Structural and molecular analysis of leech neuronal proteins, Calsensin and Filamin

Deepa V. Venkitaramani
Iowa State University

Follow this and additional works at: http://lib.dr.iastate.edu/rtd

Part of the Cell Biology Commons, Molecular Biology Commons, and the Neuroscience and Neurobiology Commons

Recommended Citation
http://lib.dr.iastate.edu/rtd/1200
Structural and molecular analysis of leech neuronal proteins, Calsensin and Filamin

by

Deepa V. Venkitaramani

A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of
DOCTOR OF PHILOSOPHY

Co-majors: Molecular, Cellular and Developmental Biology; Neuroscience

Program of Study Committee:
Jørgen Johansen, Co-major Professor
Kristen M. Johansen, Co-major Professor
Amy H. Andreotti
Donald S. Sakaguchi
Ted W. Huiatt

Iowa State University
Ames, Iowa
2004
The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleed-through, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.
This is to certify that the doctoral dissertation of
Deepa V. Venkitaramani
has met the dissertation requirements of Iowa State University

Signature was redacted for privacy.

Co-major Professor

Signature was redacted for privacy.

Co-major Professor

Signature was redacted for privacy.

For the Co-major Program

Signature was redacted for privacy.

For the Co-major Program
To my family and friends for their love and support
<table>
<thead>
<tr>
<th>TABLE OF CONTENTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
</tr>
<tr>
<td>CHAPTER 1. GENERAL INTRODUCTION</td>
</tr>
<tr>
<td>Introduction</td>
</tr>
<tr>
<td>Dissertation Organization</td>
</tr>
<tr>
<td>Background</td>
</tr>
<tr>
<td>CHAPTER 2. NMR STRUCTURE AND BACKBONE DYNAMICS OF CALSENSIN, AN INVERTEBRATE NEURONAL CALCIUM-BINDING PROTEIN</td>
</tr>
<tr>
<td>Abstract</td>
</tr>
<tr>
<td>Introduction</td>
</tr>
<tr>
<td>Material and Methods</td>
</tr>
<tr>
<td>Results and Discussion</td>
</tr>
<tr>
<td>Conclusions</td>
</tr>
<tr>
<td>References</td>
</tr>
<tr>
<td>Figure Legends</td>
</tr>
<tr>
<td>Figures</td>
</tr>
<tr>
<td>CHAPTER 3. LEECH FILAMIN AND TRACTIN: MARKERS FOR MUSCLE DEVELOPMENT AND NERVE FORMATION</td>
</tr>
<tr>
<td>Abstract</td>
</tr>
<tr>
<td>Introduction</td>
</tr>
<tr>
<td>Methods</td>
</tr>
<tr>
<td>Results</td>
</tr>
<tr>
<td>Section</td>
</tr>
<tr>
<td>-------------------------------</td>
</tr>
<tr>
<td>Discussion</td>
</tr>
<tr>
<td>References</td>
</tr>
<tr>
<td>Figure Legends</td>
</tr>
<tr>
<td>Figures</td>
</tr>
<tr>
<td>CHAPTER 4. GENERAL CONCLUSIONS</td>
</tr>
<tr>
<td>Conclusions</td>
</tr>
<tr>
<td>Future Directions</td>
</tr>
<tr>
<td>APPENDIX. ADDITIONAL DATA</td>
</tr>
<tr>
<td>REFERENCES CITED</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
</tr>
</tbody>
</table>
This thesis describes the structural and molecular analysis of two leech neuronal proteins, Calsensin and Filamin. Calsensin is an EF-hand calcium-binding protein expressed by a subset of peripheral sensory neurons which fasciculate into a single tract in the leech central nervous system. Calsensin is a 9 kD protein with two EF-hand calcium-binding motifs. Calsensin can multimerize via disulfide bridge formation involving the two cysteine residues in vitro. Furthermore, Calsensin shows differential gel migration depending on the presence or absence of calcium suggesting a conformational change upon calcium-binding. Using multidimensional NMR spectroscopy we have determined the solution structure and backbone dynamics of calcium-bound Calsensin. Calsensin consists of four helices forming a unicomate-type four-helix bundle and we show by analysis of EDTA and Ca\(^{2+}\) titrations that Ca\(^{2+}\) binding leads to conformation change in the protein. The backbone dynamics of the protein was determined by measuring the \(^{15}\)N relaxation rates and heteronuclear NOE. The internal dynamics of the protein correlate well with the three-dimensional structure and provide insight into plausible mechanisms of calcium and target protein binding. To search for putative molecular interaction partners of Calsensin, we conducted a GST-Calsensin overlay screen of a leech expression library. A probable candidate is the leech homolog of Caldesmon, which interacts with Calsensin in a calcium-dependent manner. We have mapped the binding surface of this interaction using HSQC experiments.
The Laz10-1 and Lan3-14 monoclonal antibodies recognize a 400 kD antigen expressed in all muscle cells. We have cloned and identified this antigen as a member of the filamin family of actin-bundling proteins. Leech Filamin consists of two calponin homology (CH) domains and 35 filamin/ABP-repeats. The CH domains bind actin while the ABP-repeats have been implicated in dimerization as well as protein-protein interactions. Using the Laz10-1 antibody, we have shown that the dorso-ventral flattener muscles develop as three discrete bundles. The middle bundle transiently expresses neuronal CAM Tractin concomitant with the formation of the DP nerve. This suggests that the middle dorso-ventral muscle anlagen may provide the substrate for the axonal outgrowth and nerve formation.
CHAPTER 1. GENERAL INTRODUCTION

INTRODUCTION

Axon guidance is made possible due to the ability of growth cones to detect the local cues and convert them into signal transduction changes in the cytosol that result in cytoskeletal rearrangements. In recent years, the guidance cues and their receptors that drive these processes have been extensively studied. The signal transduction events occurring downstream of these receptors are being dissected to understand how the guidance cues lead to modification of cytoskeleton. Exploring the mechanism of axon guidance and pathfinding would help advance our knowledge of neuronal development and synaptic plasticity. Various neurological disorders like Periventricular Heterotopia and Down's syndrome have been attributed to dysfunction of proteins involved in axon guidance. Hence, to better understand the importance of neuronal proteins, we have performed structural and molecular analysis of a leech calcium-binding protein, Calsensin and an actin-bundling protein, Filamin.

DISSERTATION ORGANIZATION

This dissertation has been divided into four chapters. The first chapter is a general introduction of the development of nervous system with emphasis on axon guidance and pathfinding. Following the overview, the importance of calcium-mediated signal transduction events and cytoskeletal rearrangements are discussed in detail. Some of the proteins involved in the above-mentioned processes are
surveyed. Finally, the advantages of leech as a model system are described followed by previous work on Calsensin.

The second and third chapters of the dissertation are arranged in the paper format. The second chapter is a manuscript in preparation. This paper elucidates the NMR structure and backbone dynamics of a neuronal calcium-binding protein, Calsensin. Calsensin is expressed by a subset of peripheral sensory neurons that fasciculate into single axon tract. Its structure consists of four helices arranged as a unicormate-type four-helix bundle. Calsensin undergoes a calcium-induced conformational change, thereby exposing hydrophobic residues which may be involved in target binding.

Chapter Three consists of a paper published in *Journal of Neurobiology*, September 2004. This paper describes the cloning and characterization of leech Filamin and its relationship to the non-neuronal expression of cell-adhesion molecule, Tractin. Transient expression of Tractin by the dorsoventral flattener muscles coincides with the formation of DP nerve, suggesting a role in axon fasciculation and pathfinding.

