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The Isolated Sixth Gelsolin Repeat of Villin Has a Calcium-regulated Structure and Lacks Capacity to Bind F-actin

By

Jacob A. Brockerman

Accepted in Partial Completion
Of the Requirements for the Degree
Master of Science

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MASTER’S THESIS

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Date: 5/15/2012
The Isolated Sixth Gelsolin Repeat of Villin Has a Calcium-regulated Structure and Lacks Capacity to Bind F-actin

A Thesis
Presented to
The Faculty of
Western Washington University

In Partial Fulfillment
Of the Requirements for the Degree
Master of Science

By
Jacob A. Brockerman
May 2012
ABSTRACT

Villin is a modular, epithelial, actin binding protein responsible for the regulation of microvilli in the brush border region of the small intestine and kidney. Villin is regulated by tyrosine phosphorylation, Phosphatidylinositol 4,5-bisphosphate and calcium concentration. These signal molecules control villin’s multifunctionality and allow it to switch among its several opposing functional roles. Villin can cleave, nucleate, cap and bundle F-actin. This is achieved through villin’s six gelsolin-like domains which are connected by a 40 residue linker sequence to a novel C-terminal headpiece domain (HP). It is known that the HP forms a calcium insensitive F-actin binding site that is necessary for F-actin bundling. It is unclear how villin accomplishes F-actin bundling with several proposed mechanisms under debate. In this study, we determined the isolated sixth domain (D6) of villin’s solution structure via NMR spectroscopy, its ability to bind calcium, its ability to oligomerize and its ability to bind F-actin. When referenced to the D6-HP fragment, the smallest known F-actin bundling sequence of villin, these data give insight into the mechanism of how D6-HP accomplishes F-actin bundling. Our study shows that D6 is monomeric and adopts a gelsolin like fold containing a five strand β-sheet sandwiched between a long α-helix and two smaller α-helices. Our 15N-HSQC data shows that D6 binds calcium and undergoes a conformational change when calcium is removed. Our sedimentation assay shows D6 possesses no capacity to bind F-actin. These data strongly point to a dominant role of the intrinsically disordered linker sequence in D6-HP’s ability to bundle F-actin. Furthermore, in the context of villin as a whole, the linker sequence may play an unknown role in villin’s regulation of actin structure.
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**Introduction**

*Evolutionary Role of Microvilli in Enterocyte Cells and Their Regulation by Villin*

Evolution has designed the intestinal tract of vertebrates to maximize nutrient uptake. This is accomplished through several digestive and absorptive steps. Once mechanical and chemical digestion nears completion, processed food moves into the small intestine where the majority of nutrients are most often absorbed. This difficulty of moving nutrients from the lumen through the plasma membrane and into cytoplasm of epithelial cells has been overcome by the small intestine through a remarkably simple mechanism. The small intestine accomplishes nutrient uptake for the majority of the organism by maximizing surface area contact with the lumen. This increases the number of membrane transport proteins available for nutrient uptake. Surface area is increased through multiple layers of folding starting with

![Figure 1. SEM Views of the Small Intestine.](image)

A: Circular folds of the small intestine increases surface area in contact with the lumen. B: the finger-like projections called villi are found on the cell face in contact with the lumen. C: Microvilli coat each villus, exponentially increasing the surface area in contact with the lumen.
creases of the small intestine called circular folds (Figure 1A) and ending with microvilli. The epithelial cells of the small intestine have finger-like projections, called villi, lining the interface between the intestine and the lumen (Figure 1B). These villi are typically 0.6-1.5 mm in length and form the second layer of cellular structure designed to increase surface area (Ferguson, 1977). Each villus in turn has finger-like projections called microvilli (Figure 1C). Microvilli are on average 1 μm in length and blanket each villus, exponentially increasing its total surface area (Brown, 1962). The protein target of this study, villin, is expressed almost solely in epithelial cells and has been implicated in the formation/regulation of microvilli. Its name comes from its ability to regulate and induce the formation of microvilli (Dudouet, et al., 1987).

Villin is known to govern the formation of microvilli through its interactions with actin. Actin is a cytoskeletal protein traditionally thought to act as a mechanical support. Cytoskeletal proteins are conventionally thought to provide three key functions in cells: structural support, cell motility and intracellular transport. In enterocyte cells, globular (monomeric) actin is polymerized to filamentous actin (F-actin) through the hydrolysis of adenosine triphosphate (ATP) to adenosine diphosphate (ADP). F-actin forms double helical microfilaments (Figure 2) that are regulated by salt concentrations and actin binding proteins (Wilson, et al., 1970).
Figure 2. 42 kDa globular actin monomers (monomers different colors) make up filamentous actin. F-actin is commonly thought of as a cellular structural support. The process of filament formation requires the use of ATP and is regulated by salt concentrations and actin binding proteins. Actin serves a plethora of roles in the cell ranging from muscle contraction to transcription regulation. (PDB 2Y83)
In microvilli, multiple F-actin filaments are bundled into large columns to provide structural support for the finger-like projections (Bretscher, 1978). This structural support has been viewed at low resolution via electron microscopy (Hampton et al., 2008) and modeled in silico (Brown et al., 2011) but a definitive mechanism for this arrangement is still lacking. There are several models on how villin could bundle F-actin, but because only low-resolution cryo electron microscopy images are available, the structural mechanism remains unresolved.

**The Gelsolin Domain Comprising the Structural Building Block of the Gelsolin/Villin Superfamily**

Villin is part of a family of actin regulating proteins called the gelsolin/villin superfamily. This family is characterized by ~100 residue gelsolin-like repeats (Figure 3). Representatives of this family include gelsolin (Yin & Stossel, 1979), dematin (Rana, 1993), severin (Andre et al., 1988), and supervillin/archvillin (Prestonjamasp et al., 1997). These proteins can have functions relating to cytoskeletal regulation, anti-apoptosis (Wang, et al., 2008) and, recently discovered, transcription regulation (Archer et al., 2006). If a gelsolin domain has actin binding capacity, it is most often accomplished through interactions of the long alpha helix with actin (Choe, et al., 2002). Gelsolin-like domains often flank the major binding domain and have minor interactions with actin, most often via the AB loop (Figure 3). Comparing the members of this family, it is seen that members typically contain 3 to 6 gelsolin-like repeats, and often a unique N- and/or C-terminal sequence.
In this family, the N- and C-terminal domains flanking the gelsolin-like core are currently under investigation in several labs. Of particular interest is the C-terminal headpiece domain (HP) (Figure 4) found in villin, supervillin, dematin, protovillin (Hofmann et al., 1993) and villidin (Gloss et al., 2003). The HP domain has been extensively studied as a protein folding model because of its short sequence and high stability. The HP domain can also have the capacity to bind F-actin (Glenny and Weber, 1981). Other family members like supervillin, archvillin, and flightless-1 (Straub et al., 1996), have long N-terminal (Archvillin/supervillin ~800 residues, Flightless-1 ~ 600 residues) domains. Our previous studies point to supervillin’s N-terminus being intrinsically disordered (Stanislav Fedechkin, Jacob Brockerman, Michail Lobanov, Oxana Galzitskaya, Serge Smirnov. data, in preparation for publication).

