentration of Vt Q1018K in the sample (40, 200, and 650 μM , respectively). The second panel highlights T962 and S1016, which have shifted NH resonances when Vt is dimeric (127). There is very little shift for these resonances, suggesting minimal dimer formation.

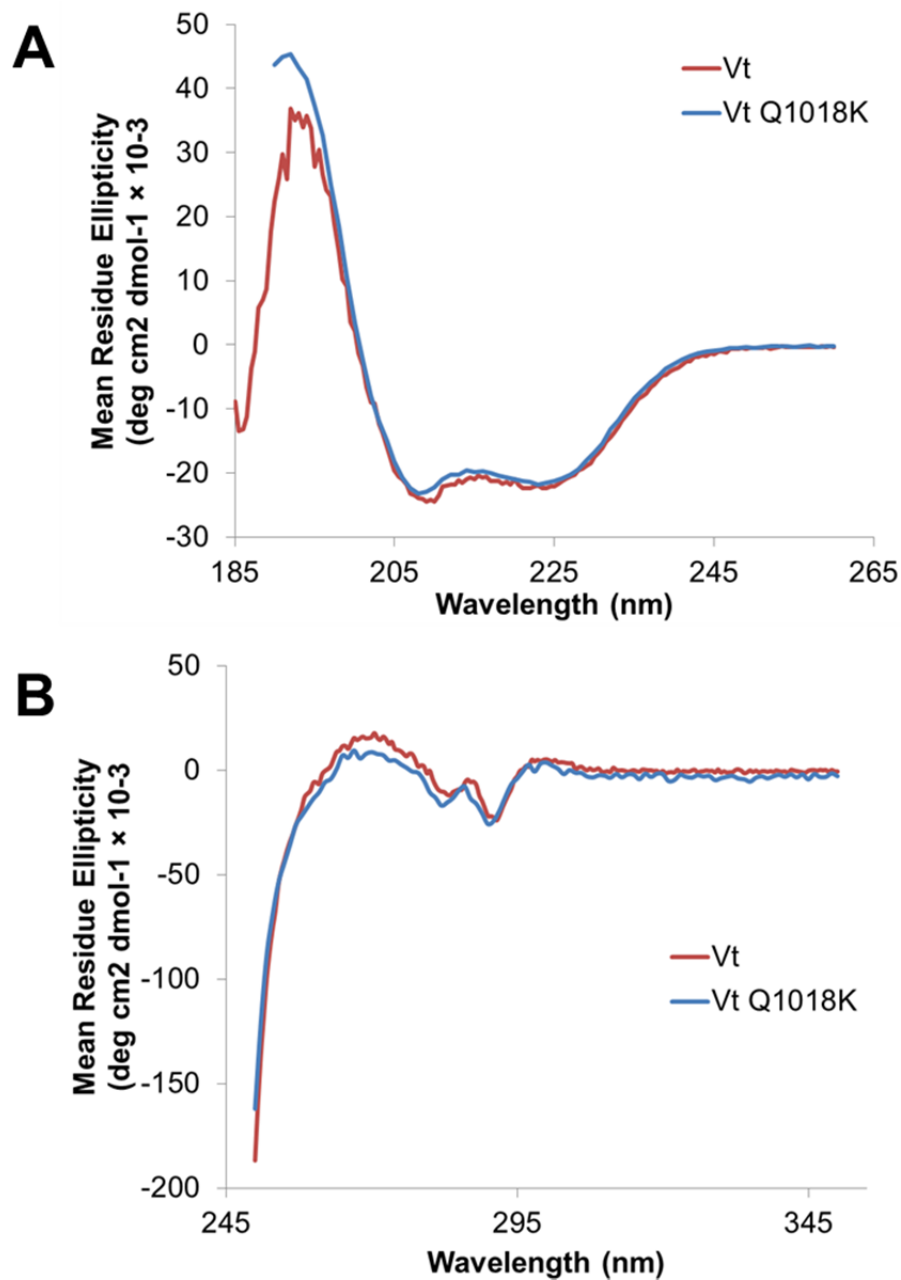


Figure 4.6. CD of Vt and Vt Q1018K.

Far- and near-UV CD spectra (A and B, respectively) of Vt and Vt Q1018K. Vt is in red, Vt Q1018K is in blue. Spectra are similar, suggesting that the structure of Vt Q1018K is very similar to Vt.

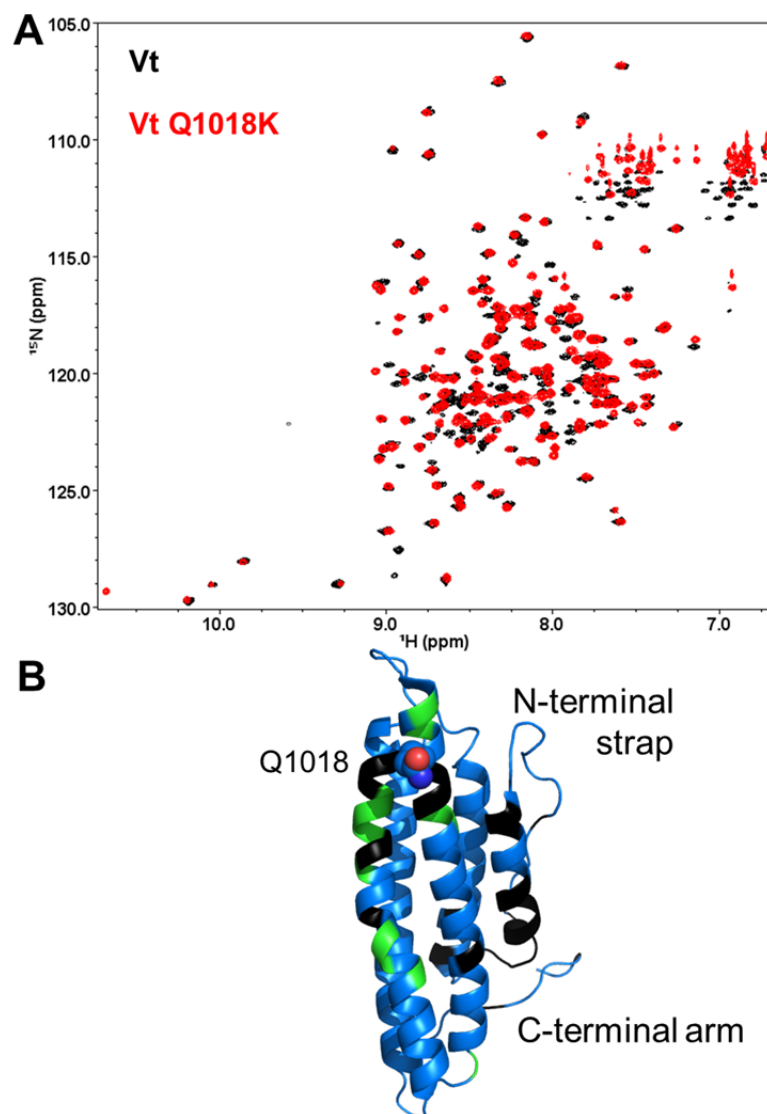
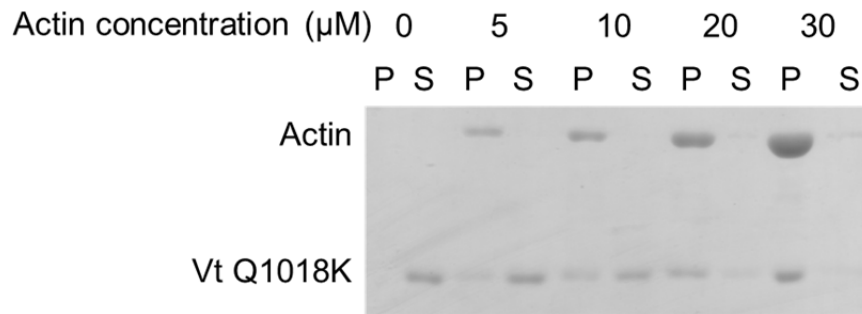


Figure 4.7. ^1H - ^{15}N HSQC overlay of Vt and Vt Q1018K.

The overlay of the ^1H - ^{15}N HSQC spectra of Vt (black) and Vt Q1018K (red) are shown in A. The residues that exhibit a significant change in chemical shift values (difference greater than twice the linewidth) are mapped onto the Vt structure (PDB code 1ST6) in green. Residues in black do not have assignments for amide hydrogens or nitrogens in Vt. The Q1018 sidechain is shown in spheres.

A



B

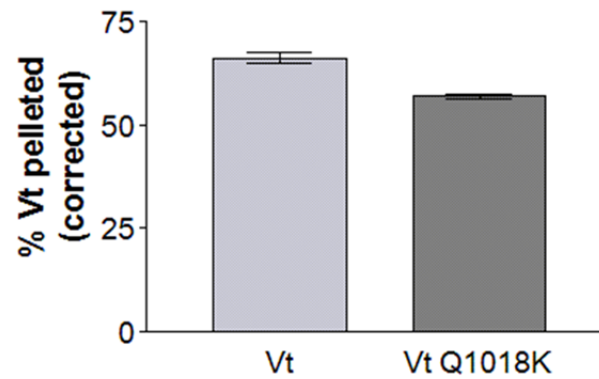


Figure 4.8. Actin binding of Vt Q1018K.

Actin co-sedimentation results with Q1018K. In A, a gel is shown with the pellet (P) and supernatant (S) fractions from a co-sedimentation assay. Vt Q1018K does bind to F-actin. In B, the relative binding amounts of Vt Q1018K to Vt at 30 μM F-actin are shown with error bars reporting standard deviation.

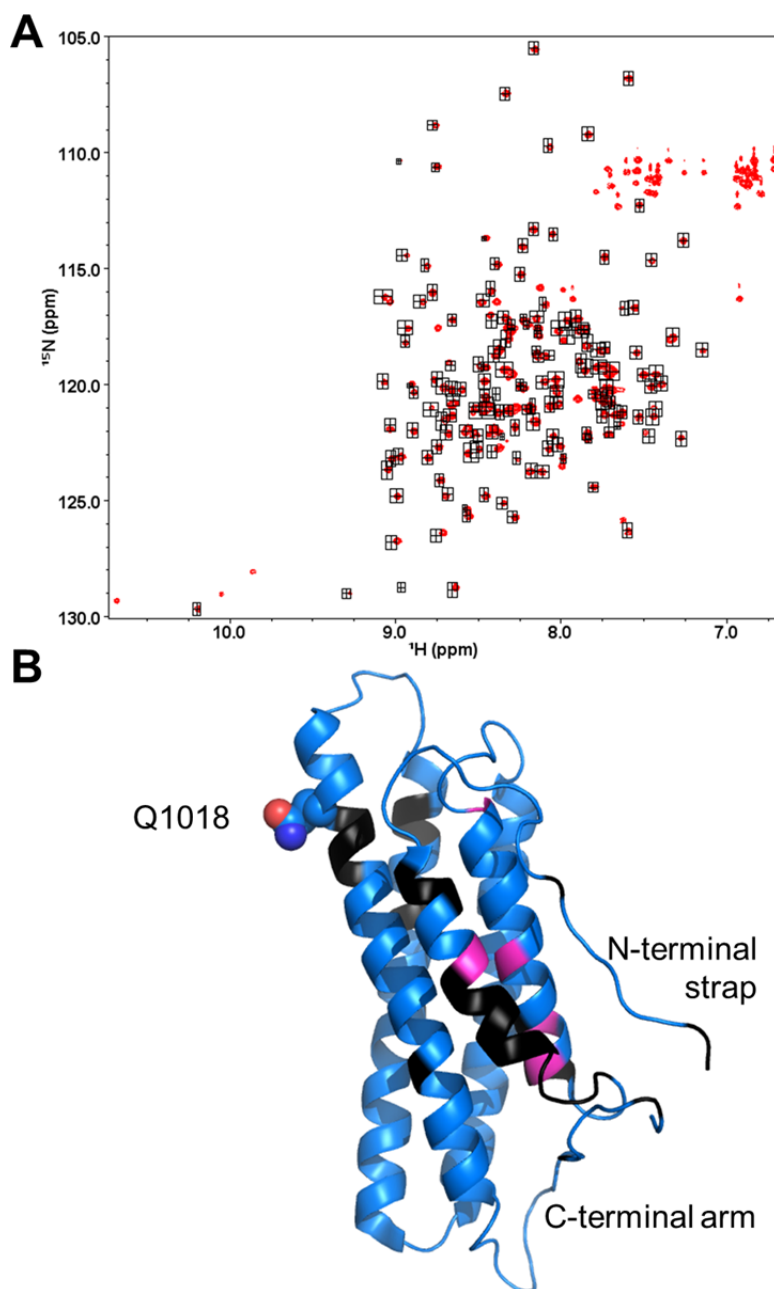


Figure 4.9. Assignment of Vt Q1018K.

NMR spectrum of Vt Q1018K showing backbone amide hydrogens and nitrogen assignments (A). Peaks with boxes have been assigned. The residues that were not assigned are shown on the Vt structure (B, PDB code 1ST6). Pink residues are assigned for Vt but not Vt Q1018K, and black residues are not assigned. The Q1018 sidechain is shown with spheres.