The fourth chapter presents the general conclusions summarizing the current work on Calsensin and Filamin. It then goes on to discuss the probable function of both proteins and propose future directions to advance our understanding of axon guidance and pathfinding.
BACKGROUND

Overview of axon guidance

The growth cones of early neurons pioneer specific axon pathways, establishing the route for the axons of later developing neurons (Dodd and Jessell, 1988). Pathfinding by growing axons in the developing nervous system is guided by diffusible and/or bound factors that attract or repel the axonal growth cones (Tessier-Lavigne and Goodman, 1996). The precise and stereotypic connections of the developing neurons with their targets are formed by specific navigation and pathway selection of the growth cones in response to various combination of guidance cues (Goodman and Shatz, 1993; Goodman, 1996; Tessier-Lavigne and Goodman, 1996; Chisholm and Tessier-Lavigne, 1999). Some of these interactions exert specific growth-promoting effects while others have inhibitory effects (Dodd and Jessell, 1988; Jessell, 1988). The cytoplasmic signaling mechanisms that trigger the responses of the growth cone to guidance factors are mostly unknown. Previous studies have shown that the level and temporal patterns of cytoplasmic calcium can regulate the rate of growth cone extension in vitro and in vivo. Recently it was reported that calcium also mediates the turning behavior of the neuronal growth cones in culture that are induced by extracellular gradients of guidance molecules. Different patterns of calcium elevation were shown to trigger attractive or repulsive turning responses (Hong et al., 2000; Zheng, 2000).
Calcium signaling in axon guidance

Increase in intracellular calcium, either through release from intracellular stores or influx through the L- and N- type calcium channels, is essential for the signal transduction mechanisms used by various guidance factors studied so far. The optimal range between 100-300 nM of intracellular calcium is an essential factor in the control of neuronal development and any fluctuation from this range results in cessation of growth cone motility (Kater and Mills, 1991). For instance, neuronal differentiation of PC12 cells and neurite outgrowth from a variety of rat neurons cultured on either N-CAM-, N-Cadherin- or L1-expressing 3T3 monolayers could be blocked by L- and N- type calcium channel blockers, suggesting that calcium plays an important role in the signaling pathway involved in neuronal differentiation mediated by L1 and N-CAM (Doherty, et al., 1991; Williams, et al., 1992; Doherty, et al., 1993; Doherty, et al., 1994). Furthermore, increases in intracellular calcium levels may be necessary for the turning behavior of growth cones (Gunderson, and Barrett, 1980; McCaig, 1989; Hong, et al., 2000; Zheng, 2000) and there is also evidence that it may be essential for fasciculation of neurites since clamping intracellular calcium at 50-70nM in rat sympathetic neurons prevented their fasciculation in vitro (Tolkovsky, et al., 1990).

The level and temporal pattern of intracellular calcium may be an important aspect of signal transduction used by both growth cone attracting and inhibiting guidance molecules (Doherty et al., 1991; Williams et al., 1992). Generation of short-lived calcium domains at the cytosolic side of open channels may provide a crucial mechanism for axon guidance during development and for promoting regeneration
of damaged axons without raising bulk cytosolic calcium concentration (Archer et al., 1999).

**EF-hand calcium-binding proteins in the nervous system**

Numerous EF-hand calcium-binding proteins are expressed specifically in the nervous system. Recent evidence has implicated calcium-binding proteins in signal transduction events in the growth cone. For example, when calmodulin function was selectively disrupted by an antagonist *in vivo* in a subset of neurons in *Drosophila*, growth cones often stalled, defasciculated, and made errors in trajectory (VanBerkum, and Goodman, 1995). These results demonstrate an *in vivo* function for calcium signaling in growth cone extension and guidance and suggest that they may in part regulate specific growth cone decisions, including when to defasciculate and whether or not to cross the midline.

In addition to their role in calcium mediated signal transduction, extracellular functions have been attributed to certain members of the S100 family of EF-hand calcium-binding proteins. Extracellular S100B has been shown to promote neurite extension in chick cortical neuronal cultures (Kligman and Marshak, 1985) and stimulate glial cell proliferation (Selinfreund, et al., 1991). S100A1 and S100B expression in PC-12 cells results in increased intracellular calcium and subsequently cell death (Zimmer, et al., 1998; Mariggio, et al., 1994). Both the neuronal trophic (neurite outgrowth) as well as toxic (apoptosis) effects of S100A1 and S100B have been suggested to be mediated via Receptor for Advanced Glycation End products (RAGE) and in several cases requiring disulfide linked dimers (Huttunen, et al., 2000).
Actin cytoskeletal events downstream of calcium signaling

Finally, many of the growth promoting molecules are associated with cytoskeletal components (Gumbiner, 1993). The calcium signal may be transduced into cytoskeletal rearrangements by EF-hand calcium-binding proteins. In fact, many of these proteins have been shown to interact with cytoskeletal components in a calcium-dependent manner. Recently, it was found that the developmental expression pattern of Xenopus Calcineurin transcripts in vivo coincides temporally with axonal pathfinding by spinal neurons, supporting a role for Calcineurin in regulating calcium dependent neurite extension in the spinal cord via actin cytoskeleton remodeling (Lautermilch and Spitzer, 2000). In addition, some of the EF-hand proteins have been proposed to function in neurite outgrowth and signal transduction events associated with vision and olfaction.

The local increase in calcium activates Ca\(^{2+}\)/calmodulin dependent kinases and other kinases (Doherty and Walsh, 1996). Regulation of cytoskeletal stability may be a major function of calcium in the growth cones. The elevated calcium levels can activate profilin and gelsolin, which in turn promote reorganization of the actin cytoskeleton (Suter and Forscher, 1998). The ability of the microtubule stabilizing agent taxol to block calcium-ionophore A23187-induced neurite retraction and the ability of the actin-stabilizing agent phalloidin to reverse both A23187-induced growth cone collapse and neurite retraction suggested that calcium may control neurite elongation at least in part by regulating actin filament stability, and support a model for neurite outgrowth involving a balance between assembly and disassembly of the cytoskeleton (Lankford and Letourneau, 1989; Welnhofer et al., 1999).
**Leech as a model system**

To understand the signaling mechanisms used by identified guidance cues *in vivo*, simple organisms with developmentally accessible nervous systems are invaluable. The developing leech nervous system provides an excellent model system for studying the mechanisms of axonal guidance and fascicle selection of the peripheral sensory neurons due to its relatively simple, segmentally iterated nervous system and accessibility to functional perturbation experiments throughout development (Müller et al., 1981; Jellies and Kristan, 1988; Zipser et al., 1989). The nervous system of the leech consists of a head brain, a tail brain, and 21 iterated segmental body ganglia that contain approximately 400 bilaterally symmetric neurons (Müller et al., 1981; Sawyer, 1986). Another major advantage of the leech nervous system is that the number of peripheral neurons continues to increase throughout the life of the animal (Peinado et al., 1990). This means that the molecules involved in axon guidance and pathfinding would persist throughout the life of the leech.

The development of the central and peripheral nervous systems occurs at E7-8 stage (E-embryonic day), and proceeds in a rostro-caudal direction. Each posterior segment is approximately 3-4 hours later in development than the immediate anterior segment, so that the leech embryo exhibits segments in various stages of development spanning over a period of 3-4 days (Stent et al., 1982). The first peripheral neurons to differentiate are the sensillar neurons at E7-8 (Johansen et al., 1992). When the growth cones of the sensillar neurons reach the ganglionic CNS, they bifurcate into rostral and caudal branches, and segregate into four well defined and stereotypically located fascicles in each of the central connectives (Johansen et
al., 1992; Johansen et al., 1994; Jellies et al., 1994; Jellies et al., 1995; Briggs et al.,
The growth cones of these neurons fasciculate in the CNS with the four fascicles
formed earlier by the peripheral sensory neurons (Johansen et al., 1992; Briggs et
al., 1993).

In order to identify the molecular markers involved in the formation of precise
connections between the neurons, a large number of nervous system specific
monoclonal antibodies were generated against adult *Haemopsis marmorata* nervous
system proteins (Zipser and McKay, 1981). The segregation of the peripheral
sensillar neurons into four distinct fascicles within the leech CNS (Johansen et al.,
1992), and the results that regenerating peripheral axons were able to re-establish
their correct axon pathways following nerve root crushing, suggested that precise
molecular guidance cues are utilized during the development of leech nervous
system (Peinado et al., 1987).