Figure 3. Domain 6 of Gelsolin representing a typical gelsolin-like domain containing 5 β-sheets sandwiched between a long α-helix and 2-3 smaller helices. (PDB 1P8Z)
Figure 4. The head piece domain found at Villin’s C-terminal has F-actin binding capacity via a KKEK motif and is implicated with villin’s F-actin bundling abilities. (PDB 2K6M)

**Gelsolin as a Model for the Gelsolin/Villin Superfamily**

Gelsolin’s is comprised of six gelsolin-like repeats with no unique N- or C-terminal sequences. These fold into six gelsolin-like domains connected by short linker sequences (Figure 5). Gelsolin has been extensively studied and crystal structures of the whole protein (Burtnick, *et al*., 1997), domains 1-3 bound to G-actin (Burtnick, *et al*., 2004), and domains 4-6 bound to G-actin (Choe, *et al*., 2002) have been published. Gelsolin is regulated by phosphatidylinositol 4,5-bisphosphate (PIP$_2$) (Janmey *et al*., 1987), tyrosine phosphorylation (De Corte, *et al*., 2002), and calcium (Kwiatkowski, *et al*., 1986). Calcium regulation is accomplished through a “latch and hinge” mechanism (Robinson, *et al*., 1999). At low calcium levels (<0.1 μM), gelsolin’s 6$^{th}$ domain adopts a bent helix which allows it to bind domain 2 and sterically hinder the F-actin binding site on domain 2 (Figure 6A). If calcium levels increase, the long α-helix in domain 6 straightens, disassociating domain 6 from domain 2 and exposing gelsolin’s F-actin binding site (Figure 6B). Domain 2 has been shown to instigate F-actin depolymerization (severing), whereas the other two actin binding domains on domains 1 and 4 are involved in F-actin capping at the barbed end and nucleation (Choe, *et al*., 2002).
Figure 5. Gelsolin Structure Function Domain Analysis. Gelsolin has 3 known actin binding sites. Two G-actin on domains 1 & 4 and 1 F-actin on domain 2. Domain 1 is associated with F-actin capping in a calcium sensitive manner. Domain 2 is associated with F-actin severing. Domain 4 is associated in a calcium independent F-actin capping. Several key regulatory sites have also been identified on gelsolin including calcium and PIP$_2$ binding.
Figure 6. Gelsolin is Regulated by a Latch and Hinge Mechanism. A. (G1-G6) Under low calcium concentration, gelsolin’s sixth domain (G6) contains a bent, long, α-helix (red) allowing for it to bind G2. This is held in place via a C-terminal helix. B. (G4-G6) Upon calcium concentration increase, G6’s helix straightens (red) and allows gelsolin to open. This exposes the F-actin cleavage site on G2. (PDB 1D0N & 1H1V)
Villin’s Regulatory Functions and Pursuit Toward Determining Mechanisms of Actin Regulation

Villin bears high sequence homology (51% identities, 64% positives) to gelsolin, as it contains 6 gelsolin-like domains. Distinct to villin is its C-terminal sequence, which contains the isolated headpiece domain (HP, Figure 5) connected to the gelsolin core via a 40-residue disordered linker sequence. Thus, villin can be viewed as a combination of two families of actin binding proteins: proteins that bind actin through gelsolin-like domains and proteins that bind by the C-terminal headpiece domains. Villin is an epithelial cell specific regulatory protein (George, 2008). Villin also shares many of the same functions as gelsolin. Villin nucleates F-actin polymerization, cleaves F-actin, and caps the barbed ends of F-actin (Walsh et al., 1984). Barbed end capping is associated with domain 1 (Janmey et al., 1988) and is regulated by PIP$_2$ and calcium (Northrop et al., 1986). Calcium promotes capping, while PIP$_2$ suppresses it. Two F-actin binding sites are known on villin. The first is residues 138-146 on domain 2 (de Arruda, et al., 1992) (Figure 7). This site’s affinity for F-actin is increased with calcium and decreased by tyrosine phosphorylation (Zhai et al., 2001). The second site is residues 816-824 on the headpiece domain which is insensitive to calcium (Friederich, et al., 1992). Based on homology to gelsolin, two G-actin binding sites are suspected on domains 1 and 4. These would serve as F-actin capping and/or to start nucleation of F-actin at the pointed end. Nucleation is calcium and tyrosine phosphorylation sensitive (reviewed in Khuranna, 2006). Tyrosine phosphorylation decreases actin nucleation, while calcium is necessary for nucleation to occur (Zai, et al., 2001).
Figure 7. Villin Structure Function Domain Analysis. By homology to gelsolin, domains 1 and 4 are thought to bind G-actin. Domain 2 has F-actin binding, is associated with F-actin severing and possibly F-actin bundling. The HP domain contains an F-actin binding site required for F-actin bundling. Regulatory calcium binding sites are thought to be on domains 1, 4 and possibly 6, based on gelsolin homology. Other calcium binding sites exist but are not known to have a regulatory function.
Villin’s ability to regulate F-actin varies based on calcium concentration and phosphorylation state. Under low calcium concentrations villin bundles F-actin (A). Tyrosine phosphorylation and the increase in calcium concentration switch villin’s activity to cleave F-actin (B).

F-actin severing occurs under high calcium concentration (100-200 μm), which is found during periods of cellular insult (Figure 8B). Severing can also be controlled by tyrosine phosphorylation which decreases the calcium concentration required for severing to the nanomolar range (Kumar, et al., 2004). This allows villin to regulate F-actin by severing during non-apoptosis states.

Villin has one novel ability that gelsolin lacks. Villin can bundle F-actin into columns under nanomolar calcium concentrations (Figure 8A). The mechanism for this activity is
currently contested and several seemingly contradictory papers have created opposing models for F-actin bundling. Until 2007, it was thought that villin bundled F-actin via both its F-actin binding sites. Domain 2 and the headpiece form a tether holding two F-actin filaments in proximity. *In vitro* cryo electron microscopy studies produced by the Taylor lab (Hampton, *et al.*, 2008) suggested villin bound to F-actin as a monomer via domains 1, 2 and the headpiece and implicated that the headpiece and domain 2 bound the same F-actin strand. This was accomplished by producing three-dimensional models from cryo electron microscopy and fitting structures based on the gelsolin homology model and the headpiece to the electron density. In late 2007, the Khurana lab produced *in vivo* data suggesting that only the headpiece and a dimerization site is needed for F-actin bundling and that villin forms a dimer using residues 21-67, 112-119 (George, *et al.*, 2007). This was accomplished using recombinant villin proteins expressed in Caci-2 cells, *in vivo* Förster resonance energy transfer, chemical crosslinking, and matrix assisted laser desorption ionization mass spectrometry. In 2007, the McKnight lab while studying the D6-HP fragment of villin discovered it could also produce F-actin bundling in a calcium sensitive manner and proposed a cryptic F-actin binding site somewhere on domain 6 and/or the core to headpiece linker. Interestingly, the D6-HP fragment regulation by calcium concentration is opposite from that for wild type villin. Under low calcium concentrations D6-HP would aggregate but when calcium concentrations increased to millimolar levels D6-HP would bundle F-actin (Smirnov, *et al.*, 2007).
Nuclear Magnetic Resonance Spectroscopy

The usefulness of nuclear magnetic resonance (NMR) has continued to increase since its development in 1945. Over the last 6 decades, the technique has advanced from experiments on simple organic molecules used to develop and characterize the key properties of the technique to being able to acquire structural, dynamic and binding data on molecules approaching megadalton sizes. Advances in methodology and technology helped spur this change which has resulted in several Nobel prizes. Some highlights are the employment of Fourier transformation allowing for simultaneous spectra acquisition, the development of superconducting magnets and cryoprobes allowing for a higher signal to noise ratios, and the development of multidimensional and heteronuclear NMR experiments.