Chapter 5: Characterization of the Role of Vt Backbone Dynamics and Proline 989 in Vt Function

Introduction

Vinculin is a scaffolding protein that plays an important role in cell adhesion and migration (9). The ability of vinculin to act as a scaffold involves ligand-induced conformational changes that separate the head and tail domain (2, 10, 11, 87, 137). Moreover, the tail domain (Vt) changes conformation upon binding to certain ligands. Both F-actin (10, 120) and phosphatidylinositol 4,5-bisphosphate (PIP₂) (10, 66) induce conformational changes in Vt upon binding which then drive oligomerization of Vt. In the case of F-actin, this oligomerization crosslinks F-actin filaments and is required for proper force transduction, focal adhesion (FA) maturation, and binding of some vinculin ligands (6, 138). F-actin is a dynamic polymer. Therefore, studying Vt binding to F-actin and its conformational change upon binding are difficult to study by high resolution structural approaches, such as X-ray crystallography or nuclear magnetic resonance (NMR). Similar problems exist for studies with PIP₂-binding and membrane insertion by Vt. Further complicating matters is the self-associative properties of Vt in the absence of ligand (127). With the development of Vt Q1018K as a Vt variant with a weaker affinity for self-association, NMR becomes an available technique to study Vt (Chapter 4). NMR is particularly useful here, as it provides high-resolution structural and dynamic information. By studying the dynamics of Vt Q1018K in solution by NMR, we hope to learn something about the conformational changes that take place upon ligand binding.

Vinculin is a highly conserved protein (139). In the middle of Vt helix 4, resides a conserved proline, P989, which generates a kink in the helix (**Figure 5.1**). Proline residues tend to disrupt helices, as they lack an amide proton and cannot form hydrogen bonds that

stabilize helical secondary structure. Moreover, prolines possess restricted backbone mobility relative to other amino acids. Prolines in the middle of α -helices are common in transmembrane proteins (140) and can also be found in some cytosolic proteins (141, 142), and are almost always associated with a kink in the helix. In some instances, the prolines are not required for the helix kink (141, 142), suggesting that other structural elements may promote kink formation. While P989 in vinculin is strictly conserved, the kink in Vt helix 4 has yet to be probed for any structural or functional significance.

Materials and Methods

Protein expression and purification

The plasmid for expression of Vt was generated by the Liddington lab (10). The gene for chicken Vt (residues 879-1066) was placed into a pET15b vector for protein expression. All mutagenesis was performed using QuikChange site-directed mutagenesis (Stratagene, La Jolla, CA, USA) and the DNA sequence was verified by sequencing (Genewiz, RTP, NC, USA). Additionally, the Vt gene was cloned into the pQLinkH vector using BamHI and NotI as the 5' and 3' restriction endonucleases, respectively. pQLinkH, like pET15b, codes for an N-terminal histidine tag, but unlike pET15b, contains a TEV protease cleavage site instead of a thrombin cleavage site.

To express Vt in bacteria, chemically competent BL21 DE3 *E. coli* with the Rosetta2 plasmid (EMD Millipore, Billerica, MA, USA) were transformed with either vector and plated on LB plates containing ampicillin and chloramphenicol. A single colony was selected and grown overnight. The overnight growth was used to inoculate flasks with 1 L Luria Broth. Cells were grown at 37°C until OD reached 0.6. Expression was induced with 250 μ M IPTG, the incubator temperature was cut to 16-18 °C, and cells were harvested after 16-20 hours. To harvest, cells were pelleted via centrifugation and then resuspended in lysis buffer (20 mM Tris, 150 mM NaCl, 5 mM imidazole, and 2 mM β -mercaptoethanol (BME)). Cells

were lysed either with sonication or homogenization and the lysate was subsequently clarified by centrifugation at $25,000 \times g$ for 45 minutes. His-tagged Vt proteins were first purified using Ni-NTA beads (Qiagen, Germantown, MD, USA). The lysate was applied to the beads, which were subsequently washed with lysis buffer. The beads were then washed with lysis buffer containing 60 mM imidazole. The protein was eluted with lysis buffer with 500 mM imidazole.

After purification by nickel column, proteins expressed in the pET15b system were dialyzed into thrombin cleavage buffer (20 mM Tris pH 7.5, 500 mM NaCl, 2 mM CaCl_2 , and 5 mM BME) and their histidine tags cleaved by addition of thrombin (Sigma-Aldrich, St. Louis, MO, USA). Proteins were then further purified by ion exchange chromatography (S-column) and size exclusion chromatography (S-200 superdex).

For proteins expressed using the pQLinkH vector, after nickel affinity chromatography the N-terminal histidine tag was cleaved by the addition of TEV protease. The mixture was dialyzed overnight at room temperature into TEV cleavage buffer (20 mM Tris pH 7.5, 50 mM NaCl, 5 mM BME). The protein mixture was then passed over Ni-NTA beads a second time to remove the histidine tag, TEV protease, and any contaminating proteins that bound to the Ni-NTA beads. After this step, ion exchange and size exclusion chromatography were used to obtain highly purified proteins samples (> 95% purity, as determined by SDS-gel electrophoresis).

Circular dichroism

Circular dichroism (CD) spectra were collected on a Jasco J-815 CD Spectrometer (JASCO, Easton, MD, USA). CD data were collected in 10 mM potassium phosphate, pH 5.5 or pH 7.5, 50 mM Na_2SO_4 , and 1 mM DTT at 25°C unless otherwise specified. For near-ultraviolet (UV) measurements, protein was concentrated to 450 μM , while far-UV measurements were collected on protein at 5 μM .

Denaturation experiments were collected by tracking the CD of a sample at 222 nm as the temperature of the sample increased at a rate of 2°C min⁻¹.

NMR spectroscopy

To make uniformly ¹⁵N-enriched Vt for NMR, Vt was expressed in M9 media with ¹⁵NH₄Cl as the sole nitrogen source. ¹⁵N-Vt was purified in the same manner as Vt from LB, exchanged into NMR buffer (10 mM potassium phosphate, pH 5.5, 50 mM NaCl, 0.1% NaN₃, 2 mM DTT, and 10% D₂O), and concentrated to 30 µM. To generate uniformly ¹⁵N- and ¹³C-enriched Vt, M9 media was used with ¹⁵NH₄Cl and ¹³C-D-glucose as the nitrogen and carbon sources (Cambridge Isotope Laboratories, Tewksbury, MA, USA). Two-dimensional heteronuclear single quantum coherence (HSQC) spectra were collected on a Varian INOVA 700 MHz spectrometer at 37°C. Three dimensional HNCACB and HNCO spectra for backbone assignments were collected on a Varian INOVA 700 MHz spectrometer equipped with a cryoprobe at 37°C. The HNCO was collected using 16 transients, 32 real increments in the ¹⁵N dimension, and 38 real increments in the ¹³C dimension. The HNCA was collected using 32 transients, 30 real time increments in the ¹⁵N dimension, and 52 real increments in the ¹³C dimension. Spectra were processed with NMRPipe (125) and analyzed with NMRViewJ (126).

NMR relaxation experiments

R₁, R₂, and heteronuclear {¹H}-¹⁵N Nuclear Overhauser Effect (hetNOE) experiments (143) were collected on Varian INOVA 600 and/or 700 MHz magnets. The 600 MHz magnet was equipped with a room temperature probe and the 700 MHz magnet with a cryoprobe. The times used in the T₁ and T₂ experiments are reported in **Table 5.1**. All experiments were performed with 200 µM protein in NMR buffer at 37°C and collected in an interleaved fashion.

The Carr-Purcell-Meiboom-Gill (CPMG) was collected using an improved relaxation-compensated sequence (144, 145) on the Inova 700 MHz spectrometer with a Bruker cold probe and console. The total CPMG period was 28 ms. In addition to a reference spectrum, 13 total HSQC-type spectra were collected, two of which were duplicates. The delay times, τ , for these spectra ranged from 0.67 to 14 ms. These spectra were collected in an interleaved manner to limit the effect of any change in the sample or magnetic fields over time.

Relaxation analysis

Model-free analysis of ^{15}N R_1 , R_2 , and NOE backbone relaxation data was performed using relxn2.2 (146, 147). The best fit for the isotropic rotational correlation time for Vt Q1018K was determined to be 11.86 ns/rad. To evaluate the effectiveness of using a model of rotational anisotropy, the program qfit (148) was employed using PDB file 1ST6. However, the data did not support the use of an anisotropic model for Vt tumbling, so an isotropic model was used. The N-H bond distance used was 1.0 Å and the ^{15}N chemical shift anisotropy value used was -170. Models were selected based on Akaike's information criterion (AIC) (149). Errors were estimated by 150 or more Monte Carlo simulations.

CPMG data were analyzed with NMRViewJ (126). Peak intensities for each timepoint were determined. $R_{2,\text{eff}}$ was calculated using the following formula:

$$R_{2,\text{eff}} = \frac{-1}{T_{\text{relax}}} \ln \frac{I}{I_0}$$

where T_{relax} is the total CPMG pulse time, I is the intensity of the peak, and I_0 is the intensity of the reference peak.

Hydrogen exchange

Samples for hydrogen exchange were prepared by exchanging Vt Q1018K into a deuterated buffer (10 mM potassium phosphate, pD 5.9, 50 mM NaCl, 0.1% NaN_3 , 2 mM DTT, 95% D_2O) using a PD-10 column (GE Healthcare Life Sciences, Piscataway, NJ, USA). NMR spectra were collected on a Varian INOVA 700 MHz magnet using a fast-HSQC (150)

over the course of ten days at times reported in **Table 5.2**. Data were processed and fit to an exponential decay function using the Rate Analysis tool in NMRViewJ (126).

CLEAN chemical exchange spectroscopy (CLEANEX) experiments (151, 152) were collected on a Varian INOVA 600 MHz magnet. Mixing times were 0, 15, 30, 55, 85, 125, 180, and 300 msec. Data were processed and fit to an exponential function using the Rate Analysis tool in NMRViewJ (126).

Actin co-sedimentation

Actin co-sedimentation assays were performed as previously described (6, 103). G-actin was polymerized by addition of 2X and 1X actin co-sedimentation buffer (10 mM Tris, pH 7.5, 100 mM KCl, 2 mM MgCl₂, and 4 mM DTT) so that the final concentration of actin is 45.6 μ M and the buffer is at 1X. Polymerization was carried out for 30 minutes. Ten μ L of 100 μ M Vt was mixed with enough actin and buffer to give the reported actin concentration in a total volume of 100 μ L.

Actin and Vt were incubated together at room temperature for 1 hour. The samples were then spun for 30 minutes at 150,000 $\times$ g. The supernatant was collected by pipetting and the pellet resuspended in an equal volume of actin co-sedimentation buffer. The supernatant and pellet fractions were run on a gel using SDS-PAGE, and band intensities were compared using ImageJ (104). The percentage of protein pelleted was calculated by dividing the intensity of the pellet band by the sum of the intensities of the pellet and supernatant bands. Values were adjusted to fit the percentages published previously (6, 43) by fitting to a binding curve and scaling the respective K_d values.

To evaluate the ability of Vt to crosslink F-actin, the same procedure was followed, using only the 20 μ M actin concentration point, and the spin was at 5,000 $\times$ g for ten minutes. The supernatant was pelleted off and the pellet resuspended. The fractions were run on a gel using SDS-PAGE and the actin bands quantified using ImageJ.

Fluorescence microscopy

Fluorescence microscopy of actin bundles formed in the presence of Vt was performed as previously described (6, 153). Briefly, samples were prepared similarly to those used for the bundling co-sedimentation assay. The samples were then diluted 20-fold by the addition of actin polymerization buffer and Alexa Fluoro-488 phalloidin (Invitrogen, Carlsbad, CA, USA) was added to a final concentration of 1.5 μ M. Samples were incubated with the fluorophore for five minutes and then 5 μ L of the samples were placed on glass slides with a glass coverslip. Slides were observed with a Zeiss Axiovert 200M microscope with a 60 $\times$ objective lens and images were collected using a Hamamatsu ORCA-ERAG digital camera.

Negative-stain electron microscopy

Samples were prepped by incubating 800 nM actin F-actin with 800 nM Vt (or Vt variant) on ice for ten minutes. Ten μ L of sample was then applied to a copper grid covered by a thin glow-charged carbon foil and allowed to incubate on the grid at room temperature for one minute. The grid was then washed with 2% uranyl acetate for one minute by dropwise addition, blotted, and air dried. Grids were examined with a Philips CM12 TEM at 80 kV and images were taken with a Gatan 2k $\times$ 2k SC200 CCD camera.

Results

Vt helices 1 and 4 are less solvent protected than other helices in the bundle

With a Vt variant, Vt Q1018K, that exhibits decreased self-association, we were able to apply NMR techniques to evaluate protein dynamics (Chapter 4). Protein dynamics can reveal networks involved in allosteric motion; Vt, which changes conformation upon binding to F-actin and PIP₂, likely has such dynamic networks. In the case of actin, we know that actin-binding involves helices 4 and 5 (Chapter 3) (103) and subsequent Vt dimerization involves the basic collar (Chapter 2) and the C-terminus (6, 115), so such a network would be expected to stretch from one surface to the other. Similar to NMR work with Vt (135), we

were unable to assign backbone resonances for the majority of residues in helix 1 in Vt Q1018K (Chapter 4). This indicates that helix 1 is undergoing conformational exchange on an intermediate NMR timescale. The lack of assignments limits any further analysis of helix 1 dynamics by NMR. However, we wanted to evaluate the dynamics of the rest of the protein to better understand how Vt undergoes conformational change.

One way to evaluate the dynamics of proteins is to measure the hydrogen-deuterium exchange (HDX) rates for backbone amides. These rates reflect the protection of the backbone amide hydrogen from solvent exchange, which is heavily influenced by the protein structure. If the protein samples a conformation in which the backbone amide hydrogen becomes less protected, such as the unfolding of a helix, this will be observed by an increase in the exchange rate. Vt Q1018K was subjected to both CLEANEX and standard HDX NMR experiments to measure these rates.

Of the 150 assigned amide nitrogen and hydrogen pairs assigned in Vt Q1018K, 77 were observable using real-time (RT) HDX techniques. Of these, 68 amide resonances fit well to an exponential decay. An additional 44 resonances were observable using CLEANEX. The remaining 29 residues could not be evaluated; either the rates of deuterium exchange were too slow for CLEANEX but too fast for HDX, or the resonances were in regions of the spectra where there was too much overlap to unequivocally determine the rate of exchange. Not surprisingly, the residues that were observable with CLEANEX reside primarily in loops or regions without defined secondary structure, while those observable by RT HDX reside primarily in the helices (**Figure 5.2**). The presence of residues in the helix bundle that are not observable by RT HDX suggests that the helices do undergo local unfolding at rates faster than 6 hr^{-1} .