The glycoepitope recognized by the monoclonal antibody Ian 3-2 is present in
all of the peripheral neurons and the four fascicles formed by their afferents
(Johansen et al., 1992). The two antigens of Ian 3-2 are Tractin, a member of the L1
subfamily of the Ig superfamily (Huang et al., 1997), and Leech CAM, a leech
homolog of NCAM/FasII/ApCAM (Jie et al., 1999a). Perturbation with Ian 3-2
antibody results in inhibition of neurite extension, truncated fascicle formation, and
decrease in synaptogenesis (Zipser et al., 1989; Huang et al., 1997; Tai and Zipser,
1998). The guidance cues used by the peripheral neurons to segregate into four
stereotypic fascicles probably are provided by the CNS, since the growth cones of
the rostral and caudal projections of peripheral neurons meet in the connectives in
perfect register (without any apparent realignment) as if they were following a
preformed tract (Briggs et al., 1993; Johansen et al., 1994). The experiments
involving the surgical removal of a part of the CNS in the developing leech embryo
provided strong evidence for the existence of a hierarchy of guidance molecules.
The growth cones of the peripheral neurons failed to reach the CNS and segregate
into four distinct fascicles when a part of the CNS was removed; instead they
fasciculated together and migrated to nephridiopore (Johansen et al., 1994). Recent
evidence demonstrates that the guidance cues provided by ectopic CNS projections
are both necessary and sufficient for the peripheral neurons to migrate into the CNS
(Jellies et al., 2000).

The entire population of peripheral neurons which segregate into four tracts is
recognized by the Ian 3-2 antibody, suggesting that the segregation of axons into
different tracts involves different sets of guidance cues which may interact in a highly
complex and temporally regulated manner (Johansen et al., 1992; Johansen et al.,
1994). A potential candidate for mediating axonal guidance and pathway selection
and/or maintenance is the Ian 3-6 antigen (Calsensin), due to its restricted
expression to a subset of peripheral neurons that segregate into a single tract within
the CNS (Briggs et al., 1993).

**Previous work on Calsensin**

Calsensin is a novel neuronal EF-hand calcium-binding protein cloned in our
laboratory by screening the leech expression library using the Ian 3-6 mAb (Briggs et
al., 1995; Zipser and McKay, 1981). It is a small 83 aa protein with a predicted
molecular mass of 9.1 kD that contains two helix-loop-helix domains (Briggs et al.,
1995). This sequence was used for in situ hybridization and for generating polyclonal antibodies Friggs and Hel, since lan 3-6 mAb does not recognize denatured native antigen on western blots. The Calsensin polyclonal antisera recognizes a ~9 kDa protein on western blots which corresponds to its predicted size. Sequence analysis revealed that Calsensin belongs to a new subfamily in a large superfamily of EF-hand containing calcium-binding proteins. The members of this family modulate the action of other enzymes and structural proteins, contain one to eight copies of the calcium-binding motif which is composed of a loop of 12 contiguous residues with oxygen atoms involved in calcium-binding and two flanking α-helices that stabilize the complex. Functional EF-hands are usually found in pairs (Persechini et al., 1989).

The two calcium-binding domains of Calsensin conform to the HLH-structure of the EF-hand family of calcium-binding proteins indicating that the domains are likely to be functional (Strynadka and James, 1989). To verify that the EF-hands are functional, partially purified Calsensin fusion protein was incubated with $^{45}$CaCl$_2$ and subsequently subjected to autoradiography (Maruyama et al., 1984). The Calsensin fusion protein was found to selectively bind $^{45}$Ca$^{2+}$ under these conditions in vitro (Briggs et al., 1995). Calsensin is most closely related to bovine ICaBP, both being 9 kDa proteins with two HLH motifs. It shares highest identity with l-Plastin (39%), a calcium dependent actin-bundling protein found in human intestine and kidney (Lin et al., 1994; Rosales et al., 1994). Calsensin shares 33% identity to protozoan Calcineurin B (Briggs et al., 1995). However, these proteins are much larger than Calsensin and most of them have more EF-motifs. Even though Calsensin shares
some identity with these EF-hand proteins, there appears to be no known homolog to this protein.

An unusual feature of Calsensin is the presence of two cysteines at the N- and C- termini, respectively, suggesting that it maybe able to form intra- or inter-molecular disulfide bridges. A change in conformation of Calsensin would be expected due to the formation of disulfide bonds, which could regulate its interactions with target molecules in vivo. Cysteine residues are usually found in reduced state in the cytosol (Branden and Tooze, 1991), but some proteins like S100β are found to form dimers in vivo. The disulfide linked S100β dimer has been found to promote neurite outgrowth from chick cerebral neurons in vitro (Kligman and Marshak, 1985; Sastry et al., 1998).

Many EF-hand calcium-binding proteins are found to be nervous system specific, where their expression is restricted to certain tissues and types of neurons like parvalbumin, calbindin, and calretinin. But, the physiological functions of most of these proteins are still unknown (Baimbridge et al., 1992). The EF-hand family members are generally classified into trigger proteins and buffer proteins based on their functions (Levine and Dalgarno, 1983; Baimbridge et al., 1992). Trigger proteins, like Calmodulin, change their conformation upon calcium-binding and interact with and regulate various enzymes and ion channels, while buffer proteins such as parvalbumin and ICaBP have no identifiable targets and are thought to regulate intracellular free calcium concentration passively. Some of these calcium-binding proteins are thought to maintain calcium homeostasis and are implicated in the pathology of various neurodegenerative disorders (Heizmann and Braun, 1992). A subfamily of small calcium-binding proteins with three EF-hands has been
identified and is thought to mediate visual and odorant signal transduction (Palczewski et al., 1994; Dizhoor et al., 1991; Yamagata et al., 1990; Nemoto et al., 1993).

The physiological function of Calsensin is still unknown. Nevertheless, the two HLH motifs are likely to be functional in vivo. Immunoaffinity purification of both Haemopis and Macrobdella CNS extracts with lan 3-6 mAb covalently cross linked to protein A-Sepharose beads results in co-immunopurification of a 200 kD protein with Calsensin (Briggs et al., 1993). This suggests that Calsensin may function as trigger protein which interacts with and/or regulates the larger protein. By immunocytochemistry, the distribution and differential expression of Calsensin during development in the PNS and CNS of the three-hirudinid leech species Haemopis, Hirudo and Macrobdella was studied (Veldman et al., 1996). The restricted expression of Calsensin in a subpopulation of peripheral neurons appears to be a common feature shared by all the three leech species, but the temporal and spatial patterns of Calsensin expression by the CNS neurons varied among the different species, suggesting that Calsensin may function as a non-essential buffer protein in maintaining calcium homeostasis along with other calcium-binding proteins (Veldman et al., 1996).

As previously discussed, intracellular calcium signaling has been implicated in neurite outgrowth, turning, fasciculation and collapse. Thus, calcium-binding proteins found in the growth cones and axons, which regulate or are regulated by change in intracellular calcium levels may modulate the calcium-dependent processes of growth cone extension and axonal fasciculation. In support of this theory, many EF-hand proteins have been implicated in signal transduction pathways associated with
neurite extension. The S100β dimer has been shown to promote neurite outgrowth in chick cerebral cortical neurons *in vitro*. In addition, blocking or overexpression of *Drosophila* calmodulin *in vivo* caused errors in fasciculation and axon guidance. CALI (chromophore assisted laser inactivation) of calcineurin in chick DRG neurons has shown that its distribution may modulate growth cone motility. Finally, the molecular features of Calsensin, its association with the 200 kD protein and its restricted expression to a subset of peripheral neurons fasciculating into a single tract in the CNS are consistent with the hypothesis that the putative Calsensin protein complex may play a role in the formation and/or maintenance of the specific axonal pathways *in vivo*. 
CHAPTER 2. SOLUTION STRUCTURE AND BACKBONE DYNAMICS OF CALSENSIN, AN INVERTEBRATE NEURONAL CALCIUM-BINDING PROTEIN

A manuscript in preparation

Deepa V. Venkitaramani, D. Bruce Fulton, Amy H. Andreotti, Kristen M. Johansen
and Jørgen Johansen

Abbreviations: EDTA, Ethylenediaminetetraacetic acid; CaM, Calmodulin; SDS-PAGE, Sodium dodecyl sulfate-Polyacrylamide gel electrophoresis; NMR, Nuclear magnetic resonance; IPTG, isopropyl β-D-thiogalactopyranoside; DTT, Dithiothreitol; PMSF, Phenylmethylsulfonyl fluoride; GST, Glutathione-S-transferase; β-ME, β-mercaptoethanol; HRP, Horseradish peroxidase; HSQC, Heteronuclear single quantum correlation; 3D, three-dimensional; NOESY, Nuclear overhauser effect spectroscopy; TOCSY, Total correlation spectroscopy; HMQC, Heteronuclear multiple quantum correlation; DQF-COSY, Double-quantum-filtered correlation spectroscopy; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate; CNS, Crystallography and NMR systems; TALOS, Torsion angle likelihood obtained from shift and sequence similarity; RMSD, root mean square deviation.