Introduction to NMR Spectroscopy

NMR spectroscopy exploits the inherent property of nuclear spin. This spin is characterized by several vector quantities, the first being its nuclear spin quantum number, $I$. This quantum number must be greater than or equal to zero and must be a multiple of $\frac{1}{2}$. This dictates the number of spin states according to the relationship

$$\text{Spin states} = 2I + 1 \quad \text{Eq. 1.}$$

Thus an atom with a spin quantum number of 0 has 1 spin state, $I = \frac{1}{2}$ has 2 spin states and $I=1$ has 3 spin states. The proton ($^1\text{H}$) and phosphorus ($^{31}\text{P}$) are spin $\frac{1}{2}$ giving them the simplest magnetically sensitive spin state of 2. These are used routinely for biological
molecule characterization. The other atom types common to biological molecules are spin 0 and thus are silent in NMR ($^{12}\text{C}$ and $^{14}\text{N}$). Fortunately, their isotopes ($^{13}\text{C}$ and $^{15}\text{N}$) are spin $\frac{1}{2}$ and methods have been developed to isotopically enrich biological samples allowing for homo- and heteronuclear experimentation.

The second characteristic of nuclear spin is its angular momentum, $P$. The angular momentum is responsible for the nuclei’s magnetic moment, $\mu$. $P$ and $\mu$ are related by the gyromagnetic ratio, $\gamma$, according to the following expression

$$\mu = \gamma P \quad \text{Eq.2.}$$

For spin $\frac{1}{2}$ nuclei there exists 2 spin states of equal distribution, but upon the application of a magnetic field this distribution polarizes with the spin state in parallel to the magnetic field having a lower energy state and thus a higher population than the spin state opposed. This magnetic field can also be thought of as applying a torque to magnetic moment causing the vector quantity to precess around the magnetic field. This is called the Larmor precession ($\nu$ in Hz, $\omega$ in rad/s) and in Hertz is related to the gyromagnetic ratio and the magnetic field ($B_0$) by the expression

$$\nu = \frac{-\gamma B_0}{2\pi} \quad \text{Eq.3.}$$

The energy difference ($\Delta E$) between the spin states is proportional to the magnetic field strength. The two are related by plank’s constant $h$.

$$\Delta E = \hbar\nu \quad \text{Eq.4.}$$
From this we see that the energy required to excite the nucleus from its parallel to opposed spin state is proportional to the magnetic field strength in which the nucleus resides. Current magnet strength on NMR spectrometers puts the photons needed to cause this excitation in the radio frequency (rf) to microwave range, but as magnetic strength continues to increase this will shift toward the microwave frequency. This distribution of energy states can also be used to predict the populations of nuclei in parallel and opposed to the magnetic field according to the Boltzmann distribution which states

$$\frac{N_\alpha}{N_\beta} = e^{\Delta E/k_B T} \quad \text{Eq. 5.}$$

Where \(N_\alpha\) is the number of nuclei with spin state parallel to the magnetic field, \(N_\beta\) is the number of nuclei with spin state opposite to the magnetic field, \(k_B\) is the Boltzmann constant and \(T\) is the temperature in Kelvin.

In order to understand how spin-active nuclei interact, a brief introduction to the quantum mechanics of nuclear spin is needed. Without going through the lengthy derivation, we can state that the Hamiltonian for nuclear spin with a magnetic field along the Z axis is

$$\hat{H} = -\gamma B_0 I_z \quad \text{Eq. 6.}$$

where \(I_z\) is the operator representing the z component of the nuclear spin angular momentum. For spin \(1/2\) systems the operator \(I_z\) can have 2 eigenfunctions represented by the wavefunctions \(+ \frac{1}{2} \Psi\) and \(- \frac{1}{2} \Psi\) (this is what really dictates the spin states listed in equation 1). These are alternatively referred to as \(\Psi_\alpha\) and \(\Psi_\beta\). When \(I_z\) acts on the wave function the eigenfunction is

$$I_z \Psi_m = m\hbar \Psi_m \quad \text{Eq. 7.}$$
$m$ is either $+\frac{1}{2}$ or $-\frac{1}{2}$. Applying this to the Hamiltonian for one spin system we get

$$\hat{H}\Psi_m = -\hbar \gamma B_0 \Psi_m \quad \text{Eq.8.}$$

Our eigenvalue here is in units of energy (joules), but can be easily converted to radians per second ($\omega$) by dividing $I_z$ by $\hbar$ and in hertz by dividing $\omega$ by $2\pi$ ($\nu$). Therefore the Hamiltonian for a one spin system written in hertz would be

$$\hat{H} = \nu I_z \quad \text{Eq.9.}$$

Applying this to multiple spin systems, the Hamiltonian for two spins is

$$\hat{H} = v_{1m} I_{1z} + v_{2m} I_{2z} \quad \text{Eq.10.}$$

This gives us four discrete eigenvalues depending on whether $m$ is $+\frac{1}{2}$ or $-\frac{1}{2}$ for the two spin systems. Upon close analysis it is seen that these four wavefunctions remain eigenfunctions of the Hamiltonian for nuclear spin. These are said to commute and is the basis for interactions in quantum mechanics. With the two spin system in place we can now describe how these spins interact through $J$-coupling.

To describe this spin transfer a third component of the Hamiltonian for nuclear spin is added. Denoted as $J_{12}I_{1z}I_{2z}$ the Hamiltonian becomes

$$\hat{H} = v_{1m} I_{1z} + v_{2m} I_{2z} + J_{12} I_{1z}I_{2z} \quad \text{Eq.11.}$$

The eigenvalues associated with this two spin system take on the form

$$m_1v_{1m} + m_2v_{2m} + m_1m_2J_{12} \quad \text{Eq.12}$$
Figure 9. The allowed transition states of spin $\frac{1}{2}$ nuclei that give rise the J-coupling in a two spin system. Each nucleus is allowed a $m\pm 1$ but only 1 is allowed at once. Homonuclear systems have $\alpha \beta = \beta \alpha$. This is often not the case for heteronuclear systems.

and are limited by the allowed transitions of $m_1 \pm 1$ or $m_2 \pm 1$. This is depicted graphically in Figure 9. It is these four transitions that give rise to the two doublets seen between two interacting atoms interactions. This through-bond interaction is the basis of nuclei assignment and can be used to give information on the conformation of the dihedral angles.