Residues that were observed by RT HDX were tracked over the course of 18 days (**Table 5.2**), and the intensities of the ^1H - ^{15}N peaks were fit to an exponential decay function, which provided the exchange rate, k_{ex} . By dividing the rate of exchange for the

amide in an unstructured protein (k_{rc}) by the rate of exchange in the folded protein, a protection factor (P_f) can be calculated (154). The free energy of exchange (and by extension, the structural change) is related to the log of the protection factor. The protection factors for residues are reported in **Table 5.3**. The average protection factor for residues in helices observable by RT HDX is reported in the final column. As shown in **Table 5.3** and **Figure 5.2**, helices 1 and 4 show the least protection of the five helices, suggesting that they undergo more frequent structural transitions to a state that de-protects the amide proton relative to helices 2, 3, and 5. This is congruent with the difficulty in assigning helix 1 residues (135) and the conformational mobility required in helices 1 and 4 to adopt the conformation seen upon binding to F-actin (Chapter 3).

Vt Q1018K ps-ns backbone dynamics

To further explore the dynamic properties of Vt, standard R_1 , R_2 , and $\{^1H\}$ - ^{15}N NOE NMR experiments were collected at two fields and the data analyzed to obtain the requisite parameters (**Figure 5.3**). These experiments probe NH backbone dynamics on a ps-ns timescale. The relaxation profiles for the majority of the residues that comprise the helix bundle, with high $\{^1H\}$ - ^{15}N NOE values and similar R_1 and R_2 values, indicate that these residues are not flexible on a ps-ns timescale. The unstructured regions have high R_1 values, and, generally, lower R_2 and $\{^1H\}$ - ^{15}N NOE values, indicative of residues with flexible backbone motions on a ps-ns timescale. The primary exceptions to this are the helix 3-helix 4 loop, which has higher R_2 rates, and the N-terminal strap. The N-terminal strap has $\{^1H\}$ - ^{15}N NOE values indicative of residues that are not highly flexible, and supports the notion that the interactions between the strap and the helix bundle are relatively stable. The larger R_2 values at the helix 3-helix 4 loop suggest that this portion of Vt Q1018K may undergo motions on a μ s-ms timescale.

The helix 3-4 loop, the C-terminal arm, and helix 1 undergo μ s-ms backbone motions

In the current models for allostery (155, 156), many proteins sample their bound/active and unbound/unbound conformations independent of the presence of the allosteric ligand. In this model, conformational selection, the ligand alters the relative populations of each state. Transitions among these states often occur on a μ s-ms timescale and may be detectable in the absence of a ligand. Vt undergoes an allosteric change upon binding to F-actin (120), and μ s-ms motions detected in the helix 3-helix 4 loop may be related to this conformational change.

The product of R_1 and R_2 is a good indicator of μ s-ms motions, and limits the influence of rotational anisotropy on the metric (157). Residues with significantly larger products likely experience chemical exchange on the μ s-ms timescale. The average product for residues in helices at 600 MHz is 15.9 s^{-2} , while the average product for residues in helices at 700 MHz is 13.2 s^{-2} (**Figure 5.4**). Residues with a product greater than the average by at least 2.5 times the error in the product at 600 MHz are K966, K970, Q971, and T979. Those that fit these criteria at 700 MHz are D882, A893, A921, L947, K970, Q971, R976, R978, N980, R1039, K1047, R1049, and D1051. The majority of these residues are clustered around the helix 3-helix 4 loop, with others in the C-terminus of helix 5, which is near the helix 3-helix 4 loop.

With R_1 , R_2 , and $\{^1\text{H}\}\text{-}^{15}\text{N}$ NOE values for Vt Q1018K at two fields, model-free analysis was performed to better characterize the protein dynamics (146, 147, 158, 159). Using relxn2.2, the six datasets were fit to 6 different models (**Tables 5.4, 5.5**) and AIC was used to select the best model. The results of the analysis are visualized in **Figure 5.5**. The order parameter, S^2 , reports on the flexibility of the backbone N-H bond vector. These values support what was seen in the NOE data, that the C-terminus of Vt Q1018K is flexible, as expected, but the N-terminus is less flexible. Additionally, the loops seem to be relatively flexible (and poorly fit), with the helix 4-helix 5 loop having the lowest average S^2 order

parameter. Some of the residues fit best to models with a R_{ex} component. These residues are mapped onto the structure of Vt in **Figure 5.5**, and are spread across the structure. However, residues with the largest R_{ex} values are near the bottom of the helix bundle, suggesting that there may be μ s-ms motions near the helix 3-helix 4 loop and the C-terminal arm.

To further confirm the presence of exchange on a μ s-ms timescale, CPMG data were collected at 700 MHz for Vt Q1018K. Put simply, CPMG pulses suppress the effects of chemical exchange on a μ s-ms timescale. The more frequent the CPMG pulses, the better the effects of μ s-ms motions are suppressed, and the effective R_2 ($R_{2,eff}$) of the nucleus decreases. Thus, if the nucleus (and its residue) is undergoing chemical exchange on a μ s-ms timescale, a decrease in the $R_{2,eff}$ is observed as the CPMG pulse frequency increases. The average $R_{2,eff}$ for the four longest delay times (five total data points) was subtracted from the average $R_{2,eff}$ for the four shortest delay times (five total data points). The dispersion curves for residues that exhibited a difference in the average $R_{2,eff}$ greater than 4 s^{-1} were evaluated and residues with strong outliers that exaggerated the average were discarded. The remaining 13 residues are shown in **Figure 5.6**. As with the model-free analysis and the R_1R_2 product, the majority of these residues reside near the helix 3-helix 4 loop. The relatively high error is due, to the low concentration (200 μ M) of Vt Q1018K, used to limit self-association, and the loss of signal to relaxation.

Mutation of P989 likely disrupts the kink in helix 4

The presence of μ s-ms motions near the lower actin binding site at the helix 3-helix 4 loop (103, 121, 123) suggested that these motions might be involved or related to the conformational change that takes place in Vt when it binds F-actin (10, 120). The kink in helix 4 caused by P989 (**Figure 5.1**) was identified as a potential structural feature that may facilitate such motions. We postulated that removal of the helix 4 kink would likely disrupt

the μ s-ms motions near the lower F-actin binding site and influence the ability of Vt to adopt the appropriate conformation for crosslinking actin filaments.

To test this, P989 was mutated to alanine (P989A) to straighten helix 4. Alanine introduces an amide proton to form the missing hydrogen bond with C985 and has the highest helical propensity of the 20 native amino acids (160). CD was the first technique applied to evaluate the structural consequences of the Vt P989A mutation. As shown in **Figure 5.7**, the far-UV spectra for Vt P989A is similar to that obtained for Vt (**Figure 5.7**), suggesting that the mutation does not significantly alter the helical character of Vt. This was somewhat expected, as altering the kink in a single helix is unlikely to significantly contribute to the relative signal for an α -helical protein. Additionally, the near-UV spectra for Vt and Vt P989A are similar as well, suggesting that the mutation does not disrupt the packing of W912 with W1058. This packing interaction indicates that the C-terminus of Vt is packing normally against the helix bundle and the helix 1-helix 2 loop; it is a reasonable proxy for proper tertiary structure (127). However, the CD thermal denaturation curve shows an increase in the Vt P989A denaturation midpoint of 4°C relative to Vt, yet it unfolds in a less cooperative manner. The decrease in cooperativity and increase in thermal stability indicate that P989A is likely adopting a slightly different conformation from Vt, supporting the expectation that helix 4 in Vt P989A is no longer kinked.

To further evaluate the structural changes induced by the P989A mutation, a ^1H - ^{15}N 2D HSQC was collected with Vt P989A/Q1018K (**Figure 5.8**). The introduction of the P989A mutation caused at least 45 (24.4%) ^1H - ^{15}N peaks to significantly shift. These shifted signals were found in helices 2-5, indicating that the effects of the P989A mutation were not restricted to residues in the immediate vicinity of 989. Because of the large number of shifts, assignments from Vt Q1018K could not be used for Vt P989A/Q1018K. Hence, 3D HNCACB and CBCACONH experiments were run to assign the HN, N, C α , and C β chemical shift values for Vt P989A/Q1018K. Only 79.5% of the non-proline C α resonance assignments were

obtained for Vt P989A/Q1018K, as many of the residues preceding A989 could not be assigned (**Figure 5.8**). The difficulty in assigning these residues is due primarily to their weak signals, suggesting that these signals are line broadened and undergo conformational exchange between two or more states on the intermediate NMR time scale at 700 MHz. Additionally, the C α and C β shifts for the residues at the end of helix 3 are significantly shifted compared to the signals in Vt Q1018K. Though these resonances shifted, they still were indicative of resonances from residues in an α -helix, suggesting that helix 3 maintains its helicity. These data suggest that the P989A mutation removes the helix 4 kink, potentially changing the dynamics at the helix 3-helix 4 loop.

Mutation of P989 changes the backbone dynamics of Vt

To further explore how the P989A mutation affects Vt backbone dynamics, R₁, R₂, and {¹H}-¹⁵N NOE data were collected for Vt P989A/Q1018K on a 700 MHz magnet (**Figure 5.9**). Upon comparison to Vt Q1018K, there are minimal changes in the relaxation rates and NOE ratios. The largest difference is the decreased R₂ values at the helix 3-helix 4 loop in Vt P989A/Q1018K, which results in a decreased R₁R₂ product for these residues (**Figure 5.9**), suggesting that the P989A mutation disrupts the μ s-ms time scale dynamics at the helix 3-helix 4 loop, likely changing the timescale of or eliminating these motions. Moreover, these results indicate that the helix 4 kink contributes to μ s-ms timescale motions associated with the helix 3-helix 4 loop. In contrast, the higher R₁R₂ values observed for residues 893 and 896 are maintained, suggesting that μ s-ms motions associated with these residues are not significantly disrupted by the P989A mutation and are unlikely coupled to motions at the helix 3-helix 4 loop.

Mutation of P989 does not significantly affect the ability to bind or bundle F-actin

P989 resides near the F-actin binding interface (103) and creates a kink in helix 4. As this helix plays a key role in actin-binding, the actin binding and bundling properties of Vt

P989 were evaluated. The ability of Vt P989A to bind or bundle F-actin is similar to that observed for Vt (**Figure 5.10**). These data provide further support for the cryo-EM model (Chapter 3) that shows helix 4 is straight when Vt binds to F-actin.

Mutation of Vt P989 changes the morphology of crosslinked actin filaments

The loss of the helix 4 kink did not change the affinity of Vt P989A for F-actin or its ability to quantitatively crosslink filaments relative to Vt (**Figure 5.10**). We wanted to test if these changes caused structural changes in the Vt:actin interactions that would not be observed by an actin co-sedimentation assay. To that end, Vt and Vt P989A were incubated with F-actin, phalloidin was added to label actin, and then the sample was evaluated with a fluorescent microscope. As expected, in the absence of Vt, the actin remains polymerized, but is not bundled. Addition of Vt resulted in actin bundles, crosslinked by Vt (**Figure 5.11**) (6). Addition of Vt P989A produced actin bundles, in agreement with the co-sedimentation results. However, the actin bundles crosslinked by Vt P989A tended to be shorter and thinner. The resolution limitations of fluorescence microscopy (hundreds of nanometers) prevented a more detailed description of the structural differences.

To overcome these limitations, negative-stain EM was used to characterize the F-actin bundles at a higher resolution. F-actin, in the absence of Vt, did not form crosslinked filaments (**Figure 5.12**). Addition of Vt resulted in actin bundles that were comprised primarily of parallel actin fibers. The addition of Vt P989A still produced actin bundles, but these bundles tended to have an increased number of cross-over events, where the filaments wound over each other. Other bundles tended to fray or splay out at the ends. These results suggest that Vt P989A slightly alters the conformation of Vt bound to F-actin and/or the Vt dimer responsible for crosslinking F-actin such that the actin filaments no longer run parallel to each other. These slight structural differences are not distinguishable by actin co-sedimentation, which relies on a difference in the size of the crosslinked actin bundles. Instead, the bundle architecture is observable by negative-stain EM. These findings suggest

that conformational sampling between two or more states on the μ s-ms timescale at the helix 3-helix 4 loop and nearby residues is involved in the Vt:F-actin interaction. One or more of these conformers may contribute to or promote the conformational changes that take place when Vt binds F-actin and subsequently dimerizes. Disruption of these conformations or the ability to sample them by the P989A mutation may cause the observed changes in the actin bundle geometry we observed.

Discussion

Protein function is regulated by both the protein's structure and dynamics. Vt's ability to bind and bundle F-actin relies on its structural plasticity (Chapter 3). Vt changes conformation upon binding to F-actin, which is required for F-actin crosslinking (10, 120), but the distinct structural and dynamic details of this transition are only beginning to be understood. The work presented here begins to address some of these gaps.

One of the most surprising observations is that, compared to the C-terminus of Vt, residues in the N-terminus have higher order parameters (S^2) and thus fewer motions on the ps-ns timescale (**Figure 5.5**). X-ray structures of Vt suggest that the strap can adopt multiple conformations (2, 10, 133) while packing against the helix bundle. These results support the notion that the N-terminal strap interacts strongly with the Vt helix bundle, and that the different conformations for the strap do not exchange on a ps-ns timescale. This is further supported by the fact that removal of the N-terminal strap increases the affinity of Vt for both F-actin and PIP_2 (43), suggesting that release of the strap from the helix bundle has an energy cost. In the case of PIP_2 -binding, release of the strap increases the exposure of the basic collar binding site (Chapter 2) (40), and in F-actin binding, it likely decreases the energy cost for helix 1 partitioning away from the helix bundle.