ABSTRACT

Calensin is an EF-hand calcium-binding protein expressed by a subset of peripheral sensory neurons which fasciculate into a single tract in the leech central
nervous system. Calsensin is a 9 kD protein with two EF-hand calcium-binding motifs. An unusual feature of Calsensin is the presence of two cysteine residues at the amino- and carboxyl-termini and we demonstrate that Calsensin can multimerize via disulfide bridge formation in vitro. Furthermore, Calsensin shows differential gel migration depending on the presence or absence of calcium suggesting a conformational change upon calcium-binding. Using multidimensional NMR spectroscopy, we have determined the solution structure and backbone dynamics of calcium-bound Calsensin. Calsensin consists of four helices forming a unicomate-type four-helix bundle and we show by analysis of EDTA and Ca$^{2+}$ titrations that Ca$^{2+}$ binding leads to a conformational change in the protein. We provide evidence that the calcium-binding to the two loops is a two-step, sequential process. Furthermore, the residues in the third helix undergo slow conformational exchange indicating that the motion of this helix is associated with calcium-binding. The backbone dynamics of the protein as measured by $^{15}$N relaxation rates and heteronuclear NOEs correlate well with the three-dimensional structure and provide insight into plausible mechanisms of calcium and target protein binding.

INTRODUCTION

Intracellular calcium concentration regulates a variety of cellular processes including neurite extension, cell motility, cell-cycle progression, cell proliferation, and apoptosis (1, 2, 3). Many of these signal transduction events are mediated by members of the EF-hand family of calcium-binding proteins via interaction with target proteins in a calcium-dependent manner (2). The EF-hand family of calcium-binding
proteins can be classified as sensor or buffer proteins based on their function (4, 5). The buffer proteins like calbindin D_{2K} maintain calcium homeostasis by regulating the intracellular calcium concentration (5). On the other hand, sensor proteins such as S100B and CaM undergo a conformational change upon calcium-binding, thereby altering their affinity for target proteins like caldesmon, tau, annexins, and various kinases (2, 3). EF-hand calcium-binding proteins have been implicated in a variety of pathological diseases including Alzheimer's, Down's syndrome and inflammatory disorders (6). Solving the structure of these proteins will be valuable for elucidating their functions.

We have previously cloned and characterized a small 9 kD neuronal EF-hand Ca^{2+}-binding protein, Calsensin (7). Calsensin is expressed in a subset of peripheral sensory neurons fasciculating into a single axon tract in the leech central nervous system (7, 8). The molecular features of Calsensin and its restricted expression in the nervous system are consistent with the hypothesis that it may participate in protein-complex mediated calcium-dependent signal transduction events in growth cones and axons (7). It has become increasingly clear that changes in intracellular calcium levels can modulate axon fasciculation and growth cone motility (9, 10) and previous studies have directly implicated calcium-binding proteins in growth cone guidance in Drosophila (11). For these reasons we have undertaken a structural study of Calsensin and its calcium dependence. We show that Calsensin can oligomerize under non-reducing conditions in vitro and that it changes its migration on SDS-PAGE upon calcium-binding. Furthermore, we have solved the structure of calcium-loaded Calsensin under reducing condition using multi-dimensional NMR spectroscopy. By analysis of the Ca^{2+} concentration dependence of NMR spectra,
we have identified the residues of Calsensin that are involved in calcium-dependent conformational change. This study is the first step in characterizing the probable mechanism of calcium-induced structural change of Calsensin and will provide a foundation for understanding its functional role in the nervous system.

MATERIALS AND METHODS

Protein expression and purification. The full-length Calsensin ORF (6) was PCR amplified and cloned into the pGEX4T3 vector (Amersham Biosciences) to generate the construct pGEX4T3-Cal. For protein purification Escherichia coli strain BL21 (DE3) was transformed with pGEX4T3-Cal and the cultures grown in 2XYT medium supplemented with 1 mM CaCl₂ or modified M9 medium supplemented with 1 mM CaCl₂ and 100 mg/ml ampicillin. For unlabeled protein expression in 2XYT medium, the cultures grown at 37°C were induced with 0.1 M isopropyl β-D-thiogalactopyranoside (IPTG) when O.D₆₀₀ reached 0.5. For ^¹⁵N- single labeled or ^¹⁵N- and ^¹³C- double-labeled protein preparations, the modified M9 minimal media contained ^¹⁵N-enriched ammonium chloride [1 g/l, Cambridge Isotope Laboratories] and/or ^¹³C- enriched glucose [2 g/l, Cambridge Isotope Laboratories] as the sole nitrogen and carbon source, respectively. Cells were grown to O.D₆₀₀ of 0.6 at 37°C, transferred to 30°C and induced with 1 mM IPTG when O.D₆₀₀ reached 0.9. The cells were harvested by centrifugation, 6 h and 12 h post-induction for unlabeled and labeled samples respectively. The cell pellets were resuspended in 50 ml of 50 mM sodium phosphate buffer (75 mM NaCl, 2 mM DTT and 0.02% NaN₃, pH 6.0) per liter of culture with lysozyme added to a final concentration of 1 mg/ml. After
freezing at −80°C overnight (12) the cells were disrupted upon thawing and protease inhibitor (1 mM PMSF) and DNase I (500 ml of 1 mg/ml stock) were added. The cell extracts were clarified by centrifugation and the supernatant loaded on a glutathione-agarose (Sigma) column. The GST-fusion proteins were eluted with 5 mM reduced glutathione in 50 mM sodium phosphate buffer, concentrated with a Millipore stirred ultrafiltration cell, and separated on a size-exclusion column (Sephacryl S-100 HR, Amersham Pharmacia Biotech) equilibrated with 50 mM sodium phosphate buffer. Fractions containing the fusion proteins were pooled, the NaCl concentration increased to 150 mM, and the GST-tag cleaved off with thrombin by incubation at room temperature for 12-16 h. The GST-tag was subsequently removed from the recombinant Calsensin protein using a glutathione-agarose column. The Calsensin protein was further purified by gel-filtration (Sephacryl S-100 HR). The collected fractions were analyzed by SDS-PAGE for purity, pooled, and concentrated to 1-2 mM for NMR experiments. The final NMR samples contained 10% ²H₂O.

Oligomerization and mobility shift assays. Full-length recombinant protein was separated on 16% SDS-PAGE either in the presence or absence of 5% β-mercaptoethanol according to standard procedures (13). Recombinant Calsensin was incubated with 5 mM EDTA or 5 mM CaCl₂ and 1 mM EDTA at room temperature for 20 min, separated on 16% SDS-PAGE using the Bio-Rad Mini PROTEAN II system, and electroblotted onto nitrocellulose (14). The Calsensin protein was labeled with the mAb Ian 3-6 (Briggs et al., 1995) and visualized using anti-mouse HRP-conjugated secondary antibody (Bio-Rad) (1:3000) diluted in Blotto.
The signal was detected with the ECL chemiluminescence kit (Amersham) and digitized using Photoshop software (Adobe) and a flatbed scanner (Epson Expression 1680).

*NMR spectroscopy.* All NMR data were acquired at 298 K on a Bruker DRX500 spectrometer operating at $^1$H frequency of 499.867 MHz. A 5 mm triple-resonance ($^1$H/$^{15}$N/$^{13}$C) probe with XYZ field gradients was used for all experiments. A gradient-enhanced HSQC experiment with minimal water saturation (15) was used for all $^1$H-$^{15}$N correlation experiments. 3D $^{15}$N-edited TOCSY, $^{15}$N-edited NOESY (16) and $^{15}$N-HMQC-NOESY-HMQC (17) spectra were collected for $^{15}$N-labeled Calsensin sample using mixing times of 80 ms for TOCSY and 125 ms for NOESY experiments. For $^{15}$N/$^{13}$C doubled labeled samples 3D CBCA(CO)NH, HNCACB (18), CCONH (18), HCCH-TOCSY (19) and $^{13}$C-edited NOESY (20) spectra were acquired using standard experimental procedures. The backbone coupling constants ($^3J_{NH-NH}$) were measured by a HNHA (21) experiment. Additionally, 2D homonuclear $^1$H-$^1$H TOCSY (22), NOESY (23), as well as DQF-COSY (24) data were obtained. Deuterium exchange experiments were performed as described by Roberts (25). The proton chemical shifts were referenced to DSS (26) and $^{15}$N and $^{13}$C chemical shifts were referenced indirectly. The data were processed on a Linux workstation using NMRPIPE software package (27) and assignments were carried out using NMRView (28).