The ability to manipulate spin systems has progressed to the point where we can selectively excite groups of spin systems (e.g. aromatic or aliphatic protons) or selectively view coupling constants pertaining to a particular interaction as seen with various versions of TOCSY and COSY experiments. This is all accomplished through pulse sequences which exploit the change in coupling constants and relaxation rates in different types of nuclei or in different local environments.

The second interaction seen between spin active nuclei is the Nuclear Overhauser Effect (NOE). This is a through-space interaction that is the result of dipole-dipole or cross...
relaxation. A simple representation of this is the bar magnet representation of the nucleus. Each nucleus can be thought of as having its own local magnetic field. These fields interact with each other and, as the molecule tumbles, their relative positions to each other change. This results in a change in local magnetic field, which induces a torque on the adjacent bar magnet (Figure 10). This is actually a form of spin transfer but this simple illustration is all that is needed to conceptualize distance restraints required for solution structure determination of large molecules. Dipole-dipole relaxation is dependent on distance according to the relation

\[ \sigma \approx \frac{1}{r^6} \quad \text{Eq.13} \]

where \( \sigma \) is the dipole-dipole relaxation constant and \( r \) is the distance. Nuclei separated by over 5 Å typically do not engage in dipole-dipole relaxation. This immensely useful interaction is used in NOESY experiments and is the main source of distance restraint used in structural determination of biological molecules. It is valuable because it can show interactions between nuclei separated by many atoms/bonds in the amino or nucleic acid sequence or between interacting molecules.
In order to easily visualize the effect of pulse sequence, the rotating reference frame approach is used. The rotating reference frame rotates at the Larmor precession around the direction of the magnetic field (Z). Using an rf pulse applied along the x axis as an example, it is easy to see the advantage of this simplified model. Looking at the laboratory reference frame at time $T_1$, 2 vectors representing the rf pulse branch out in opposite precession at the Larmor frequency (Figure 11B). In the rotating reference frame one vector is frozen to the x’ axis while the other precesses at $-2v$. The latter vector is typically ignored allowing for the torque applied on the nuclei to be viewed along the $Z’ Y’$ plane (Figure 11C). From this viewpoint, it is easy to see the effect of a 1-D pulse sequence using this vector model.

![Figure 11. Comparison of the laboratory reference frame to the rotating reference frame. In the laboratory reference frame, as time passes the single magnetic vector at $T_0$ (A) is broken into to vectors traveling opposite directions (B). The rotating reference frame allows for easy analysis of spin vectors by matching the speed of one of these vectors.](image)

The basic design of pulse sequence is a three-step process (Figure 12), equilibrium of the sample with the magnetic field allowing the increase in population parallel to the magnetic field, the excitation of the nuclei in study by an rf wave (the pulse) of the appropriate wavelength, and acquisition period ($t$) where the nuclei relax back to their lower energy state.
Several additions can be made to this pulse sequence selecting for J-coupling, NOE interactions, and other homo/hetero nuclear interactions. In order to resolve unidentified nuclei’s chemical shifts, in D6, we employed three J-coupling experiments to identify backbone and side chain assignments. We also employed two additional versions of the NOESY experiment in order to increase our distance restraints of side chains atoms (Reviewed in Keeler, 2005, Claridge, 2009 and Cavanagh, et al., 2007).
Figure 12. The basic NMR pulse sequence involves 3 phases. In phase one the spin vector is in equilibrium with the magnetic field. In phase two an rf pulse is applied exciting the spin vector into the X’Y’ plane. In phase 3 the spin vector relaxes back into equilibrium with the magnetic field emitting an observable decaying rf signal.
One of the modern workhorse experiments of protein NMR is HBHA(CO)NH (Grzesiek, 1992). This J-coupling experiment, when combined with the data from another J-coupling experiment, the HNCACB (Wittekind, 1993), allow for the chemical shift assignment of the amide backbone as well as some protons on the side chain. Three dimensional J-coupling experiments typically are named by stating their order of coupling. For the HBHA(CO)NH, initially the protons attached to the beta and alpha carbons are excited and allowed to interact via J-coupling. This is then moved through the carbonyl and nitrogen amide of the peptide bond and finally observed on the amide proton of the next residue (Figure 13).

Figure 13. J-coupling of the HBHA(CO)NH pulse sequence. This pulse sequence is used for backbone assignments and transfers spin magnetization from the i-1 β protons to the proton of the amide in residue i.
A common problem of protein NMR is the lack of assignments of side chain atoms. This often leads to undefined side chain arrangements resulting in a higher root mean square difference (RMSD) of the determined structures. To account for this, several J-coupling experiments have been developed to increase the chemical shift assignment coverage of side chain atoms. Two experiments that have proven very useful in these assignments are the HCCH-COSY (Clore, et al., 1990) and the HCCH-TOCSY (Cavanagh, et al., 2007) (Figure 14). These two experiments observe proton J-couplings between proton-proton and proton-carbon. In the HCCH-TOSCY this produces three dimensional data where each carbon chemical shift shows all the proton chemical shifts of the side chain. A cleaner version of this is obtained by the HCCH-COSY where only the protons attached to the adjacent carbons are displayed for each carbon chemical shift. Combining these experiments allows for a large

![HCCH-TOCSY and HCCH-COSY](image)

**Figure 14.** In the HCCH-TOCSY and HCCH-COSY J-coupling is used to transfer spin magnetization from the protons attached to the beta carbons through the amide bond and ending on the proton amide of the next residue.
percentage of the side chain atoms to be assigned. This, when combined with NOE data produces a higher number of structural restraints.

Two NOE-based experiments helpful in restraining side chain residues are the $^{13}$C-NOESY-Aromatic and $^{13}$C-NOESY-aliphatic. Carbon based NOE restraints are particularly useful because the resulting spectra are less crowded allowing for unambiguous cross peak assignments. These spectra also typically improve in quality as the mass of the protein is increased. This is the result of the experiment not being scalar based (Bertini, et al., 2004). Another benefit is the ability to selectively observe different distances based on increasing mixing times and classes of carbons by selected excitation.

Modern solution structural determination is highly automated process with several commonly used software packages designed to expedite the process. The PINE server (Bahrami, et al., 2009) automatically assigns chemical shifts based on inputted spectral data and the protein sequence. Dihedral restraints can be generated through software packages like TALOS (Cornilescu, et al., 1999) and PREDITOR (Berjanskii, et al., 2006). These programs, developed by the Wishart lab, utilize chemical shift of backbone and side chain chemical shifts predict secondary structure. Automated NOE cross peak assignments and Structural determination can be accomplished iteratively using the program CYANA (Guntert, 2004).
Specific Aims

The first goal of this project is to determine the atomic-resolution solution structure of isolated domain 6 (started by Danielle Pfaff). This was accomplished through the acquisition and assignment of additional heteronuclear NMR spectra and resolving undefined nuclei missing from Pfaff’s assignments. These were then used to amend the resonance assignments of D6 previously proposed. Structural calculations were performed and assignments were again refined. This process was iteratively repeated until the D6 structure was resolved to atomic resolution (<1.0Å). Structural information gained from this study will be a scaffold used to propose mechanisms from the functional aspects of the project.