Helices 1 and 4 of Vt appear to be the most dynamic of the five helices in the helix bundle. The majority of residues in helix 1 have eluded observation by NMR, likely due to conformational exchange of helix 1 on an intermediate NMR timescale. This is supported by

the observation that helix 1 is partitioned from the helix bundle when Vt binds to F-actin (Chapter 3). Indeed, it is quite possible that this unfolding event occurs in the absence of actin and that the partitioned conformation is one of the conformations being sampled by helix 1 that prevents backbone assignment. Direct observation of this event has been difficult, however, and more work is required, possibly by electron paramagnetic resonance or fluorescent studies, to confirm this hypothesis. Helix 4 is also dynamic. Though the majority of its residues are observable by NMR, its backbone amides have the lowest average protection factor compared to residues in the remaining helices as measured by HDX. This indicates that helix 4 is undergoing more frequent local unfolding events than the helices 2, 3, or 5. Additionally, backbone dynamics on the μ s-ms timescale at the helix 3-helix 4 loop, which are ablated by the P989A mutation, suggest that the N-terminal portion of helix 4 undergoes conformational exchange that is not an unfolding event. It is quite intriguing that these two dynamic helices, 1 and 4, feature prominently in the conformational changes that take place when Vt binds F-actin (Chapter 3). As mentioned earlier, helix 1 partitions from the helix bundle upon actin binding and helix 4 significantly straightens, losing its kinked conformation.

Both the model-free analysis and the CPMG data suggest that many other residues near and in the C-terminal arm possess backbone μ s-ms motions. The presence of these motions provide for the possibility of a dynamic network that extends from the helix 3-helix 4 loop back to the helix 1-helix 2 loop. The decreased R_1R_2 product of the helix 3-helix 4 loop residues in Vt P989A suggests that such a network is also dependent upon the kink in helix 4 and the flexibility it provides. Further characterization by collecting CPMG data at additional field strengths is required to better quantitate these backbone motions and the existence of a possible dynamic network.

The CPMG experiment, in addition to reporting on internal protein motions, may also be reporting on the dimerization of Vt (described in Chapter 4) (10, 127). Its ability to see

very small populations means that, even using Vt Q1018K, there may be signal coming from a monomer/dimer equilibrium. However, the data do not support this. The $R_{2,\text{eff}}$ values for residues at the dimer interface are not significantly different across the CPMG frequencies used. Specifically, S1016 and T962, residues with HN chemical shifts that report on dimerization, have flat dispersion curves. This suggests that, at the low concentrations used, the contribution to relaxation dispersion from the monomer/dimer equilibrium is negligible. With the collection of dispersion data at other field strengths, this potential complication will be addressed further.

The similarities between sites that show μs -ms backbone dynamics and those that show lower protection factors with sites where Vt changes conformation upon binding to F-actin are intriguing. These similarities suggest that these μs -ms backbone dynamics may play a role in actin-binding and that Vt may sample the actin-bound conformation in the absence of F-actin. That this conformation has only recently been observed, and only in the presence of F-actin suggests that, if Vt does sample the actin-bound conformation in the absence of F-actin, only a small fraction of Vt samples it at a given time. If this is the case, it would mean that this conformation could be studied in the absence of F-actin by techniques that are sensitive to small populations of a sample, such as NMR or electron paramagnetic resonance.

Helix 4 and the C-terminal arm, sites of local unfolding and μs -ms backbone motions, mediate the autoinhibitory interaction between vinculin head and Vt (10, 28). These motions may be suppressed when vinculin is in its autoinhibited conformation, decreasing the entropy of the closed conformation and priming vinculin for activation. The kink in Vt helix 4 is a perfect candidate for such a mechanism, as we have now observed it in two distinct conformations. In the context of the autoinhibited vinculin structure, the proline-induced kink allows for Vt helix 4 to participate in two different head:tail interfaces (**Figure 5.13**) (2). However, upon binding to F-actin, the kink is no longer present in helix 4 (Chapter 3).

Given these findings, we would predict that vinculin P989A, which should remove the kink and straighten helix 4, will show weaker autoinhibitory interactions with the head domain, thereby increasing the population of active vinculin in cells. In addition to mediating different conformations in helix 4, the kink also plays a role in allowing μ s-ms backbone dynamics at the helix 3-helix 4 loop. However, the dramatic difference in F-actin bundling architecture induced by Vt P989A was somewhat surprising. The affinity of Vt P989A for F-actin and its propensity to crosslink actin filaments remains unchanged, suggesting that the Vt conformation induced by the P989A mutation is conducive to these functions. Yet, the actin bundles formed by Vt P989A exhibit an altered structure; the filaments in these bundles do not appear parallel to each other. This altered geometry suggests that the P989A mutation somehow disrupts or alters the conformational change in Vt that drives actin-bundling geometry. This could be the result of disrupting the μ s-ms backbone motions at the helix 3-helix 4 loop (**Figure 5.9**), a function of changes in the helix bundle structure (**Figures 5.7, 5.8**), both, or some other mechanism. A better understanding of the Vt dimer responsible for crosslinking is likely required before this question can be fully addressed. However, the conservation of P989 indicates that the kinked nature of helix 4 is important in vinculin function.

Order	T1 (s)		T2 (ms)	
	600 MHz	700 MHz	600 MHz	700 MHz
1	1.304202	0.114202	110.5664	7.968
2	0.024202	3.514202	47.3856	103.584
3	0.114202	1.454202	63.1808	39.84
4	1.604202	0.369202	31.5904	15.936
5	0.304202	2.854202	15.7952	71.712
6	1.004202	1.854202	78.976	47.808
7	0.704202	0.654202	71.0784	31.872
8	0.854202	2.354202	134.2592	55.776
9	0.554202	0.984202	94.7712	23.904
10	1.304202	0.114202	110.5664	39.84
11	0.114202	2.854202	31.5904	7.968
12	0.704202	0.984202	71.0784	71.712

Table 5.1. Times of the relaxation periods used in R_1 and R_2 NMR experiments.

Experiment number	time (min)	time (hr)
1	19.5	0.325
2	29.13333	0.485556
3	40	0.666667
4	55	0.916667
5	70	1.166667
6	85	1.416667
7	100	1.666667
8	120	2
9	140	2.333333
10	165	2.75
11	190	3.166667
12	220	3.666667
13	260	4.333333
14	310	5.166667
15	370	6.166667
16	460	7.666667
17	550	9.166667
18	670	11.16667
19	816	13.6
20	962	16.03333
21	1108	18.46667
22	1254	20.9
23	1400	23.33333
24	1460	24.33333
25	1845	30.75
26	1880	31.33333
27	2333	38.88333
28	2873	47.88333
29	4311	71.85
30	8963	149.3833
31	13067	217.7833
32	15821	263.6833
33	25994	433.2333

Table 5.2. Hydrogen exchange times.

The times since addition of Vt Q1018K to a PD-10 column to the midpoint for each fast-HSQC experiment are listed above. These times were used when fitting the data to an exponential decay curve.

Residue	$k_{ex} (10^3 \text{ min}^{-1})$	$k_{rc} (\text{min}^{-1})$	P_f	$\ln(P_f)$	Average $\ln(P_f)$
884	0.35 ± 0.04	10.64	30789 ± 3125	10.33 ± 0.1	
Helix 1					8.69 ± 0.13
900	8.7 ± 0.49	47.35	5440 ± 306	8.6 ± 0.06	
901	7.41 ± 0.55	48.46	6536 ± 480	8.79 ± 0.07	
Helix 2					10.85 ± 1.44
917	2.09 ± 0.24	171.93	82444 ± 9657	11.32 ± 0.12	
921	0.54 ± 0.02	37.61	69656 ± 2322	11.15 ± 0.03	
923	0.81 ± 0.09	34.30	42273 ± 4697	10.65 ± 0.11	
924	1.02 ± 0.84	59.61	58446 ± 48132	10.98 ± 0.82	
925	1.35 ± 0.21	9.89	7321 ± 1116	8.9 ± 0.15	
928	1.87 ± 0.15	48.46	25907 ± 2044	10.16 ± 0.08	
931	0.1 ± 0.04	48.46	504756 ± 189284	13.13 ± 0.38	
932	3.03 ± 0.43	13.50	4457 ± 634	8.4 ± 0.14	
934	0.43 ± 0.01	113.59	261494 ± 8307	12.47 ± 0.03	
935	1.13 ± 0.02	90.23	79568 ± 1684	11.28 ± 0.02	
937	7.12 ± 0.14	4.63	650 ± 13	6.48 ± 0.02	
938	4.76 ± 0.11	32.76	6885 ± 161	8.84 ± 0.02	
Helix 3					9.95 ± 2.29
948	17.38 ± 0.55	4.32	249 ± 8	5.52 ± 0.03	
949	16.32 ± 1.31	25.43	1558 ± 125	7.35 ± 0.08	
950	0.9 ± 0.02	248.51	275944 ± 5331	12.53 ± 0.02	
951	0.13 ± 0.02	133.46	1059209 ± 151316	13.87 ± 0.14	
952	0.62 ± 0.1	34.30	54975 ± 8458	10.91 ± 0.15	
953	0.28 ± 0.01	26.79	94407 ± 4191	11.46 ± 0.04	
955	0.26 ± 0.01	22.15	86655 ± 4679	11.37 ± 0.05	
956	2.04 ± 0.05	34.30	16816 ± 396	9.73 ± 0.02	
957	1.6 ± 0.1	49.59	30952 ± 1855	10.34 ± 0.06	
960	14.72 ± 0.2	9.46	643 ± 9	6.47 ± 0.01	
961	0.12 ± 0.01	5.92	49302 ± 5177	10.81 ± 0.11	
962	0.28 ± 0.01	23.19	83128 ± 3039	11.33 ± 0.04	
963	2.02 ± 0.06	71.67	35552 ± 1058	10.48 ± 0.03	
964	0.53 ± 0.05	16.42	30748 ± 2764	10.33 ± 0.09	
966	0.3 ± 0.03	34.30	114349 ± 11435	11.65 ± 0.1	
967	0.97 ± 0.02	17.79	18417 ± 458	9.82 ± 0.02	
968	0.05 ± 0.02	5.92	128057 ± 44903	11.76 ± 0.35	
969	0.1 ± 0.01	27.25	262516 ± 24279	12.48 ± 0.09	
970	3.9 ± 0.11	34.30	8801 ± 260	9.08 ± 0.03	
971	40.43 ± 6.89	56.93	1408 ± 240	7.25 ± 0.17	
972	0.13 ± 0.01	248.51	1872389 ± 164284	14.44 ± 0.09	
Helix 4					8.57 ± 2.05

977	13.65 ± 1.52	11.62	852 ± 95	6.75 ± 0.11	
980	2.67 ± 0.06	184.23	68999 ± 1551	11.14 ± 0.02	
981	4.75 ± 0.28	20.67	4354 ± 253	8.38 ± 0.06	
983	8.45 ± 0.42	26.63	3153 ± 156	8.06 ± 0.05	
984	0.83 ± 0.07	11.89	14418 ± 1154	9.58 ± 0.08	
985	0.86 ± 0.02	113.59	132319 ± 3610	11.79 ± 0.03	
986	16.43 ± 1.78	47.88	2915 ± 316	7.98 ± 0.11	
988	5.99 ± 0.5	11.62	1942 ± 163	7.57 ± 0.08	
991	8.16 ± 0.27	11.10	1361 ± 45	7.22 ± 0.03	
992	13.33 ± 0.54	51.92	3896 ± 157	8.27 ± 0.04	
997	22.13 ± 3.44	9.23	417 ± 65	6.03 ± 0.16	
998	10.59 ± 0.16	5.83	550 ± 9	6.31 ± 0.02	
999	0.24 ± 0.05	54.37	227003 ± 48326	12.33 ± 0.21	
Helix 5					10.66 ± 2.05
1017	0.79 ± 0.12	26.93	34259 ± 5230	10.44 ± 0.15	
1018	3.41 ± 0.23	27.04	7931 ± 540	8.98 ± 0.07	
1024	8.02 ± 0.16	4.63	577 ± 11	6.36 ± 0.02	
1027	0.36 ± 0.02	78.59	218298 ± 14553	12.29 ± 0.07	
1028	3.28 ± 0.07	43.19	13176 ± 283	9.49 ± 0.02	
1029	1.01 ± 0.14	184.23	181683 ± 25801	12.11 ± 0.14	
1030	0.08 ± 0.01	20.67	275608 ± 28663	12.53 ± 0.1	
1031	5.19 ± 0.24	22.66	4364 ± 202	8.38 ± 0.05	
1032	1 ± 0.05	55.64	55524 ± 2992	10.92 ± 0.05	
1033	2.34 ± 0.05	139.75	59722 ± 1225	11 ± 0.02	
1035	0.02 ± 0.01	24.85	1294349 ± 687623	14.07 ± 0.53	
1037	0.12 ± 0.01	25.24	211367 ± 21243	12.26 ± 0.1	
1038	0.04 ± 0.01	11.89	275340 ± 57362	12.53 ± 0.21	
1039	0.15 ± 0.01	32.76	215815 ± 17060	12.28 ± 0.08	
1040	0.53 ± 0.02	22.40	41943 ± 1885	10.64 ± 0.04	
1041	0.13 ± 0.01	29.65	221611 ± 21863	12.31 ± 0.1	
1042	0.53 ± 0.01	13.50	25589 ± 611	10.15 ± 0.02	
1043	15.31 ± 0.28	29.65	1936 ± 35	7.57 ± 0.02	
1044	10.78 ± 0.84	37.61	3491 ± 272	8.16 ± 0.08	

Table 5.3. Protection factors for Vt Q1018K derived from NMR hydrogen exchange data.