*Structure Calculation.* The NOE and distance restraints were generated using resonance assignments from 2D and 3D datasets analyzed with NMRView.
Additionally, the deuterium exchange as well as the backbone dynamics data was interpreted using NMRView. The peak volumes obtained from NOEs were classified as strong, medium, weak and very weak restraints, corresponding to upper bound interproton distances of 2.8, 3.4, 4.3, and 5.0-6.0 Å. Pseudo-atom corrections were added for methylene and methyl protons (29). The interproton distances and backbone torsion angle constraints served as input for structure calculations using distance geometry and simulated annealing with CNS version 1.1 (30). Hydrogen bond constraints of rNH-O 1.5-2.8 Å and rN-O = 2.4-3.5 Å were introduced during structure calculations based on $^2$H$_2$O exchange data, and in the regions of secondary structure having characteristic NOEs. The refinement of 200 structures yielded several structures (>150) with no distance violations greater than 0.4 Å and no dihedral angle violations greater than 5°. The final 20 structures were selected on the basis of lowest total energies and having minimal restraint violations. The statistics of the 20 lowest energy structures are represented in Table 1 and the coordinates have been deposited in the Protein Data Bank (accession number xxxx). All the structures were visualized and rendered using MOLMOL (31). The complete resonance assignments have been deposited into BioMagRes Bank as accession code xxxx.

**EDTA and calcium titrations.** The effect of calcium-binding on the structure of Calsensin was studied by titrating with EDTA and/or Ca$^{2+}$ and monitoring the changes with $^1$H-$^{15}$N HSQC experiments. The titrations were carried out by adding appropriate aliquots of 1 M CaCl$_2$ or 0.5 M EDTA to the Calsensin protein solution.
Relaxation data analysis. Measurement of $^{15}$N longitudinal relaxation rates $R_1$, transverse relaxation rates $R_2$ and $\{^1\text{H}\}$-$^{15}$N NOE were obtained as previously described (32). The auto relaxation rate constants $R_1$ and $R_2$ were calculated by non-linear optimization using the rate analysis tool in NMRView. The heteronuclear NOEs were obtained as a ratio of steady-state intensities measured with and without saturation of proton magnetization. The global correlation time $\tau_c$ was calculated using 53 residues after excluding residues for which the resonance frequencies overlap, or those that undergo large-scale internal motions and/or conformational exchange (33). The rotational diffusion tensor was determined with the energy minimized average structure and the $^{15}$N relaxation parameters using TENSOR2 (34). The data were analyzed using extended model-free formalism with the statistical model selection of Mandel et al., (35) as implemented in TENSOR2.

RESULTS AND DISCUSSION

Oligomerization and mobility shift assays. Calsensin has two cysteine residues, one at each terminus. To determine whether the two cysteine residues can form disulfide bridges recombinant Calsensin was resolved on SDS-PAGE in the presence or absence of reducing agents and detected by immunoblotting with the Calsensin mAb Ian 3-6. Figure 1A shows that the antibody recognized Calsensin in bands of 9 kD, 18 kD and 28 kD, respectively, when reducing agent was absent. The sizes of the bands correspond closely to those predicted if Calsensin migrated as a monomer, dimer and trimer under these conditions. Thus, these experiments provide evidence that the cysteine residues in Calsensin can form inter-molecular
disulfide bonds. The predominant multimeric forms were dimers and trimers; however, a few higher molecular weight oligomers could also be detected (Figure 1A).

The functional character of neuronal calcium sensor proteins like NCS-1, VILIP-3 was indicated by their ability to bind calcium and migrate faster on SDS-PAGE (36). To assess the ability of Calsensin to bind calcium, recombinant Calsensin protein was incubated with 5 mM EDTA or 5 mM CaCl$_2$/1mM EDTA, separated on SDS-PAGE, and immunoblotted with mAb lan3-6. Figure 1B shows that migration of Calsensin during SDS-PAGE is relatively faster in the presence of calcium than in its absence. These results indicate that Calsensin undergoes a calcium-dependent conformational change.

3D solution structure of Calsensin. In order to determine the 3D solution structure of Calsensin, sequence-specific resonance assignments were performed according to standard protocols (29) using 3D $^{15}$N-edited TOCSY and NOESY as well as 3D HNCACB, CBCACONH CCONH, and HCCH-TOCSY (37). The backbone amide resonances were assigned for all but six N-terminal and three C-terminal amino acids (Figure 2). The 513 intra- and 1016 inter-residue NOE assignments were obtained by analyzing 2D NOESY as well as 3D $^{15}$N-edited and $^{13}$C-edited NOESY spectra. The aromatic resonances were assigned based on 2D DQF-COSY and 2D NOESY data. The $^{3}J_{NH-NH_a}$ scalar coupling constants from HNHA data were used to obtain the $\phi$ angle constraints according to the Karplus equation (29). The $\phi$ angles for the remaining residues and the $\psi$ angles were obtained using TALOS (38). Hydrogen bond restraints were introduced corresponding to slowly exchanging
amide protons observed in the deuterium exchange data. A total of 1529 NOE, 44 hydrogen bond (there are two distance restraints for each hydrogen bond), and 78 dihedral angle constraints (Table 1) were used for final calculations with CNS. The 20 lowest energy structures have no distance violations greater than 0.4 Å, angle violations greater than 5°, and the RMSDs from the experimental constraints and idealized covalent geometry are low. The pairwise RMSD as well as RMSD to the mean structure of these structures are relatively small (Table 1). Furthermore, the backbone dihedral angles of the majority of residues in the minimum average structure (85.1%) fall inside the most favorable regions of the Ramachandran plot (Table 1).

The $^1$H-$^15$N HSQC spectrum of Calsensin is well dispersed suggesting that the protein is in a folded three-dimensional conformation (Figure 2). The major species observed in the HSQC spectrum (Figure 2) is the calcium-loaded form, and the calcium-free or apo form is observed only as a minor species. The tertiary structure is relatively well-ordered except for the unassigned N- and C-termini and the hinge region connecting the two EF-hands (Figure 3A). Calsensin is monomeric under the experimental conditions due to the presence of reducing agent and as verified by a lack of concentration effect on the HSQC spectrum. The structure of Calsensin consists of two helix-loop-helix motifs arranged as a unicomate-type four-helix bundle (Figure 3B) (39). $\beta$-strands connecting the calcium-binding loops are observed in many calcium-binding proteins (40, 41). In Calsensin, downfield shifted H$_\alpha$ protons, slower amide proton exchange rate, and large $^3$J$_{NH-H\alpha}$ scalar coupling constants consistent with $\beta$-strands were observed for residues Y23-T25 in calcium-binding loop I and K65-S67 in loop II. However, inter-strand NOEs characteristic of
The higher RMSD of the third helix (H3) as compared to the other three helices reflects the lower number of intra-helix NOE restraints in this region and is consistent with the observation that H3 reorients upon calcium-binding in the related S100 family of EF-hand proteins. The extent of this movement varies among the different S100 signaling proteins. For example, the S100B protein shows a large conformational change of the third helix upon calcium-binding as compared to S100A6 (44, 45). The flexibility of H3 in Calsensin suggests it may be important for promoting conformational exchange between calcium-bound and unbound states. Most of the hydrophobic residues that are in the hinge region as well as in the second and fourth helices are exposed on the surface as would be expected for the calcium-bound form (Figure 3D). The hydrophobic residues have been implicated in target binding in other calcium-binding proteins (46, 47).