The second goal of this project is to determine whether isolated domain 6 is sensitive to calcium. This was accomplished through a back titration of calcium by ethylenediaminetetraacetic acid (EDTA) and monitoring changes in D6’s backbone by a series of $^{15}$N-HSQC spectra. This binding data in the context of D6-HP provides insight into the mechanism of how D6-HP has the ability to bundle F-actin under high calcium concentrations (Smirnov et al., 2007). Also, noting the residues whose chemical shift is susceptible to calcium levels could identify the calcium binding site(s) and give evidence for large domain rearrangements.

The third goal of this project is to see whether D6 can dimerize or aggregate. D6-HP was unstable in low calcium levels and aggregated within 24 hours of calcium removal. To see if this propensity to oligomerize is an inherent property of D6, we compared through size exclusion chromatography, a D6 sample in a calcium containing buffer to a D6 sample where calcium has been removed from solution by buffer exchange or EDTA addition. This
data in the context of D6-HP narrowed the structural features responsible for Ca\textsuperscript{2+} binding. It also suggested the identity of the structural elements responsible for D6-HP’s intolerance to low calcium environments.

*The final goal of this project is to test D6’s ability to bind F-actin.* D6-HP’s unexpected ability to bundle F-actin led to the hypothesis that a cryptic F-actin binding site existed somewhere on D6 and/or the linker sequence. To test this hypothesis, we examined D6’s ability to bind F-actin in isolation by employing a sedimentation assay. G-actin was polymerized and then incubated with isolated D6. F-actin was then pelleted out of solution by ultra-centrifugation. The pellet was analyzed for D6 by reverse phase high pressure liquid chromatography. D6 is likely responsible for actin binding because it has a gelsolin-like fold which is a domain known, in some cases, to bind F-actin.
Results

D6 in Isolation Folds in the Same Manner as in D6-HP

From the $^{15}$N – HSQC spectrum we can see that D6 in isolation contains one species. The total number of cross peaks agrees with the D6 amino acid sequence. The $^{15}$N-HSQC cross peak overlap between D6 resonances in isolation and D6 in D6-HP was very high with a few exceptions. All cross peaks were within .04ppm in the proton dimension and .34 ppm in the $^{15}$N dimension, except residues 84, 105, 106, 107 with residues 84, 106 and 107 violating both dimensions. This C-terminal difference is easily explained by the lack of linker sequence in isolated D6. This high peak overlap shows that D6 in isolation adopts the same conformation as in D6-HP. The chemical shift difference of residue 84 between D6 and D6-HP is unexplained.

D6 Adopts a Gelsolin-like Fold

The solution structure of D6 was determined based on the distance and dihedral restraints generated by NMR data. The exhaustive list of spectra acquired for structural determination is: HNCACB (Wittekind et al., 1993, Muhandiram et al., 1994), CBCA(CO)NH (Grzesick, et al., 1992), HNHA (Vuister et al., 1993), HNHB (Archer et al., 1991), $^{15}$N-TOCSY, TOCSY (in 100% D$_2$O), TOCSY (in 10% D$_2$O), 3D $^{15}$N-NOESY, 2D NOESY (in 100% D$_2$O), 2D NOESY (in 10% D$_2$O), HCCH-COSY, HCCH-TOCSY, HBHA(CO)NH, $^{13}$C-NOESY-aliphatic, $^{13}$C-NOESY-aromatic, $^{15}$N-NOESY, with HCCH-COSY, HCCH-TOCSY, HBHA(CO)NH, $^{13}$C-NOESY-aliphatic, $^{13}$C-NOESY-aromatic being
newly acquired spectra since Danielle Pfaff’s graduation. Chemical shifts were assigned using the server PINE (Bahrami, et al., 2009), dihedral angles were predicted and restrained by PREDITOR (Berjanskii, et al., 2006) and the solution structure determined utilizing the CYANA (Guntert, 2004) algorithm, which assigns NOE cross peaks as it minimizes energy levels. Statistical data for the 10 best structures is listed in Table 1. D6 adopts a typical gelsolin-like fold containing a 5 strand β-sheet sandwiched between a long α-helix and two shorter α-helices. This long alpha helix is straight in high calcium concentrations (Figure 15), a feature also seen in the Homo Sapiens D6 crystal structure with no calcium bound. Our structure bears a high structural similarity to Homo Sapiens D6 with a RMSD of ~ 1 Å as determined by Chimera (Pettersen, et al., 2004). Residues 65-71 and 104-107 appear to be

![Figure 15. Ensemble of 10 best D6 structures as determined by CYANA. Heavy-atom RMSD <1.0 Å.](image)
the most disordered between the 10 structures. Residues 65-71 (Figure 16) form the loop that connects the C-terminal of the long α-helix to the 5th β-stand. The next disordered region is the last 3 residues of the structure leading to the intrinsically disordered linker sequence. The C-terminal helix is not stabilized by non-covalent interactions with the core of domain 6. To verify that this sequence indeed had propensity to form a helix, the structure was redetermined with removed dihedral restraints for residues 99-107 (Figure 17). The resulting structure still showed a stabilized helix.

Figure 16. The unstructured linker between C-terminal of the long α-helix and the 5th β-strand (Residues 65-71) is the most unresolved central portion of the D6 fragment.
Table 1. Statistics of the NMR-based solution structure calculations.

* Average values

Figure 17. The unexpected C-terminal helix still retains its helical structure in the absence of dihedral restraints.
**D6’s Solution Structure Finishes the Structural Determination of D6-HP**

Having completed the structure of isolated D6 we can infer the solution structure of D6-HP based on $^{15}$N-HSQC spectra of D6 in isolation, D6-HP, and HP in isolation. Both D6 and HP (Figure 5) adopt the same fold when linked and in isolation (smirnov, et al., 2007). The inter domain sequence is intrinsically disordered and the two can be thought of as two independent stable domains tethered to each other.