Model	Parameters Fit
1	S^2 , τ_e
2	S^2 , τ_e , τ_m
3	S^2 , τ_e , CSA
4	S^2 , τ_e , τ_m , CSA
5	S^2 , R_{ex}
6	S^2 , τ_e , R_{ex}

Table 5.4. Models used for Lipari-Szabo model-free fitting of R_1 , R_2 , and $\{^1H\}$ - ^{15}N NOE datasets.

The parameters fit for each of the different models using the R_1 , R_2 , and $\{^1H\}$ - ^{15}N NOE datasets are shown. S^2 is the order parameter and describes the rigidity of the N-H bond vector relative to the protein, τ_m is the tumbling time of the molecule, τ_e is the rate of internal motion on a ps-ns timescale, chemical shift anisotropy (CSA) describes the anisotropic variation in the surrounding magnetic field of the ^{15}N nucleus, and R_{ex} is the relaxation component due to conformational exchange, often on a μ s-ms timescale.

Residue	Model
881	3
882	2
884	3
885	3
887	4
888	4
889	2
890	2
891	2
892	2
893	6
894	3
895	3
896	4
900	6
901	5
902	1
915	2
916	1
917	4
921	5
923	3
924	4
925	5
926	5
928	4
929	1
930	3
931	5
932	3
933	5
934	3
935	3
937	3
938	3
939	3
940	3
941	3
943	2
944	3

945	3
946	4
947	6
948	2
949	3
950	1
951	2
952	2
953	3
954	3
955	4
956	2
957	4
958	3
959	4
960	2
961	6
962	1
963	2
964	3
965	4
966	3
967	2
968	5
969	4
970	6
971	6
972	2
973	2
974	2
975	5
976	6
977	2
978	4
979	4
980	2
981	3
982	4
983	3
984	3
985	4

986	2
987	3
988	3
990	4
991	1
992	3
993	4
996	4
997	3
998	3
999	4
1003	3
1004	4
1005	2
1006	3
1007	3
1008	2
1009	1
1010	3
1011	2
1012	3
1013	4
1014	2
1015	2
1016	1
1017	2
1018	2
1020	5
1023	3
1024	5
1025	5
1026	4
1027	5
1028	2
1029	2
1030	5
1031	5
1032	2
1033	6
1034	2
1035	1

1037	2
1038	5
1039	6
1040	5
1041	1
1042	2
1043	2
1044	4
1045	1
1046	2
1047	1
1048	2
1049	3
1050	2
1051	2
1052	3
1053	2
1054	2
1055	2
1056	3
1057	3
1059	4
1061	2
1062	2
1064	2
1065	4
1066	2

Table 5.5. Models chosen by AIC to fit each residue.

Each residue for which NMR data was obtained was fit to one of six models (Table 5.4). The model was chosen based on the AIC for the fit of each model.

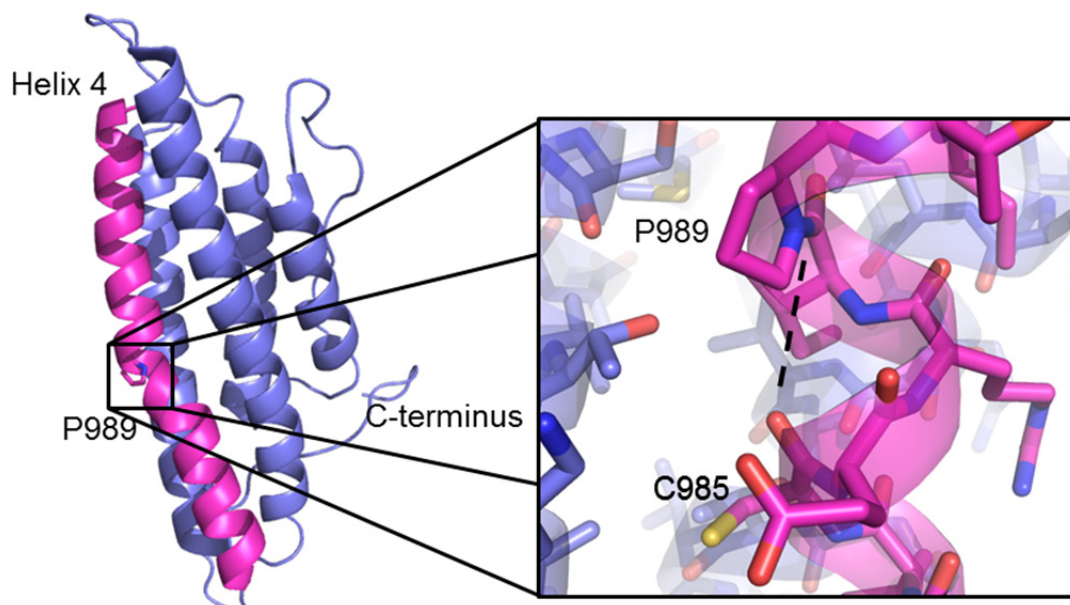


Figure 5.1. Vt helix 4 is kinked by the presence of proline 989.

The presence of proline 989 in the middle of helix 4 induces a kink in the helix. On the left is Vt, with helix 4 in pink and P989 in sticks. On the right is a magnified image of P989. Also shown is cysteine 985, and the missing hydrogen bond between P989 and C985 is shown with a dashed line.

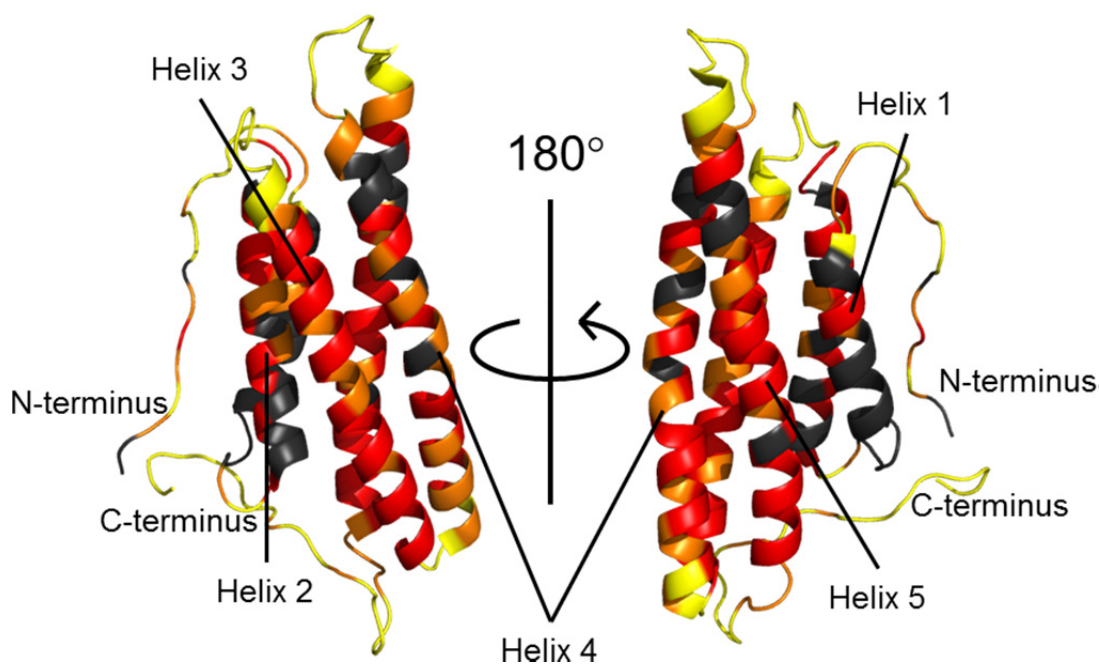


Figure 5.2. Hydrogen exchange rates for Vt Q1018K.

Vt residues are grouped according to hydrogen exchange rate. Residues in red undergo amide proton-deuterium exchange on a time scale slow enough to be observed by RT HDX. Residues in yellow exchange fast enough to be detected by CLEANEX. Residues in orange exchange too quickly to be observed by RT HDX but too slowly to be observed by CLEANEX. Residues in black are unassigned.

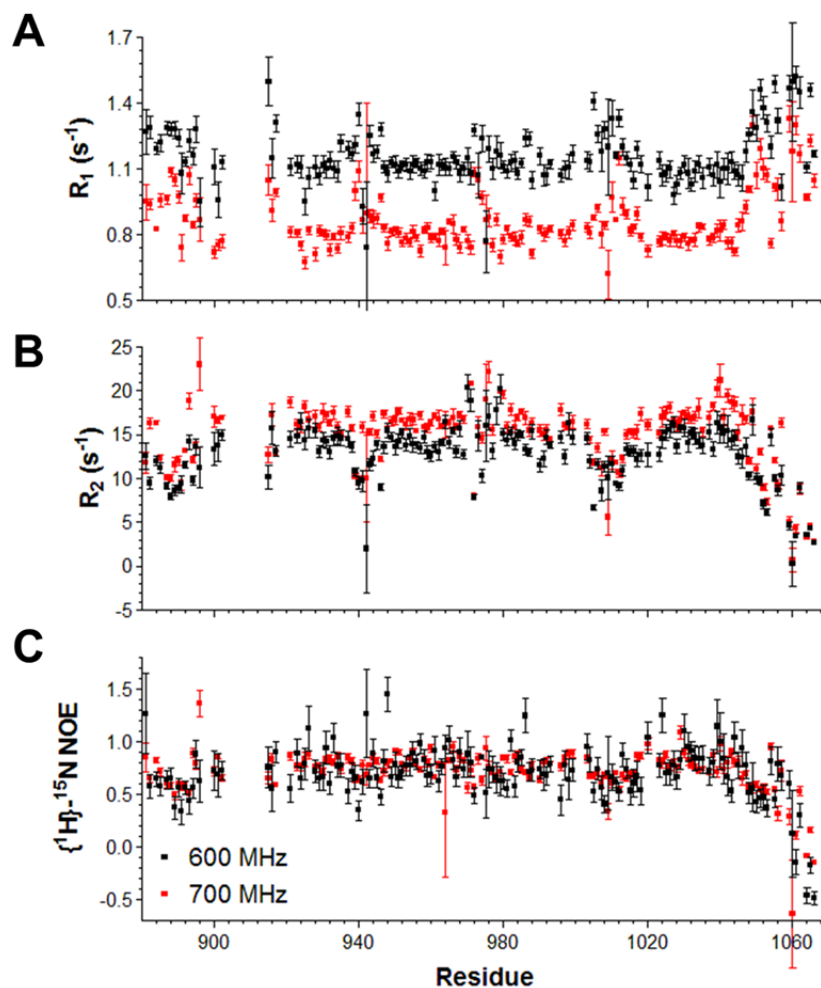


Figure 5.3. R_1 , R_2 , and $\{^1H\}-^{15}N$ NOE values for Vt Q1018K at two separate fields.

The results of the R_1 (A), R_2 (B), and $\{^1H\}-^{15}N$ NOE (C) NMR experiments are plotted above as a function of the Vt residue. Increases in R_1 occur at loops, as do the decreases in R_2 . Heteronuclear NOE values decrease at the C-terminus, as expected, but are higher than would be expected for the unstructured N-terminus.

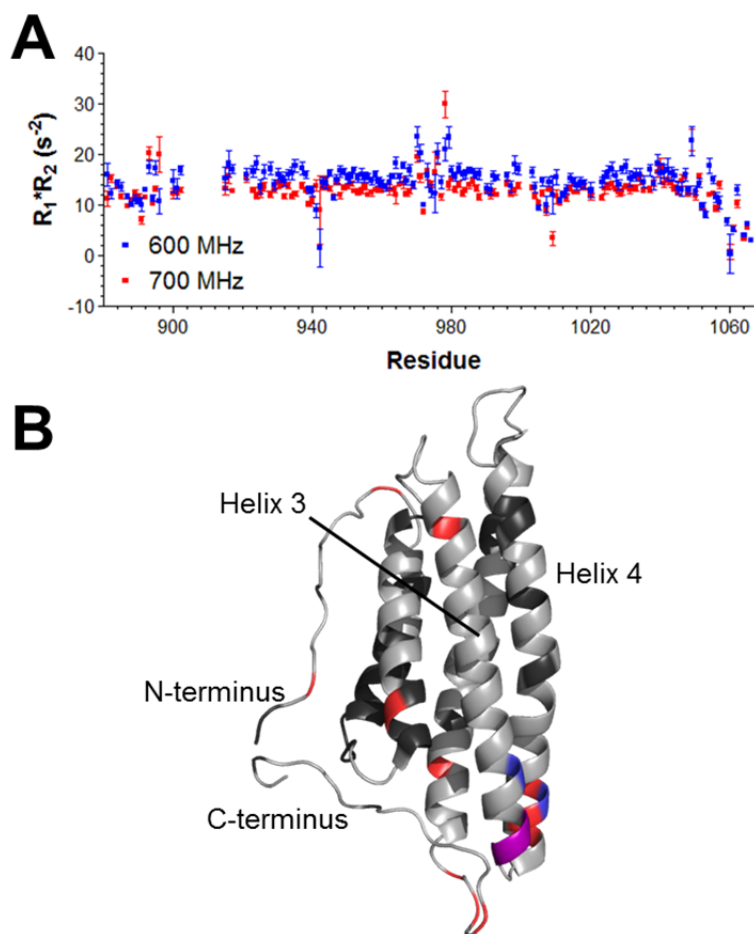


Figure 5.4. The R_1R_2 product for Vt Q108K at two fields.

The R_1R_2 product as a function of Vt Q1018K residue is plotted above (A). Residues with significantly larger products are mapped onto the Vt structure (B, PDB file 1ST6). Those with a significantly larger product at 600 MHz are highlighted in blue, at 700 MHz in red, and at both fields in purple. The majority of these residues are located near the helix 3-helix 4 loop.