**EDTA and Ca^{2+} titrations.** In order to determine the effect of calcium-binding on the structure of Calsensin, EDTA titrations were carried out to sequester the
calcium. Changes in the secondary structure were monitored using HSQC spectra. Addition of EDTA resulted in the appearance of new peaks indicating an apo conformation in slow exchange with the original conformation (Figure 4B). Increasing the concentration of EDTA shifted the population towards the apo form (Figure 4A). The spectrum is dispersed even in the absence of calcium implying a folded apo form (Figure 4A). Addition of calcium restored the protein to the original form suggesting a reversible calcium-dependent effect on the conformation (Figure 4B) and further addition of calcium completely depleted the apo form (Figure 4C). The appearance of duplicate peaks corresponding to residues of the first calcium-binding loop suggests that the initial calcium-binding is a slow event. In contrast, the finding that further addition of calcium produced chemical shift perturbation of residues in the second calcium-binding loop indicate a fast exchange event (Figure 4C). Thus, the calcium-binding properties of the two EF-hands are qualitatively different with the binding affinity of the second calcium-binding loop being lower than that of the first loop. In the presence of excess calcium, the residues at position 8 of both the EF-hands are deshielded (Figure 4C). This indicates that Calsensin is calcium-loaded under the buffer conditions used for obtaining the structure. Furthermore, as inferred by the presence of major and minor species in the spectrum, the first calcium-binding site is likely to be fully occupied while the second site is in a conformational equilibrium between the bound and unbound states.

Backbone dynamics of Calsensin. The global correlation time $\tau_c$ of Calsensin is 7.00± 0.04 ns and the molecule has a statistically significant prolate rotational diffusion tensor $\left( \frac{D^\parallel}{D^\perp} = 1.137 \right)$ which is consistent with other calcium-binding
proteins (48, 49). All 74 residues with assigned backbone amide resonances had their corresponding $^{15}$N relaxation data fitted to one of five models describing modes of backbone dynamics (35). A majority of the residues (46 out of 74) were satisfied by model 1, 13 by model 2, 3 by model 3, 11 by model 4 and 1 by model 5 using the nomenclature of Mandel et al (35). The fitted order parameters ($S^2$) (Figure 5E) reveal that the regions of high order correlate with the presence of a-helical secondary structural elements. The average order parameters for the four a-helices H1, H2, H3, and H4 are summarized in Table 2. The third helix, suggested to be involved in calcium-induced conformational change in most S100 proteins (50, 51, 52), was best fitted by the model having a ms timescale exchange term for 4 out of 8 residues (Figure 5G). This helix also has lower order parameters as compared to the other three helices.

In order to define flexible regions of the molecule the order parameters were averaged within the secondary structural elements. The lowest average order parameters are observed for the hinge region between the two EF-hand motifs (Figure 5E). Most of the EF-hand calcium-binding proteins exhibit flexibility in the hinge region and the third helix to a varying extent (42, 48, 53); hence, the differences in the flexibility can be attributed to the sequence variability observed among them (Figure 6). The hinge region of Calsensin shows ms-timescale conformational exchange, which may indicate concerted motion of the third helix (43). This suggests that Calsensin is in an exchange between the open and closed conformation similar to that of the calcium-free C-terminal domain of CaM (48). Recent evidence has shown that calcium-loaded CaM can be crystallized in the closed conformation (54). The residue D63 in the second EF-hand of Calsensin is
not oriented to bind calcium under the experimental conditions and undergoes ms
time-scale motion (Figure 3C). Consequently, the calcium-binding at the second site
could be destabilized by the presence of the lysine residue at position 68. Previous
studies have found that the calcium-binding affinity of Calbindin D9K decreases
dramatically below pH 7.0 due to protonation of carboxylate sidechains (55).

Comparison of Calsensin with other EF-hand calcium-binding proteins. The
highest sequence identity between Calsensin and other members of the EF-hand
superfamily is in the calcium-binding loops (Figure 6). Sequence alignment further
shows that the calcium-binding loops of Calsensin are most similar to those of two
members of the polcalcin family of pollen EF-hand calcium-binding proteins (56, 57).
Although, Calsensin can form dimers via oxidation of cysteine residues, it is
monomeric in solution under reducing conditions. Furthermore, Calsensin, unlike the
members of S100 family (41, 51), does not appear to form non-covalent dimers
under experimental conditions. Bet v 4 exists as a monomer whereas another
member of the polcalcin family, Phl p 7, revealed a domain-swapped dimer structure
(56, 57). In general, the EF-hand calcium-binding proteins are known to exist as
monomers, dimers or oligomers depending on their amino acid composition and
function (49). Most members of the S100 and polcalcin family are highly acidic (2,
58). In contrast, the isoelectric point (pI) of Calsensin is close to physiological pH
and hence could be modulated by small changes in the pH. The antiparallel packing
of the helices in Calsensin is comparable to the open conformation of the N-terminal
domain of Ca²⁺-loaded CaM (59) and monomer of S100 proteins (40, 41). The
packing in polcalcins Bet v 4 and Phi p7 are slightly different due to the extra Z-helix (51, 52).

_Calcium-dependent conformational change_. The EF-hand family of calcium-binding proteins that function as buffer proteins have similar structures in both the apo- and calcium-bound form (47, 60). In contrast, calcium sensors that mediate signal transduction undergo a significant calcium-dependent conformational change (47, 60). The mechanism of the calcium-dependent changes for these proteins have been extensively studied using calcium titrations (61) as well as by solving the apo- and calcium-loaded structures (62). For example, the calcium-induced structural changes for S100B suggest a large conformational change in the orientation of H3 (51). This reorientation in turn alters the structure of the hinge region and second calcium-binding site. In Calsensin, the second site shows a high degree of flexibility in the ms timescale (Figure 3C). Hence, the binding of calcium to the first site might enable a conformational change at the second site allowing calcium-binding at this site. The $^{15}$N chemical shifts of a number of EF-hand proteins has been shown to change drastically upon calcium-binding and are indicative of a conformational change (43). The EDTA and calcium titration data suggests that Calsensin undergoes a similar conformational change upon calcium-binding.

_Calsensin mediated signal transduction_. The residues in the hinge region and the C-terminal loop of S100 have been shown to be involved in target binding (63). The binding of calcium leads to a conformational change exposing the hydrophobic residues on the surface (52) modulating target binding (46, 48). The hinge region
and C-terminal helices of Calsensin consist mostly of hydrophobic residues. This suggest that the calcium-induced structural changes could expose these hydrophobic residues on the molecular surface thereby allowing interaction with target proteins. The amino acid sequence of EF-hand calcium-binding proteins differs considerably within the hinge region and C-terminal loop and this sequence divergence could account for the difference in target specificity.

CONCLUSIONS

Calsensin is a member of the two EF-hand calcium-binding protein family that includes the S100 and polcalcin families. Molecules like Calsensin that are expressed selectively in certain neurons are candidates to function as signal transducers during axon fasciculation and growth cone guidance (7). We have used multidimensional NMR to solve the structure of calcium-loaded Calsensin. The structure of Calsensin reveals an antiparallel stacking of the two helices of each EF-hand. EDTA and Ca\(^{2+}\) NMR titration data suggest that Calsensin undergoes a conformational change upon calcium-binding. The two calcium-binding sites are qualitatively distinct with respect to their binding affinities and rate of calcium-binding. The relatively higher disorder of H3 in the solution structure as compared to other helices is due to the presence of ms timescale conformational exchange. The observed flexibility of H3 could be attributed to chemical exchange between calcium-loaded and free states and/or closed and open conformations. This indicates that the third helix is important for the calcium-induced conformational changes and that it may be implicated in target protein interactions.
ACKNOWLEDGEMENTS

The authors wish to thank Dr. Monica Sundd for valuable discussions during the preparation of this manuscript. We also appreciate the technical help provided by Jayandran Palaniappan for structure calculations.

REFERENCES


Backbone dynamics of a free and phosphopeptide-complexed Src homology 2 domain studied by $^{15}$N NMR relaxation, *Biochemistry* 33, 5984-6003.


$^1$H NMR assignments of apo-calciycin and comparative structural analysis 
with calbindin D$_{9k}$ and S100$\beta$, *Protein Sci.* 5, 2162-2174.