**D6 Binds Calcium**

To check whether D6 in isolation binds calcium, we performed a calcium back-titration monitored by a series of $^{15}$N-HSQC spectra. The initial D6 spectra were recorded at 5 and 1 mM calcium concentrations. The chemical shifts of each cross peak varied little between the two spectra with only 18 residues having a proton chemical shift changes of more than 0.045 ppm in the proton dimension (Figure 18A). Of these changes the largest was only 0.093 ppm. The only residue unassignable in the 1.0 mM calcium $^{15}$N-HSQC spectrum by homology to the 5.0 mM calcium $^{15}$N-HSQC spectrum was residue 52. Calcium was then sequestered from the 1.0 mM calcium sample by EDTA. Spectrum were recorded at free calcium levels of 750, 500, 250, 0.0 μM. The chemical shifts resulting from this removal of calcium are so significant that assignments for the majority of cross peaks cannot be determined based on the 5.0 mM Calcium D6 $^{15}$N-HSQC spectrum (Figure 18B). The 5.0 mM Ca$^{2+}$ spectrum was restored from the no Ca$^{2+}$ solution by buffer exchange into a Ca$^{2+}$ buffer showing reversibility.
Figure 18. A. Comparison of D6’s $^{15}$N-HSQC spectra: 5 mM Ca$^{2+}$ (black) & 1 mM Ca$^{2+}$ (red). B. Comparison of D6’s $^{15}$N-HSQC spectra: 5 mM Ca$^{2+}$ (black) & no Ca$^{2+}$ (red). C. Chemical shifts differences between D6 5 mM Ca and 1 mM Ca mapped onto the backbone of isolated D6 (differences in red).
**D6 in Isolation Exists as a Monomer**

D6-HP’s propensity to oligomerize in the absence of calcium led to the hypothesis that D6 dimerizes or oligomerizes in a calcium dependent manner. To test this, we performed a size exclusion chromatography study on isolated D6 (MW 12.5 kDa) in a 5.0 mM calcium solution and in a solution containing no calcium. The elution volumes for D6 in 5.0 mM calcium and in the calcium free environment were 13.1 and 12.8 ml respectively. Lysozyme (MW 14.7 kDa) eluted at 11.8 ml. This demonstrates that D6 is monomeric regardless of calcium concentration (Figure 19).

![Figure 19. Size exclusion chromatogram of isolated D6 (MW 12.5 kDa) in 5 mM Ca\(^{2+}\) solution (bottom) compared to isolated D6 in 0 mM Ca\(^{2+}\) solution (top)
**Isolated D6 has no Capacity to Bind F-actin**

Fragment D6-HP has the ability to bundle F-actin under high calcium concentrations. One F-actin binding site is expected on the HP domain, whereas the other is proposed on D6 using a cryptic F-actin binding site. In order to test this cryptic binding site hypothesis, we utilized an F-actin sedimentation assay. D6 appears to have no detectable F-actin binding capacity (calibration curve Figure 20A, binding curve Figure 21A, chromatograms figure 22). The control HP domain displayed a hyperbolic binding shape, characteristic of specific binding, when total HP was graphed versus HP bound (calibration curve Figure 20B, binding curve Figure 21B, chromatograms Figure 23).
Figure 20. Calibration curves of D6 and HP. Absorbance measured at 222 nm.
Figure 21. A. Bound D6 pulled down in the F-actin sedimentation assay versus total D6 concentration. B. Bound headpiece pulled down in the F-actin sedimentation assay versus total headpiece concentration.
Figure 22. Representative chromatograms of D6 bound to F-actin eluting from the HPLC (λ = 222 nm, elution volume 28.5 ml). As total D6 concentration increases bound D6 peak area remains undetectable.
Figure 23. Chromatograms of HP bound to F-actin eluting from the HPLC ($\lambda = 222$ nm, elution volume 30.3 ml). As total HP concentration increases bound HP peak area also increases. When peak area is graphed vs. total HP concentration a hyperbolic curve is generated characteristic of specific binding.
Discussion

Domain 6 Contains One or More Calcium Binding Sites that Regulate Structural Conformations

Villin is an extraordinarily adaptable protein. Its multifunctionality is controlled by several co-regulating signaling mechanisms. Calcium binding, tyrosine phosphorylation and PIP2 binding regulate the many roles of villin. Understanding the mechanisms whereby these roles are accomplished is becoming increasingly important given the usefulness of villin as a diagnostic agent for malignant epithelial cells and as a target to inhibit the interactions of villin with Shigella flexneri as an entry mechanism for the pathogen into the body.

Our study aimed to look into the characteristics of 6th gelsolin-like domain in isolation. Previous studies showing the unexpected bundling ability and propensity to aggregate of the D6-HP fragment, led to the hypothesis that some of these characteristics were dependent upon D6. Our first functional aim was to investigate the calcium binding potential of D6. Putative calcium binding sites have been suggested based on homology to gelsolin and tryptophan quenching data, but no definitive data has been presented to prove these sites exist. Our $^{15}$N-HSQC back-titration data conclusively establishes at least one calcium binding site on domain 6 in, what is most likely, an analogous position and coordination site with side chain residues of that of gelsolin’s sixth domain. It is feasible that two calcium binding sites exist on D6. These would flank either end of the long $\alpha$-helix. If this were the case, D6 would adopt a similar calcium binding pattern to that of domain 1 of villin, also referred to as 14T (Markus, et al., 1997). Furthermore, residues sensitive to calcium concentration primarily reside along the major $\alpha$-helix suggesting a rearrangement of
the helix with respect to the rest of the domain. Other residues sensitive to calcium reside on β-stands 1 and 2 (Figure 18C). This is expected if the long helix were to bend away from the core. This supports the notion that villin is too controlled by a “latch and hitch” mechanism where under low calcium concentrations domain 6 contains a bent α-helix allowing it to bind to another domain of villin sterically hindering villin’s actin binding sites. We propose that, at high calcium concentrations, the long α-helix in D6 straightens. This releases it from the other domains and exposes villin’s actin binding sites. Noteworthy, because it is in disagreement with our results, is the hypothesis presented in the same paper as the crystal structure of human domain 6 (Wang, et al., 2009). This study, by the Burtnick lab, suggested that the long α-helix is straight regardless of calcium concentration. This was supported by the crystal structure of human domain 6 in the absence of calcium and computational studies. Interestingly in this work, the fragment of villin reported contained domains 4, 5 and 6 but only domain 6 gave X-ray diffraction (Wang, et al., 2009). Possible explanations for this straight helix in a low calcium environment may be due to the influence of the other attached domains, the presence of actin in solution, or simple crystal packing distortions of the structure. To resolve this discrepancy, a solution or crystal structure should be determined of isolated D6 in a calcium-free environment.
The Linker Sequence is Involved in a Calcium Concentration Dependent Aggregation

The second characteristic investigated in this study was the stability of domain 6 in varying calcium concentrations. In the low calcium solution, D6-HP aggregated within 24 hours of calcium removal. Our size exclusion chromatography data indicates that the isolated D6 is stable as a monomer regardless of calcium concentrations. Knowing that HP is insensitive to calcium we can infer that the linker sequence is responsible for oligomerization of D6-HP and could cause villin oligomerization under the right circumstances. What still remains to be determined is how the linker sequence is regulated by calcium and how it interchanges between oligomerization and F-actin binding.

The Linker Sequence is Necessary for F-actin Binding

The most surprising characteristic of the D6-HP fragment was its ability to bundle F-actin under high calcium concentrations. This is particularly intriguing because villin as a whole bundles F-actin under low calcium concentrations. This makes D6-HP the smallest known F-actin bundling fragment. We speculated that D6-HP accomplished F-actin bundling through two F-actin binding domains tethered together. It was already known that villin HP contains a calcium insensitive F-actin binding site. We proposed that D6 contained a cryptic F-actin binding site that is activated in high calcium concentrations. This was reasonable because gelsolin-like domains can have F-actin binding capability. Interestingly, no F-actin binding ability was observed for D6 regardless of the calcium concentration. This
suggests that elements of the 40-residue linker sequence, an intrinsically disordered region (IDR), play a part in F-actin bundling.