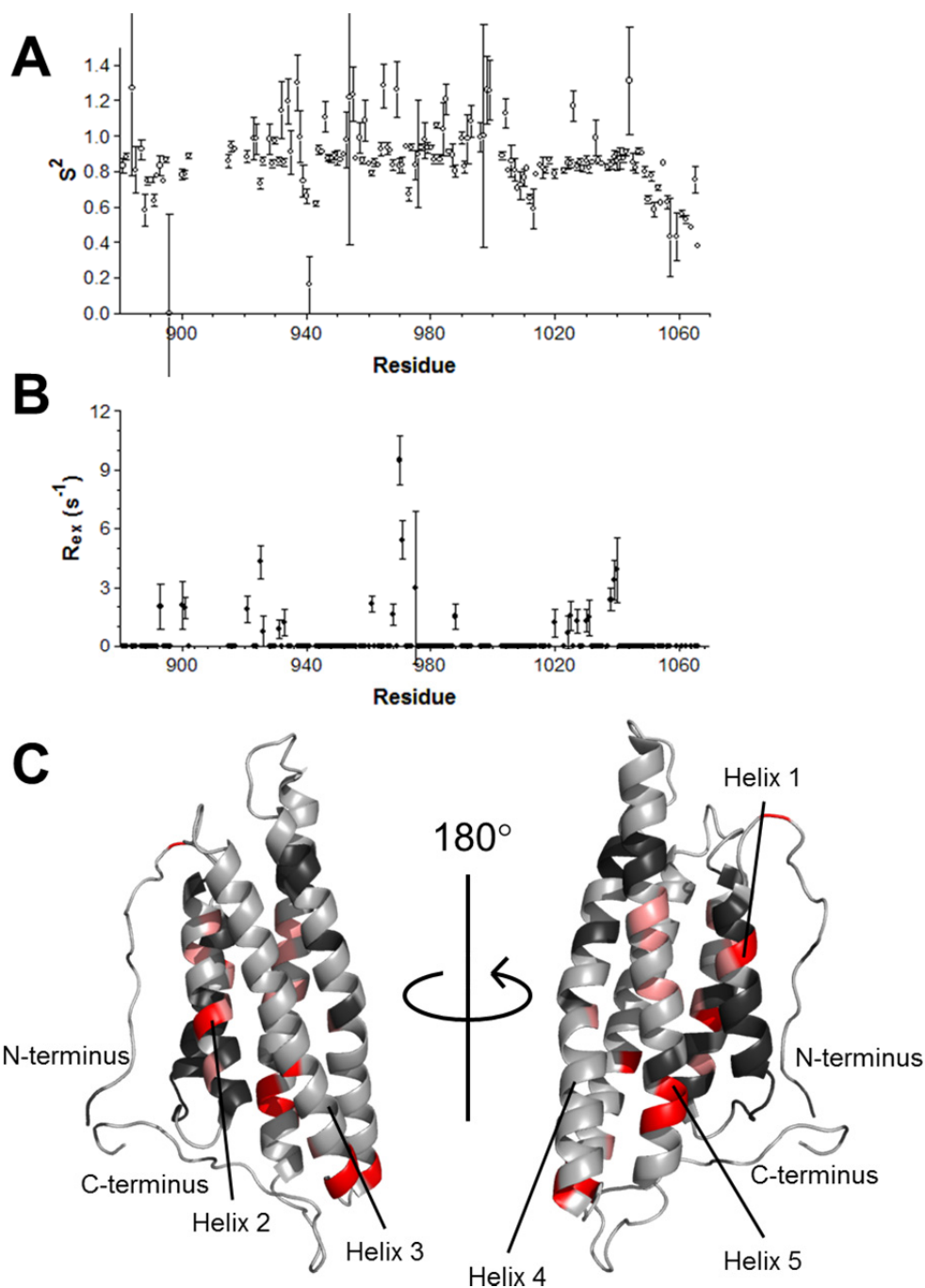


Figure 5.5. Analysis of relaxation data by mode-free formalism.

The results of the analysis with `relxn2.2` are shown. The S^2 parameter provides information on backbone flexibility on the ps-ns timescale (A). A value of 1 corresponds to a completely rigid bond, while a value of 0 corresponds to a vector that is completely free to sample any orientation. R_{ex} is plotted for residues whose data are fit best using model 5 or 6

(B). These residues are highlighted on Vt (C, PDB file 1ST6). Residues with $R_{\text{ex}} > 2 \text{ s}^{-1}$ are colored in bright red, while those that show $R_{\text{ex}} < 2 \text{ s}^{-1}$ are pink. Unassigned residues are colored in black.

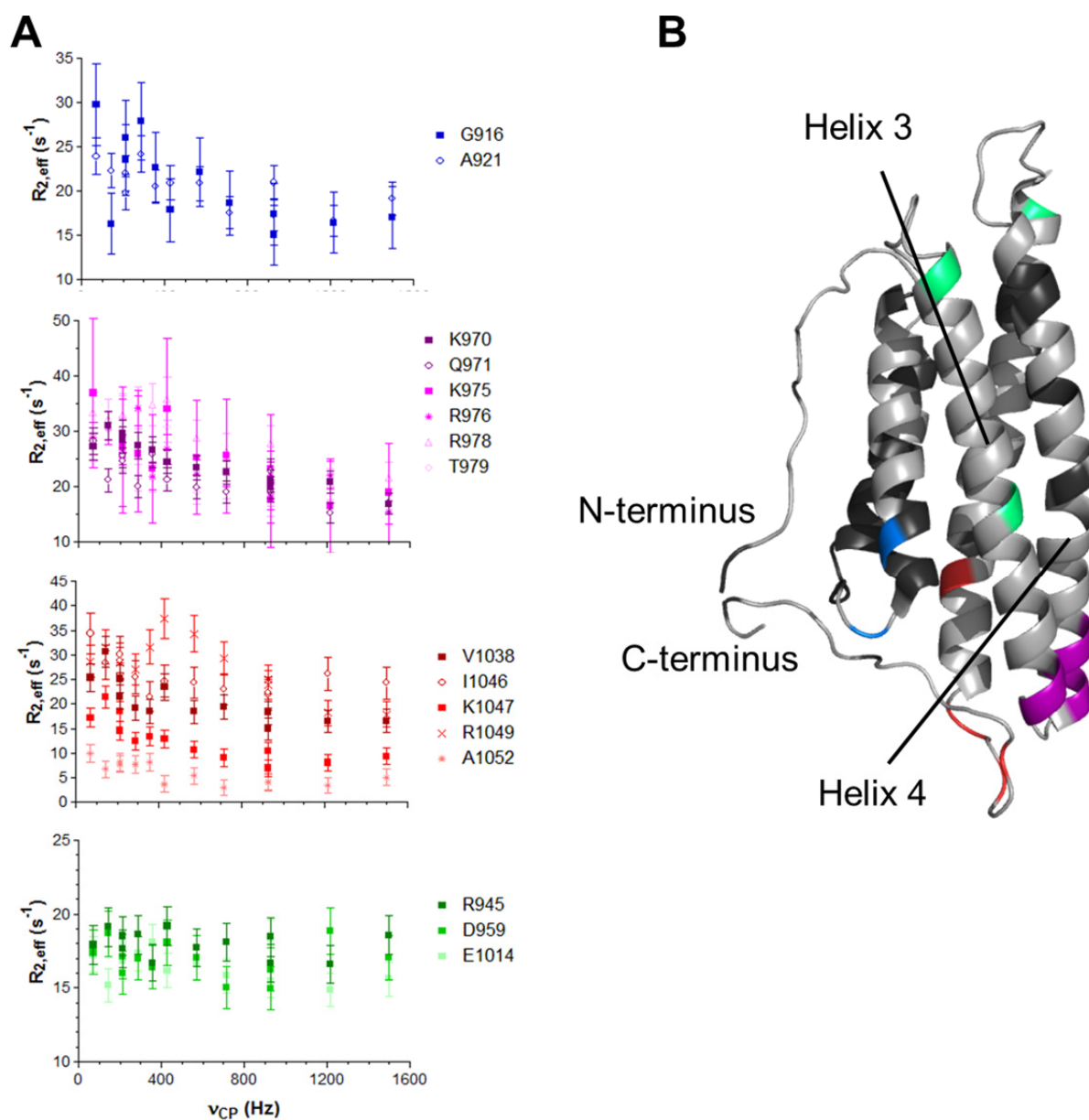


Figure 5.6. NMR CPMG results for Vt Q1018K.

Dispersion curves for different groups of residues collected on a 700 MHz magnet are shown (A). Blue, red, and purple residues all show changes in $R_{2,\text{eff}}$ as a function of the frequency of the CPMG pulse train. Green residues show no change in $R_{2,\text{eff}}$. These residues are mapped onto Vt (B, PDB file 1ST6).

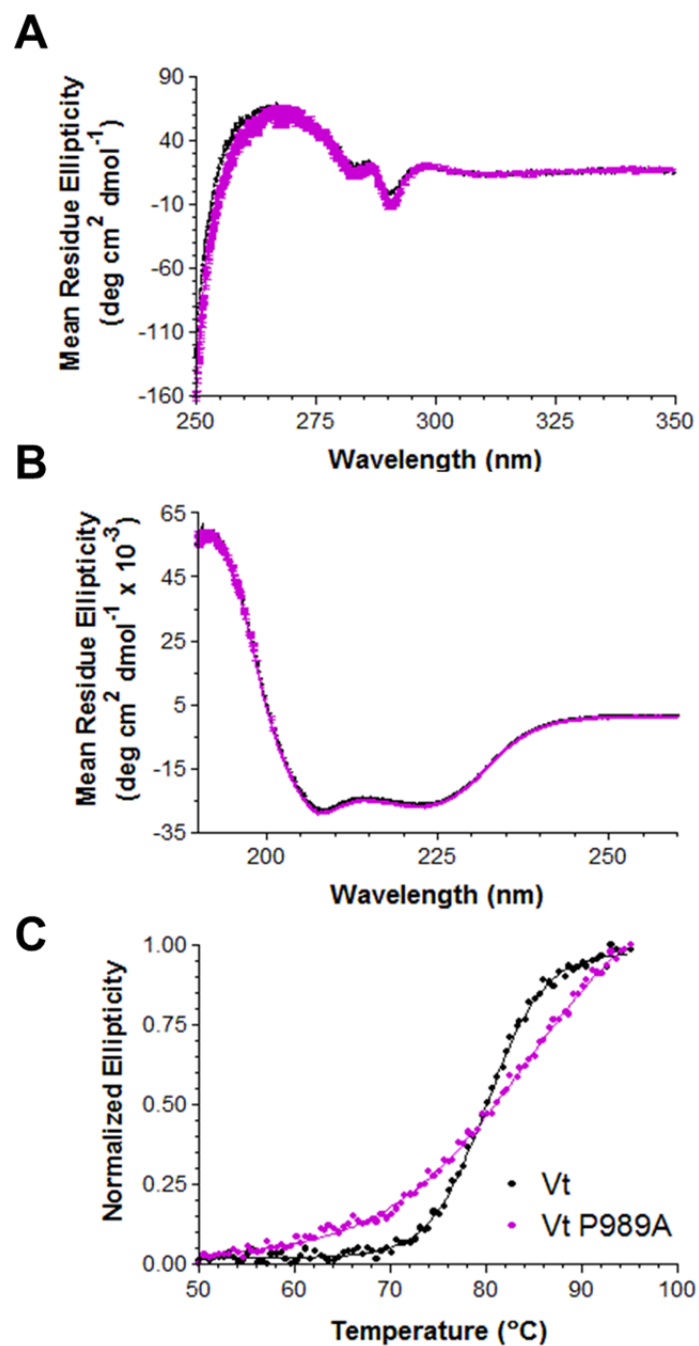


Figure 5.7. CD data for Vt P989A.

Shown are the near-UV CD (A), far-UV CD (B), and thermal denaturation (C) data for Vt and Vt P989A. Vt is in black, with Vt P989A in purple. While near- and far-UV CD data

for WT Vt and P989A Vt significantly different, thermal denaturation profiles indicate that Vt P989A unfolds in a less cooperative manner than Vt, yet has a slightly higher denaturation midpoint.