41. Vallely, K. M., Rustandi, R. R., Ellis, K. C., Varlamova, O., Bresnick, A. R., and 
Weber, D. J. (2002) Solution structure of human Mts1 (S100A4) as 
determined by NMR spectroscopy, *Biochemistry* 41, 12670-12680.

dynamics of the regulatory domain of calcium vector protein, studied by $^{15}$N 
relaxation at four fields, reveals unique mobility characteristics of the 

(1998) Ca$^{2+}$ coordination to backbone carbonyl oxygen atoms in calmodulin 
and other EF-hand proteins: $^{15}$N chemical shifts as probes for monitoring 
individual site Ca$^{2+}$ coordination, *Biochemistry* 37, 7617-7629.

dipolar couplings for determining the solution structure of rat apo-S100B ($\beta\beta$), 
*Protein Sci.* 8, 800-809.

structure of apo calciycin and structural variations in the S100 family of 

46. Ikura, M., Clore, G. M., Groneborn, A. M., Zhu, G., Klee, C. B., and Bax, A. 
(1992) Solution structure of a calmodulin-target peptide complex by 


Table 1: NMR-Derived Restraints and Statistics of 20-NMR structures

<table>
<thead>
<tr>
<th>Experimental restraints used for structure calculation</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Total No. of NOEs</td>
<td>1529</td>
</tr>
<tr>
<td>Intraresidue NOEs</td>
<td>513</td>
</tr>
<tr>
<td>Interresidue NOEs</td>
<td>1016</td>
</tr>
<tr>
<td>Hydrogen Bonds (two distance restraints each)</td>
<td>22</td>
</tr>
<tr>
<td>Dihedral angles ((\psi/\phi))</td>
<td>78</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Refinement statistics¹</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Energies (kcal/mol)</td>
<td></td>
</tr>
<tr>
<td>Overall</td>
<td>333.94 ± 15.45</td>
</tr>
<tr>
<td>Bond</td>
<td>21.97 ± 1.48</td>
</tr>
<tr>
<td>Angles</td>
<td>115.16 ± 6.56</td>
</tr>
<tr>
<td>Improper</td>
<td>19.77 ± 1.79</td>
</tr>
<tr>
<td>VDW</td>
<td>4.92 ± 1.61</td>
</tr>
<tr>
<td>NOE</td>
<td>171.56 ± 10.39</td>
</tr>
<tr>
<td>cdih</td>
<td>0.54 ± 0.34</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>RMSDs from distance contraints and dihedral restraints (Å)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>NOE</td>
<td>0.0380 ± 0.0012</td>
</tr>
<tr>
<td>cdih</td>
<td>0.3272 ± 0.0876</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>RMSDs from idealized covalent geometry</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Bond (Å)</td>
<td>0.0041 ± 0.00014</td>
</tr>
<tr>
<td>Angles (deg)</td>
<td>0.5400 ± 0.0160</td>
</tr>
<tr>
<td>Impropers (deg)</td>
<td>0.4500 ± 0.0205</td>
</tr>
<tr>
<td>% of residues in favorable region of Ramachandran plot²</td>
<td>85.10%</td>
</tr>
<tr>
<td>% of residues in favorable region of Ramachandran plot³</td>
<td>100.00%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>RMSDs to the mean structure (Å)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Secondary structure backbone²</td>
<td>0.545 ± 0.14</td>
</tr>
<tr>
<td>Secondary structure heavyatoms²</td>
<td>0.911 ± 0.21</td>
</tr>
<tr>
<td>Overall backbone³</td>
<td>0.842 ± 0.25</td>
</tr>
<tr>
<td>Overall heavyatoms³</td>
<td>1.278 ± 0.29</td>
</tr>
<tr>
<td>Helix I (E8-L16)</td>
<td>0.14 ± 0.05</td>
</tr>
<tr>
<td>Helix II (A26-T35)</td>
<td>0.33 ± 0.12</td>
</tr>
<tr>
<td>Helix III (K48-I55)</td>
<td>0.26 ± 0.10</td>
</tr>
<tr>
<td>Helix IV (K68-L79)</td>
<td>0.26 ± 0.10</td>
</tr>
</tbody>
</table>

¹ - Calculated using CNS for the 20 lowest energy structures. None of the structures has distance violations > 0.4 Å and dihedral angle violations > 5°.
² - Includes residues in the helices E8-L16, A26-T35, K48-I55, & K68-L79.
³ - Obtained for residues A7-C80 since no long-range NOEs were identified for amino acids 1-6 and 81-83.

Table 2: Order parameters averaged over the secondary structural element

<table>
<thead>
<tr>
<th>Secondary structure*</th>
<th>S²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Helix I (H1)</td>
<td>0.896</td>
</tr>
<tr>
<td>Helix II (H2)</td>
<td>0.904</td>
</tr>
<tr>
<td>Helix III (H3)</td>
<td>0.845</td>
</tr>
<tr>
<td>Helix IV (H4)</td>
<td>0.904</td>
</tr>
<tr>
<td>Strand I</td>
<td>0.924</td>
</tr>
<tr>
<td>Strand II</td>
<td>0.929</td>
</tr>
</tbody>
</table>

FIGURE LEGENDS

FIGURE 1: (A) Calsensin forms multimers under non-reducing conditions. Purified recombinant Calsensin was incubated with SDS-PAGE loading buffer with or without 5% β-mercaptoethanol (βME). The immunoblots were labeled with the Calsensin specific lan3-6 antibody (lane 1). The antibody recognizes a 9 kD band in the presence of reducing agent. In the absence of reducing agents the protein migrates as 9 kD, 18 kD, and 27 kD bands (lane 2, indicated by the arrows). The migration of molecular weight markers are indicated to the left. (B) The mobility of Calsensin on SDS-PAGE is altered by the presence or absence of calcium. Recombinant Calsensin was incubated with 5 mM EDTA (lane 1) or 5 mM Ca^{2+} and 1 mM EDTA (lane 2), separated on SDS-PAGE, and immunoblotted with lan 3-6 antibody. The migration of Calsensin is faster in the presence of calcium than in the presence of chelating agents (denoted by the arrows). The migration of molecular weight markers are indicated to the left.

FIGURE 2: NMR solution structure of Calsensin. \(^{1}\)H-\(^{15}\)N HSQC spectra of 1.7 mM uniformly \(^{15}\)N-labeled Calsensin with residue assignments. The sample was prepared in 50 mM sodium phosphate, 75 mM NaCl, 2 mM DTT and 0.02% NaN\(_3\) buffer (pH 6.0). The peaks connected by horizontal lines correspond to the asparagine and glutamine sidechain amide groups.
FIGURE 3: (A) Overlay of the 20 lowest energy structures of Calsensin. The structures were superimposed using all the backbone residues in the secondary structural elements and rendered using MOLMOL. The N-terminal residues M1-K6 and C-terminal Q81-K83 were not assigned due to lack of sequential NOEs. All the helices are well-defined except for H3, which shows chemical exchange and higher RMSD as compared to the mean structure. (B) Stereo view of the energy minimized average ribbon structure of Calsensin. The labeling of the secondary structural elements follows the standard nomenclature used for EF-hand calcium-binding proteins. (C) Expanded view of the two calcium-binding loops in Calsensin. The backbone atoms in the calcium-binding loop are represented in blue and the sidechain atoms directly involved in calcium coordination are indicated in red. (D) Surface plot of Calsensin rendered using MOLMOL. The regions colored in blue have positive electrostatic potential, those colored in red have negative electrostatic potential, and the regions in white are non-polar. The hydrophobic residues in the helices 2, 3, and 4 as well as in the hinge region are exposed to the surface.

FIGURE 4: $^1$H-$^15$N HSQC spectra of Calsensin with EDTA or with Ca$^{2+}$. The HSQC spectra of 1.8 mM $^{15}$N-Calsensin after the addition of 2 equivalents of EDTA (A), followed by 2 equivalents (B) and 4 equivalents of Ca$^{2+}$(C). The peaks in (A) represent the apo-form and those in (C) the calcium-loaded form of Calsensin.

FIGURE 5: Backbone dynamics of Calsensin correlate with the observed structural features. (A) The secondary structural elements of Calsensin are shown
corresponding to the residue number. (B) The transverse relaxation time ($T_1$), (C) longitudinal relaxation time ($T_2$), (D) heteronuclear NOE, (E) order parameters ($S^2$), (F) internal motions, and (G) exchange rates are plotted as a function of residue number.