Based on an aggregation study of D6-HP published in 2007 (Smirnov et al., 2007), it is unlikely that D6-HP bundles through dimerization or aggregation. This would point to the linker sequence containing, at least part of, a cryptic F-actin binding site. If both D6 and the linker form a binding site, its regulation by calcium can be explained through D6. The other option presented is that the linker sequence alone is sufficient for F-actin binding. This model is not easily regulated by calcium, as no calcium binding has been observed for the linker. This would indicate that the linker is controlled by D6. Both of these hypotheses would most likely be the result of an induced fit mechanism for the linker sequence. This is supported by the cyro electron microscopy images of villin bundling D6. Looking at these images it is seen that the flexible linker is likely compacted allowing domains 1,2, the HP and possibly 4 interact with F-actin (Hampton, et al., 2008). This bundling arrangement would also explain the absence of detectable F-actin binding by D6 and the linker. In this arrangement D6 is equidistant between the two F-actin filaments and thus has no chance of interacting with either. To elucidate the F-actin binding domain, a future study could employ a D6-HP fragment containing the supervillin headpiece (D6-svHP). This domain does not bind F-actin yet has the identical size and fold of villin’s headpiece. By comparing this to a GST-svHP fragment one could determine which domains are responsible for F-actin binding. The D6-svHP fragment would establish whether both D6 and the linker are sufficient for F-actin binding. The GST-HP would establish whether the linker is sufficient for F-actin binding (Figure 23). GST is a suitable replacement domain because it is stable and does not bind actin.
Figure 23. Proposed study to resolve the second F-actin binding site on D6-HP. Left: A D6-svHP fragment containing supervillin headpiece would remove the F-actin binding site on the HP. The remaining F-actin binding would be associated with D6 and the linker. Right: domain six of D6-svHP is replaced by GST. Any F-actin binding would be solely associated with the linker.
Materials and Methods

Protein Expression and Purification

D6 for the F-actin binding assay was produced according to the procedures previously adapted by Lucian Burns, Alex Nelson and Danielle Pfaff. Briefly, the E. coli strain BL21-(DE3) was transformed with the pET-24a plasmid containing the D6 sequence using heat shock transformation (ice then 42 °C); the cells were plated on Kanamycin Luria-Bertani broth (LB) plates. An isolated colony was used to inoculate seed cultures (~100 ml), which were used to start a large scale growth of the transformed strain. The strain was grown in 6 L of LB, containing 10 μg/ml of kanamycin, to an optical density of ~0.6. Protein expression was then induced with isopropyl β-D-1-thiogalactopyranoside (IPTG) and expressed for 5 hours at 37 °C. Cells were then harvested by centrifugation at 4,000 g for 30 minutes. Pellets were suspended in lysis buffer (0.50 μg/ml Lysozyme, 20 mM sodium Phosphate, 200 mM NaCl, 50 mM DTT, 0.2 mM PMSF, 0.1 mM Benzamidine, 1.0 mM EDTA, pH 7) and lysed using sonication. The lysed pellets were centrifuged at 40,000g for 30 minutes and the supernatant collected.

Isotopically labeled D6 for 13C/15N NMR studies was also produced according to methods adapted by Lucian Burns, Alex Nelson, Terry Webb and Danielle Pfaff. Outlined briefly, transformed BL21-(DE3) cells with the pET-24a plasmid encoding for domain 6 were grown at 37 °C in 6 liters of LB up to an optical density of 0.6. Cells were then pelleted from the solution via centrifugation (20 minutes at 4,000g) and respuspended/washed in minimal media (MT9) containing no nutrient sources. The cells were again centrifuged and
put into a minimal media containing only $^{15}$N and $^{13}$C labeled nutrient sources ($^{15}$NH$_4$Cl, $^{13}$C-D-glucose). The cells were equilibrated in the media for 1 hour and then induced with 0.80 mM IPTG. Cells were harvested after 5 hours of expression by centrifugation (30 minutes at 4,000g). The pellet was resuspended in Lysis buffer and lysed using sonication. The lysed pellets were centrifuged at 40,000g for 30 minutes and the supernatant collected.

D6 was initially purified from the supernatant using gravity flow size exclusion chromatography. A 2.5 cm x 100 cm column from Kontes Chromaflex Chromatography was loaded with sephadex G-50 resin from General Electric Healthcare. The mobile phase was 5mM CaCl$_2$, PIPES buffer (20mM 2-[4-(2-sulfoethyl)piperazin-1-yl]ethanesulfonic acid (PIPES), 5 mM dithiothretol (DTT), 5 mM calcium dichloride (CaCl$_2$), 150 mM sodium chloride (NaCl), .02% sodium azide (NaN$_3$), pH 7). 6 ml fractions were collected over 3 hours. High purity D6 was obtained by repurifying the fractions containing D6 using GE Healthcare Superdex 75 10/300 column. The mobile phase was 5mM CaCl$_2$ PIPES buffer (pH 7) and was run at a flow rate of 0.5 ml/min. The major peak was collected and protein purity analyzed using 15% SDS-PAGE. The $^{13}$C/$^{15}$N labeled D6 sample and the $^{15}$N labeled D6 sample used for NMR spectroscopy were prepared by Lucian Burns, Alex Nelson, Terry Webb and Danielle Pfaff.

**F-actin Binding Assay**

Lyophilized rabbit skeletal actin was purchased from Cytoskeleton Inc (Denver, CO). Samples were prepared by resuspending lyophilized actin to a concentration of 24 μM (G-
actin) in 5.0 mM tris(hydroxymethyl)aminomethane (Tris-HCl), 0.1 mM CaCl₂, 0.20 mM ATP, 0.50 mM DTT and pH 7.3. Samples sat for 1 hour at 4 °C to respsend actin. The samples were then clarified by centrifugation at 14 Krpm for 15 minutes in a tabletop microcentrifuge at 4 °C. The upper 90 - 95% (~1 ml) of the supernatant was transferred to a new container. 60.00 μL aliquots were transferred to ultra-centrifuge tubes and 6.00 μL of 10X F-actin buffer (500.0 mM potassium chloride (KCl), 20.0 mM magnesium dichloride (MgCl₂), pH 7.3, 10.0 mM ATP) was added. Samples were polymerized for 1 hour at room temperature. The solution was brought to 120 μL with final concentration being: Actin 12.0 μM, 4.1 mM Tris-HCl, ATP, 1.1 mM, 45 mM KCl, 1.8 mM MgCl₂, 0.4 mM DTT, pH 7.3 and incubated with either D6 or HP67. Concentrations of D6, HP67 and calcium chloride varied between runs. Calcium chloride concentrations were 0.1, 1 or 5 mM, D6 and HP67 concentrations were 5, 10, 25, 50, 100 and 200 μM. After 1 hour of incubation at 4 °C or room temperature, samples were pelleted by centrifugation for 1 hour at 150,000 g (centrifuge model Thermo Scientific Sorvall mX 150). The supernatant was removed, the pellet washed with 1X F-actin buffer and the pellets resuspended in 7% acetic acid.