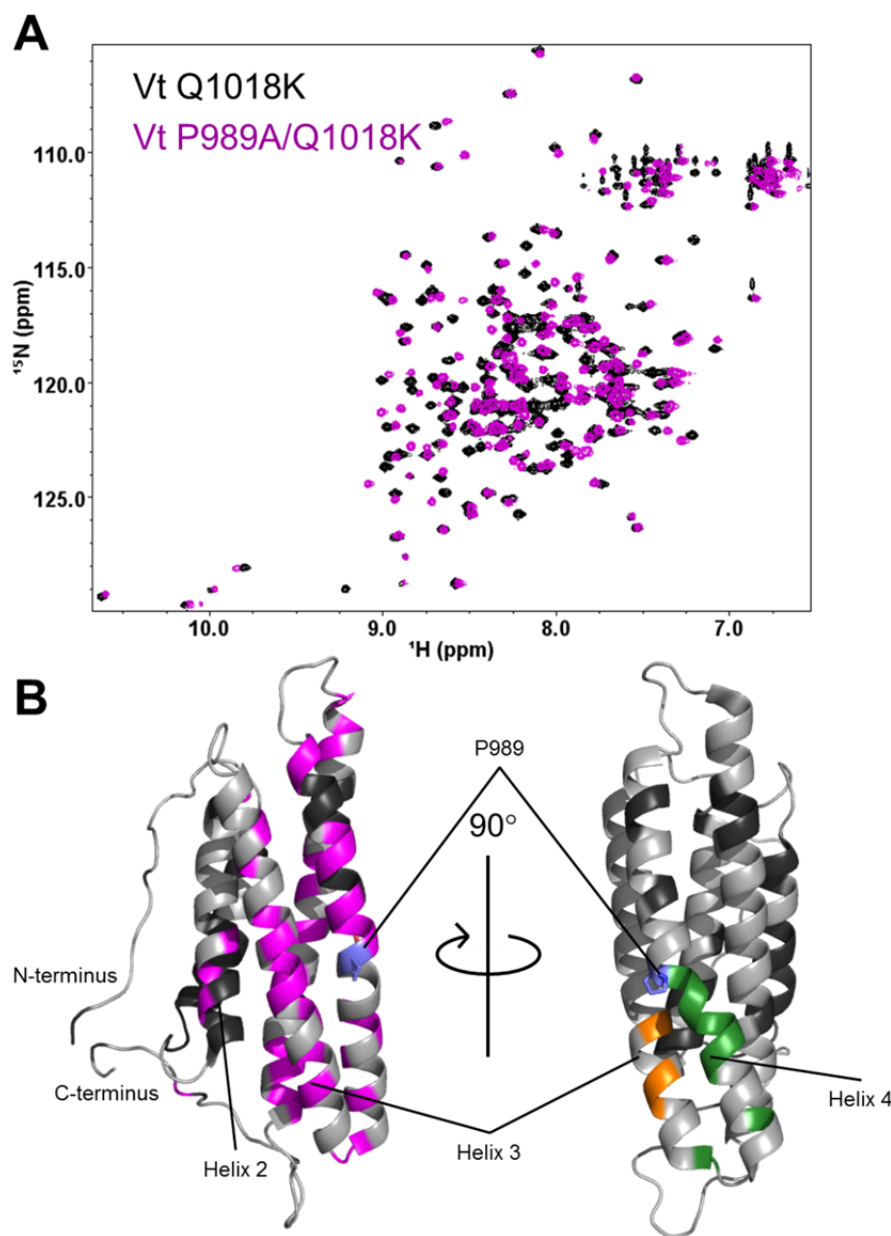


Figure 5.8. NMR of Vt P989A.

Shown is a 2D ^1H - ^{15}N HSQC of Vt P989A/Q1018K (purple) overlaid with Vt Q1018K (black) (A). Vt Q1018K NH peaks that shift in the context of a P989A mutation are mapped onto the Vt structure (PDB file 1ST6) on the left in magenta (B). Unassigned residues are in black, and P989 is in blue. The ribbon diagram on the right shows residues with a significant change in their $\text{C}\alpha$ and $\text{C}\beta$ shifts (orange). Residues lacking assignments for Vt P989A/Q1018K but were assigned in Vt Q1018K are shown in green.

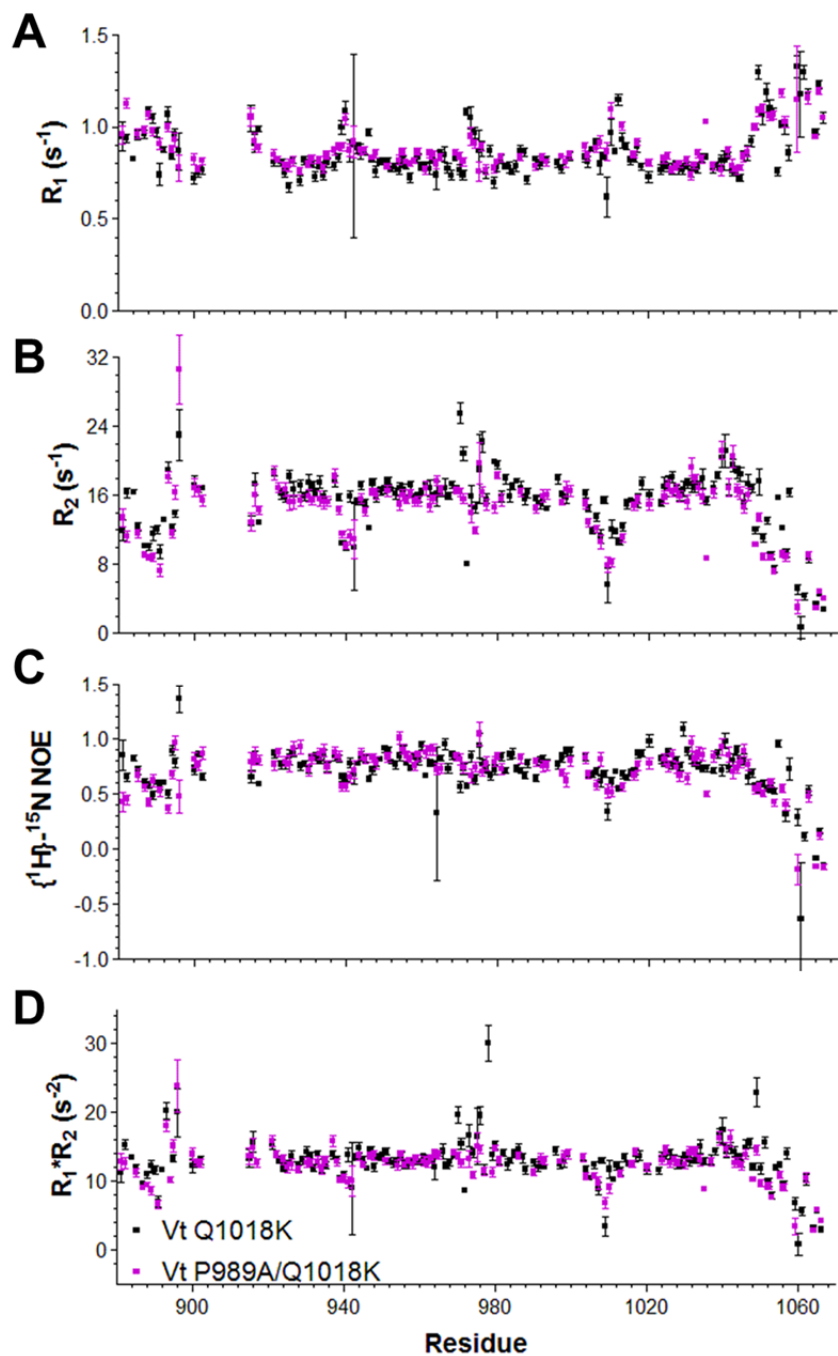


Figure 5.9. R_1 , R_2 , $\{^1\text{H}\}-^{15}\text{N}$ NOE, and R_1R_2 values for Vt P989A/Q1018K.

The R_1 (A), R_2 (B), $\{^1\text{H}\}-^{15}\text{N}$ NOE (C), and R_1R_2 (D) values for Vt P989A/Q1018K collected at 700 MHz are shown compared to those for Vt Q1018K. These data are quite similar for the two constructs for most residues. However, for residues 970-980, the R_2 rates are lower for Vt P989A/Q1018K than for Vt Q1018K.

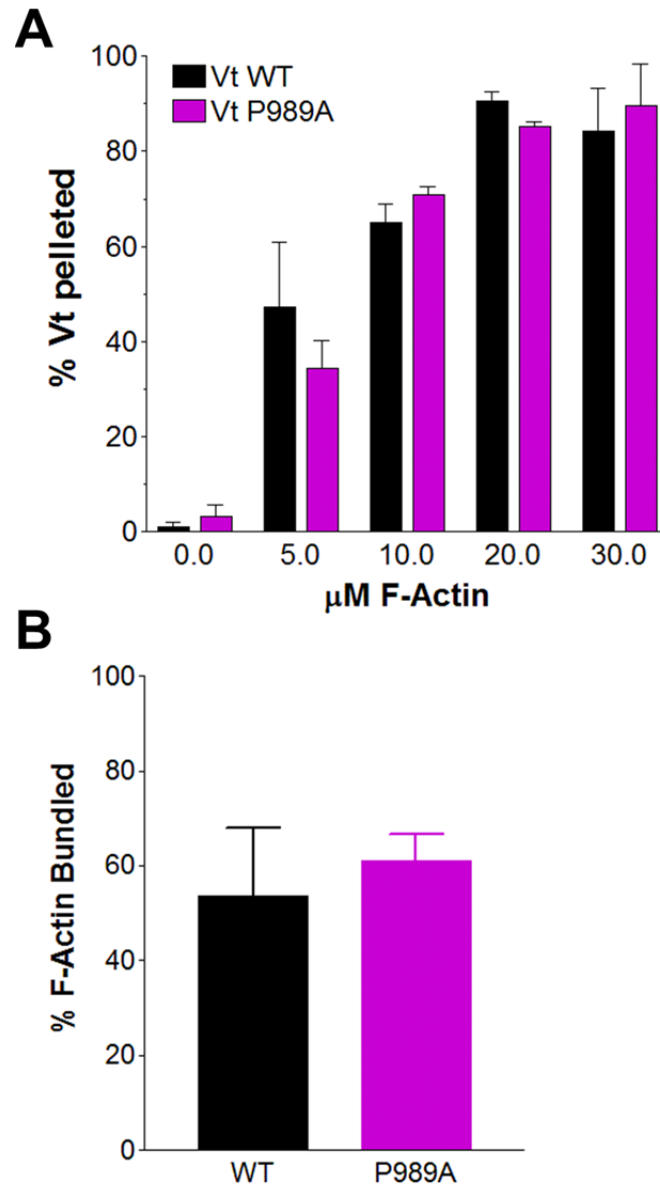


Figure 5.10. Actin binding and bundling by Vt P989A is similar to Vt.

Vt P989A (purple) is able to bind (A) and bundle (B) F-actin similarly to Vt (black) when evaluated by standard actin co-sedimentation assays. Averages are shown ($n=3$) and the errors are the standard deviations.

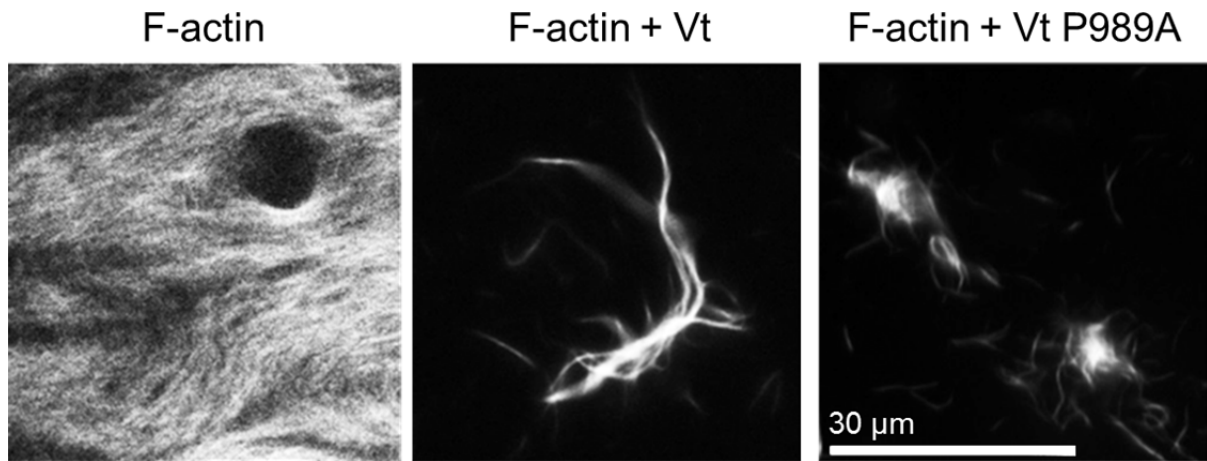


Figure 5.11. Fluorescent microscopy of F-actin bundles formed by Vt and Vt P989A.

Shown are representative images of F-actin stained with phalloidin. The addition of Vt results in crosslinking of the actin filaments into bundles. The addition of Vt P989A results in crosslinked filaments, though the bundles are generally shorter in appearance. The scale bar is 30 μm in length.

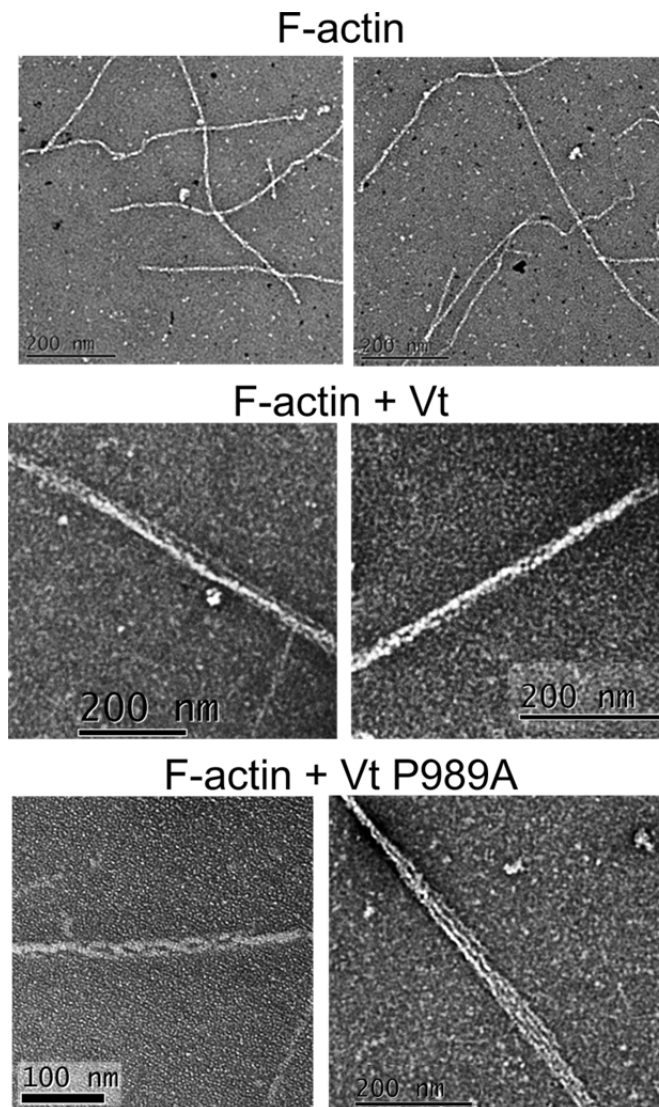


Figure 5.12. Negative-stain EM of F-actin crosslinked by Vt and Vt P989A.