Pellets were analyzed using reverse phase high performance liquid chromatography. A linear gradient from 20-80% acetonitrile was developed at a flow rate of 1 ml/min through a Beckman Ultrasphere Analytical Column over 85 minutes. The ion pairing agent was trifluoroacetic acid (0.1%) and eluting protein was monitored using UV-vis spectroscopy (λ=222 nM). Peak area and retention time were visualized using the Varian Star Chromatography Workstation software (version 5.51) and analyzed in Microsoft Excel. Peak area versus concentration calibration curves were constructed individually for HP67 and D6 using standardized protein samples.
**Calcium Binding Assay**

A previously prepared $^{15}$N labeled D6 sample in Ca$^{2+}$ NMR buffer (10% D$_2$O, 90% H$_2$O, 5 mM CaCl$_2$, 20 mM PIPES, 10 mM DTT, .02% NaN$_3$, pH 7) was used to acquire an $^{15}$N-HSQC spectrum on the in-house Varian Inova 500 MHz spectrometer using the Varian triple resonance probe. The sample was then titrated with NMR buffer containing 0.50 M EDTA and lacking CaCl$_2$. $^{15}$N-HSQC spectra were taken at free calcium levels of 5.00, 1.00, 0.750, 0.500, 0.250, 0.00 mM. To test reversibility, after the 0.00 mM calcium spectrum was collected the sample was allowed to sit for 24 hours. A buffer exchange to Ca$^{2+}$ NMR buffer then was accomplished using Amicon centrifugal filter units (MW limit 3 kDa) and another $^{15}$N-HSQC spectrum was acquired.

**Dimerization Assay**

Two D6 samples, one in 5.0 mM CaCl$_2$ PIPES buffer (pH 7), the other in 0.0 mM CaCl$_2$ PIPES buffer (pH 7) were analyzed on a GE Superdex 75 10/300 column. The samples were eluted at a flow rate of 0.5 ml/min and absorbance of the eluent was monitored at 280 nm. Raw chromatogram data was exported from the GE ÄTKA PRIME PLUS FPLC and processed in Excel. Elution time and peak area were analyzed in Excel.
Nuclear Magnetic Resonance Acquisition and Analysis

NMR Data Collection and Processing

NMR samples contained 0.5-1.0 mM \(^{13}\text{C}/^{15}\text{N} \text{D6}\) in 10\% \(^2\text{H}_2\text{O}\), 5 mM CaCl\(_2\), 10 mM d\(_{10}\)-DTT, 0.01\% NaN\(_3\), and 20 mM d\(_{18}\)-PIPES \((\text{pH} \ 7.0)\). D\(_2\)O was not corrected for in pH adjustment. 2D and 3D NMR spectra were acquired at 25 °C on a Varian INOVA 720 MHz spectrometer with Triple Resonance Probe located at the National High Magnetic Field Laboratory at Florida State University \((\text{Tallahassee, FL})\) and with a 500 MHz Vairain INOVA spectrometer with a Triple Resonance Probe \((\text{Bellingham, WA})\). All spectra were processed using NMRPipe \((\text{Delaglio, et al., 1995})\).

NMR Resonance Assignment of D6

NMR resonance assignments for the D6 fragment were produced from the following data sets. Backbone assignment was accomplished through the 2D heteronuclear spectra: \(^{15}\text{N}-\text{HSQC}\), \(^{13}\text{C}-\text{HSQC}\) and the 3D spectra: HNCACB, CBCA(CO)NH, HNCO, HN(CA)CO, HNHA, HNHB, HBHA(CO)NH. Aliphatic side chain assignments were produced from the 3D spectra: HCCH-COSY, HCCH-TOSCY, CC-(CO)-NH and HC(CO)NH. Aromatic side chain assignments were produced from the spectra: \(^{13}\text{C}-\text{HSQC}\) (aromatic), \(^1\text{H}-\text{NOESY}\) and \(^1\text{H}-\text{TOCSY}\). The PINE \((\text{Bahrami, et al., 2009})\) server was used for automated assignment which was then manually verified and corrected as necessary. All samples were referenced internally using 3-(trimethylsilyl)tetradepsidropropionate \((\text{TMSP})\). Visualization, peak picking and analysis of the NMR spectra were achieved using NMRVIEW \((\text{Johnson, 1994})\).
**Structural Determination of D6**

CYANA 2.1 (Guntert, 2004) was used to produce and refine the solution structure of isolated D6. The restraints of CYANA were NOE distance and dihedral angles. 2D and 3D NOESY data provided the distance restraints. The PREDITOR server (Berjanskii, et al., 2006) was used for dihedral angle restraints determination based of chemical shift values of $^1\text{H}$ backbone, $^{15}\text{N}$ backbone, $^{13}\text{Ca}$, $^{13}\text{CO}$, and $^{13}\text{C}\beta$. 3D NOESY, $^{15}\text{N}$-NOESY, $^{13}\text{C}$-NOESY aliphatic and $^{13}\text{C}$-NOESY aromatic processed crosspeak sets were first loaded into CYANA and assigned concurrently with the iterative structural calculations. NOE assignment was maximized using the `noeassign` macro of CYANA. Simulations typically used 600 initial conformers and employed 6000 or 10000 steps of torsion angle dynamics. The 10 lowest were manually checked prior to the next cycle. After structural calculation was completed, NOE assignment was manually checked for violated distance restraints greater than 0.5 Å. These were then unassigned and the next simulation carried out. This procedure continued until the bulk of the 3D NOESY cross peaks had been assigned (assignment > 90%). The 3D NOESY assignments were then locked and 2D $^1\text{H}$ NOESY crosspeak sets were added. Any resulting violations (2D and 3D) greater than 0.5 Å were unassigned for the next run. All runs were completed at least twice using different seed values to ensure convergence of assignments. The final structure had 1076 upper distance restraints and a backbone RMSD of <0.6 Å for residues 1-99 in the 10 best structures (directory path: home/smirnos/nmr/d6/cyana/d6_REFINEMENT/ d6__qu2).
Appendix 1: CYANA File Format Examples

These figures are intended as examples to cover all CYANA file formats. For information on CYANA macros and program updates see http://www.cyana.org/wiki/index.php/Main_Page.

Figure A1. CYANA calculation file. (d6_calc.cya) File assigning NOE crosspeak files, atom resonance frequencies file, dihedral angles file as well as CYANA macros run (in this case noeassign & overview).

Figure A2. CYANA initiation file. (init.cya) file assigning the run name and RMSD range. The file also retrieves the CYANA residue library, the protein sequence, and the resonance frequencies files for use in the calculation file.

Figure A3. CYANA prot file. (d6_manual__d6__b.prot) File containing resonance frequencies for all assigned nuclei.
Figure A4. CYANA dihedral file. (d6___pb__preditor.aco) File containing dihedral restraints.

Figure A5. CYANA sequence file. (d6.seq) File containing protein sequence.
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