Shown are representative negative-stain EM images of F-actin, F-actin crosslinked by Vt, and F-actin crosslinked by Vt P989A, respectively. Without Vt, F-actin filaments are not crosslinked. Vt or Vt P989A are able to crosslink actin filaments. However, bundles of F-actin crosslinked by Vt P989A are more likely to be wound (left) or to splay out (right) than those crosslinked by Vt.

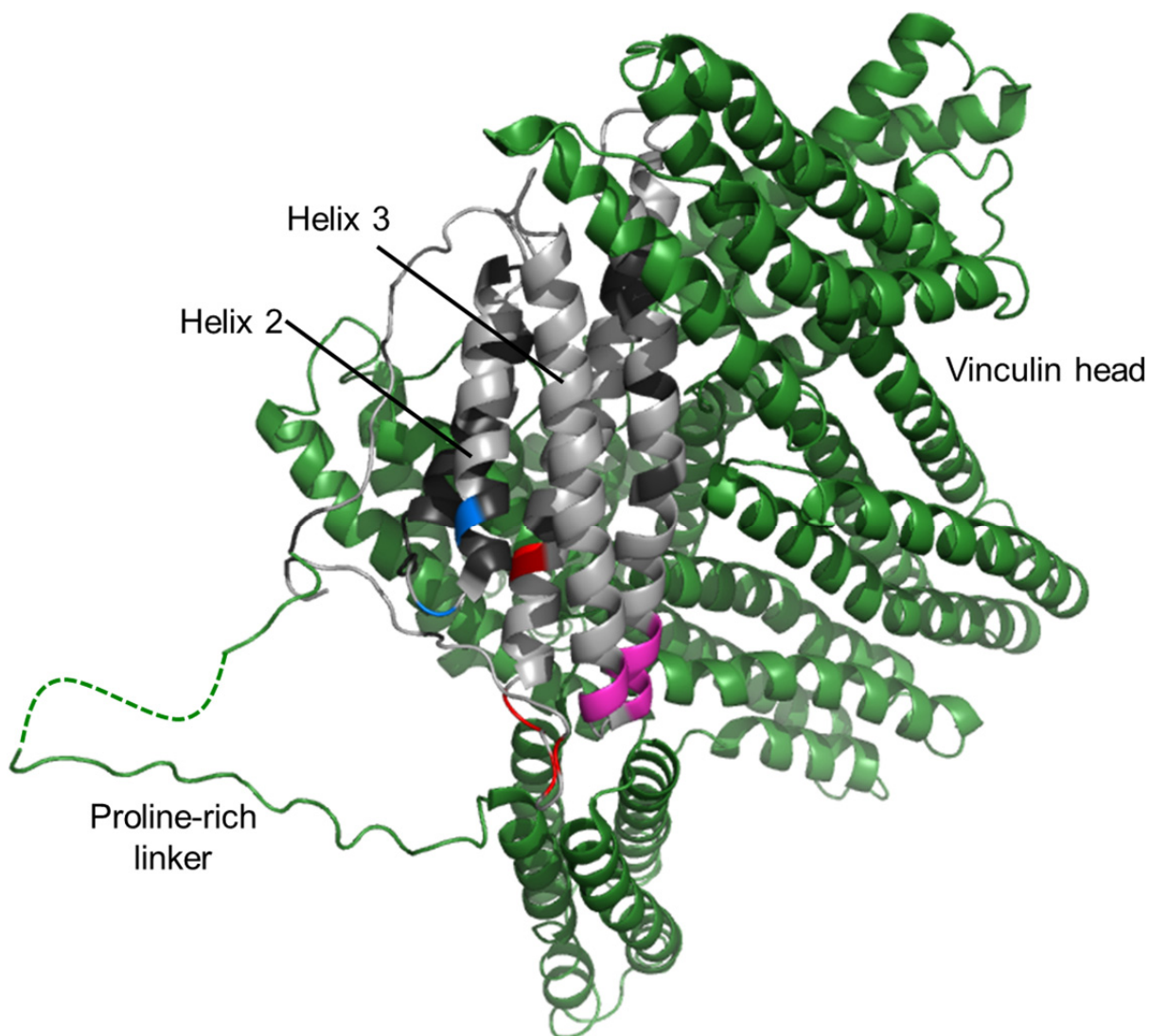


Figure 5.13. Residues with μ s-ms backbone dynamics are present at the vinculin head:tail interface.

Shown is the structure of full-length vinculin (PDB file 1ST6) in green, with Vt in the color scheme from Figure 5.6. Residues in black are unassigned, and residues in magenta, red, and blue exhibit significant changes in their $R_{2,\text{eff}}$ values as a function of the frequency of the CPMG pulses and are sites of μ s-ms backbone motions. These sites are close to the lower vinculin head:tail interface.

Chapter 6: Conclusions and Future Directions

As a scaffold protein with a litany of binding partners, vinculin provides a difficult challenge for scientists wishing to understand the biological function of each interaction it undergoes. Deletion of a domain disrupts all of the binding interactions facilitated by the deleted domain. Deletion of significant portions of a domain may perturb a nearby binding site or alter the structure of the domain, making it incapable of binding a ligand at a site distant from the deletion. Mutations are the best tool, but they can cause the same problems as a partial domain deletion if the appropriate controls are not performed. The use of mutations is made easier when a good structural model exists from which to design specific point mutations. Further complicating this problem for vinculin are the technical difficulties associated with developing good structural models with two of the tail ligands, phosphatidylinositol 4,5-bisphosphate (PIP₂) and F-actin.

To overcome some of these challenges, we applied a variety of biophysical, biochemical, and computational techniques to develop better models for the interaction of Vt with F-actin and PIP₂ (103). Through the serendipitous discovery of the actin-binding deficiencies of Vt I997A and V1001A and the application of negative-stain EM and discrete molecular dynamics (103), vinculin tools were developed to study the biological consequences of F-actin-binding by vinculin (103, 124). By using these tools, we have shown that the vinculin:actin interaction is one of, but not the only way in which vinculin regulates actin flow, both at focal adhesions and in the lamellipodium (124). This influence over the flow rate of F-actin at focal adhesions and in the lamellipodium regulates the number, growth, and size of these focal adhesions (103, 124). Additionally, the binding of vinculin to

F-actin is required for effective cell spreading, reinforcement at focal adhesions (103), generation of traction forces, and cell migration (123).

Similarly, defining the PIP₂ binding site on Vt has required the utilization of both biochemical and computational approaches. These data support the model outlined in Chapter 2, in which PIP₂ binds to Vt at the basic collar while the basic ladder mediates interactions with the lipid bilayer. A crystal structure of Vt bound to a short chain PIP₂ molecule was recently published that calls these findings into question (40), proposing that both the basic collar and basic ladder are involved in direct PIP₂ binding. However, the structure was determined with a Vt mutant, R1060A, in the absence of a lipid bilayer; it provides little help in understanding what a Vt:PIP₂ complex may look like at a membrane. Our data agree well with the biochemical data from Chinthalapudi *et al.* (40), but we think a structural model that utilizes a lipid bilayer is a better interpretation of the data.

Both the Vt:actin and Vt:PIP₂ models described in this dissertation have room for improvement. In the case of the Vt:actin model, cryo-EM will likely prove to be the most useful approach. An even higher-resolution model of the Vt:actin interaction would, ideally, provide information about specific sidechain interactions at the interface and resolve the location of the residues that make up the N-terminal strap, helix 1, and the C-terminus of Vt. Such a model would require the use of the latest cryo-EM imaging equipment and technology, which have been used to develop structural models at near-atomic resolution (< 4Å) (161-164). Another technical barrier limiting the resolution of the Vt:actin model is the tendency of Vt to disengage from the actin filament during freezing of the sample. By changing buffer conditions and possibly the freezing technique, actin filaments should remain more saturated with Vt. This greater saturation will provide more complete data sets, leading to a higher-resolution model.

Such a high-resolution model would provide a strong starting-point towards modeling a structure that has been extremely elusive: the Vt dimer responsible for crosslinking F-actin

filaments. A higher-resolution model that defines the position of helix 1, the N-terminal strap, and the C-terminus, all of which are involved in actin bundling (6, 103, 127), would provide clues as to how these components may be involved in forming the interface for the Vt dimer. Additionally, recent improvements in cryo-EM data collection should make direct observation of the crosslinked F-actin structure more tractable. Single-particle analysis techniques, which provide the highest-resolution cryo-EM models, are not applicable to such structures. However, cryo-EM tomography would be applicable. Normally a low-resolution technique, application of the best cameras (165), direct electron detectors (166), and averaging, in conjunction with the high-resolution model of the binding interaction, cryo-EM tomography may be applicable in developing a medium-resolution (~15-30 Å) model for the Vt:Vt dimer bound to F-actin (167). At this resolution, sidechains and secondary structure would not be resolved. However, using the binding model, flexible fitting, and computational modeling, a strong model could be developed that makes testable predictions about the Vt dimer interface.

As improvements in cryo-EM technology hold great promise for better models of Vt:actin interactions, improvements in developing lipid structures for structural biology applications now make a much better Vt:PIP₂ model possible. The development of smaller nanodiscs for use in solution NMR (168) has great promise for revealing how Vt binds, since its backbone has already been assigned. Nanodiscs can also be used with cryo-EM to visualize protein:lipid interactions (169). While cryo-EM would not reveal exactly what part of Vt binds the PIP₂ headgroup, it would shed light on how Vt associates with the lipid bilayer. Additionally, the use of the lipid cubic phase in generating crystals of protein:lipid complexes provides another exciting structural technique (170) that may show how Vt binds PIP₂ at a lipid membrane and reveal the conformational changes that take place upon PIP₂-binding (10). The beauty of these technologies is that all of them provide high-resolution structures of protein:lipid complexes in the context of a lipid bilayer, a more biologically

relevant interaction than with a short-chain phospholipid molecule. With such a wide variety of techniques available, it will be only a matter of time before a better high-resolution model of the Vt:PIP₂ interaction is published.

These pending improvements in the structural models of Vt bound to two of its ligands will provide key tools by allowing for rational design of vinculin mutants with defects for specific interactions. These tools can then be used to answering the more important question: how do vinculin's specific interactions drive specific biological responses? Work from the Adamson lab in generating vinculin knockout mice and embryonic fibroblasts provided the foundational tools and understanding for vinculin's biological function (8, 9). However, limited funding and the sheer number of vinculin's binding partners continue to make deciphering the specifics of vinculin function extremely difficult. In addition to these challenges, vinculin is posttranslationally modified at many sites, and these modifications can have different regulatory functions when vinculin is localized to different structures (171). With this many variables, generating a rigorous model to bridge the gap from biochemistry to biology remains a distant goal.

Yet, despite the numerous challenges, much of the foundation for addressing this gap in our knowledge is in place. The structural biology techniques that will provide high-resolution models for vinculin interactions with binding partners are now being established. Fluorescent microscopy techniques have improved such that protein localization within an adhesive structure can be determined (55). Förster resonance energy transfer (FRET) sensors to measure vinculin activation (172) and tension across vinculin (173) within cells now exist and can provide key readouts on vinculin activity. Advances in traction force microscopy (174) have pushed the technology into three dimensions, providing better biological readouts on cellular behavior. Coupling these improvements with more traditional techniques, including FRAP, immunoprecipitation, cellular migration assays, and live-cell

fluorescent imaging generates a substantial toolbox with which scientists can probe the consequences of individual vinculin:ligand interactions.

The remaining challenges, therefore, are less technical and more nuanced. The amount of time and effort required to evaluate so many different combinations of ligands and posttranslational modifications is daunting. The best way to address this challenge, short of a National Institutes of Health mandate, is to leverage the strengths of different labs in collaborative projects. This has begun to happen with greater frequency, as evidenced by many recent publications that employ biochemical, biophysical, and cell biology techniques to address vinculin function (*40, 103, 115, 123, 171, 175*). Such collaboration is vital to understanding vinculin function. More of these partnerships will arise in the coming years, especially if the current collaborations continue to be as productive and influential. This more complete understanding of vinculin is required to address the conflicting reports of vinculin's role in cancer progression.

In addition to the structural work described in this thesis, I have also reported on probing the conformational dynamics of Vt. Studying these dynamics further inform the field in how proteins move and provide valuable information for testing of molecular dynamics simulations. In the case of vinculin, these conformational dynamics also play an essential role in its biological function. They allow vinculin to crosslink actin filaments (*120*), which is essential for mechanotransduction (*6*). Additionally, conformational changes take place upon binding to PIP₂ (*10*), which may play a role in allowing vinculin to oligomerize at the plasma membrane (*40*). However, connecting Vt dynamics in the absence of ligands to these conformational changes is not straightforward and will require further dynamic data and a better understanding of the bound conformations.

If vinculin does play a significant role in cancer development, these conformational dynamics may provide a way to target vinculin function. Compounds could be developed to

target specific conformations of vinculin, enhancing the signaling response associated with that particular conformation or signaling when and where that conformation is present. One of the simplest conformations to target would be vinculin in its inactive form. A drug that stabilized the Vh:Vt interaction would be able to significantly impair vinculin function. Other, more specialized conformations could potentially be targeted to tune vinculin function. Such applications are in the distant future, however. Vinculin is ubiquitously expressed, so efficient targeting of the drug to cancer cells would be essential to limiting negative side effects. Additionally, identifying these conformations and developing compounds to target them would require development of high-throughput assays to evaluate the conformation being selected for or against.

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