FIGURE 6: Alignment of Calsensin with other members of the EF-hand family of calcium-binding proteins. The sequence of Calsensin was aligned with CaM, Plastin-1, as well as members of the S100 and the polcalcin family. The residues in Calsensin that are identical are highlighted in yellow (polcalcin family) or cyan (other EF-hand members), while similar residues are shown in red. The two highly conserved aspartic acid residues are shown in red and highlighted in gray.
Fig. 1

Fig. 2
Fig. 3

Apo form

Calcium-binding loop 1

Calcium-binding loop 2

Apo and holo forms

Holo form

Fig. 4
Fig. 6
CHAPTER 3. LEECH FILAMIN AND TRACTIN: MARKERS FOR MUSCLE DEVELOPMENT AND NERVE FORMATION

A paper published in the *Journal of Neurobiology*

Deepa V. Venkitaramani, Dong Wang, Yun Ji, Ying-Zhi Xu, Liliana Ponguta, Katie Bock, Birgit Zipser, John Jellies, Kristen M. Johansen, and Jørgen Johansen

**ABSTRACT**

The Lan3-14 and Laz10-1 monoclonal antibodies recognize a 400 kDa antigen that is specifically expressed by all muscle cells in leech. We show that the antigen recognized by both antibodies is a member of the filamin family of actin binding proteins. Leech filamin has two calponin homology domains and 35 filamin/ABP-repeat domains. In addition, we used the Laz10-1 antibody to characterize the development of the segmentally iterated dorsoventral flattener muscles. We demonstrate that the dorsoventral flattener muscle develops as three discrete bundles of myofibers and that CNS axons pioneering the DP nerve extend only along the middle bundle. Interestingly, the middle dorsoventral muscle anlage is associated with the only non-neuronal expression of the L1-family cell adhesion molecule Tractin. This expression is transient and occurs at the precise developmental stages when DP nerve formation takes place. Based on these findings we propose that the middle dorsoventral muscle anlagen provides a
substrate for early axonal outgrowth and nerve formation and that this function may be associated with differential expression of distinct cell adhesion molecules.

**Keywords:** leech; filamin; muscle formation; axon guidance; monoclonal antibodies; Tractin

**INTRODUCTION**

Because of its simple organization, the leech nervous system has been used to generate monoclonal antibodies (mAbs) to mixed antigens from whole nervous tissue extracts (Zipser and McKay, 1981). An advantage of this approach is that mAbs to unknown proteins that serve as markers for specific subsets of neurons or for particular cellular structures of interest can readily be identified (Zipser and McKay, 1981). Two mAbs generated in this way, Lan3-14 and Laz10-1, specifically label all muscle cells in leech (Zipser and McKay, 1981; Thorey and Zipser, 1991). This includes myotubes in the major body wall muscle layers (circular, longitudinal, and oblique) as well as the myotubes within the connective tissue of the CNS. For this reason these antibodies have been extensively used in leech for studies of muscle development (Torrence and Stuart, 1986; Jellies and Kristan, 1988a; 1991; Thorey and Zipser, 1991) and nerve/muscle interactions (Kuwada, 1985; Jellies and Kristan, 1988b; Braun and Stent, 1989). However, the molecular nature of the antigen(s) has not been determined. Here we provide evidence that both Lan3-14 and Laz10-1 recognize the same antigen, which is a muscle specific member of the filamin family of actin binding proteins. Filamins are generally large proteins that
organize filamentous actin into three-dimensional networks, and several muscle specific isoforms have previously been described in vertebrates (Stossel et al., 2001; van der Flier and Sonnenberg, 2001). In addition, we characterize the development of one of the major muscle systems, the dorsoventral flattener muscles, which has not previously been described. Using the Laz10-1 mAb we show that the anlagen of the dorsoventral flattener muscles initially form as three discrete muscle bundles, but only the middle bundle, which transiently expresses the cell adhesion molecule Tractin, is likely to serve as a substrate for nerve/muscle interactions.

METHODS

Animals

_Haemopis marmorata_ leeches were purchased from a commercial supplier (Washburn Point Lodge). _Hirudo medicinalis_ embryos were obtained from a laboratory breeding colony. Breeding, maintenance, and staging were as previously described (Fernández and Stent, 1982; Jellies et al., 1987) at 22-25°C, except that embryos were maintained in water that was made as sterile-filtered solutions of 0.0005% sea salt, wt/wt. _Haementeria officinalis_ embryos were obtained from animals captured in the wild. Dissections of nerve cords, muscle tissue, and embryos were performed in leech saline solutions with the following composition (in mM): 110 NaCl, 4 KCl, 2 CaCl₂, 10 glucose, 10 HEPES, pH 7.4. In some cases 8% ethanol was added and the saline solution cooled to 4°C to inhibit embryonic muscle contractions. Embryonic day 10 (E10) for _Hirudo_ embryos is characterized by the first sign of a tail sucker, and E30 is the termination of embryogenesis.
Immunocytochemistry

Five monoclonal antibodies were used in these studies. The Lan3-14 antibody (Zipser and McKay, 1981) and the Laz10-1 antibody (Thorey and Zipser, 1991) were used to label muscles and muscle anlagen whereas a monoclonal antibody directed against acetylated tubulin (ACT) (Sigma) was used to label central neurons and their axonal projections (Jellies et al., 1996). In addition, two mAbs, Laz6-56 and 1H4 (Xu et al., 2003) that bind to the extracellular domains of the L1-family CAM Tractin were applied.

Dissected Hirudo and Haementeria embryos were fixed overnight at 4°C in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. After fixation the embryos were incubated overnight at room temperature with diluted antibody in PBS containing 1% Triton X-100, 10% normal goat serum, 0.001% sodium azide, and washed in PBS with 0.4% Triton X-100. Double labeled preparations were obtained by a subsequent incubation in the other primary antibody and by using fluorescently conjugated subtype-specific secondary antibodies. A rabbit anti-mouse IgG TRITC-conjugated secondary antibody (Cappel) was used for Laz10-1, Lan3-14, or Laz6-56 and a rabbit anti-mouse IgG2B FITC-conjugated secondary antibody (Cappel) for the ACT-antibody. Fluorescently labeled preparations were mounted in glycerol with 5% n-propyl gallate. For confocal analysis of double labeled preparations a separate confocal series of images for each fluorophor were obtained simultaneously with the Leica confocal TCS NT microscope at 0.5 μm z-intervals using the krypton and argon laser lines and the appropriate filter sets. A maximum projection image for each of the image stacks was obtained using the NIH-Image software. In some cases individual slices or projection images from only two to three slices were
obtained. These were subsequently imported into Photoshop where they were pseudocolored, image processed, and merged. Dynamic 3D-representations as well as stereo pairs of images at -7.2 and +7.2 degree angles, respectively, were generated using the Leica TCS 3D-reconstruction software.

The procedure for Tractin perturbation experiments was essentially as in Huang et al. (1997). In brief, 1-2 µl of purified Laz6-56 and/or 1H4 antibody and purified mouse IgG1 (Sigma) control antibody from a 0.2 mg/ml stock solution were injected beneath the germinal plate of E8 Hirudo embryos. In addition to antibody the stock solution contained 10% Ringer and 0.2% fast green (Sigma) allowing for visual confirmation of pressure injected antibody under the stereo microscope. For the injections the embryos were immobilized in crevices in Sylgard coated tissue culture dishes while anesthetized with 8% ethanol in 10% Ringer solutions. After the injection the embryos were transferred to embryo water without ethanol and allowed to develop for 24 h at 25°C. At this time the embryos were dissected, fixed, processed for antibody labeling, and examined for defects in DP nerve formation.

**Molecular cloning and sequence analysis**

Hybridoma supernatant from the Laz10-1 antibody was used to screen a random primed Hirudo central nervous system-enriched cDNA lambda-ZAP II expression library (Huang et al., 1997) essentially according to the procedures of Sambrook et al. (1989) at a density of 30,000 plaque-forming units/150 mm plate. Positive clones were tested for immunoreactivity with the Lan3-14 antibody, plaque purified, and in vivo excised to generate pBluescript phagemids according to the method provided by the manufacturer (Stratagene). Several partial cDNAs
recognized by both the Laz10-1 and Lan3-14 antibodies were identified in these screens. To identify additional clones in order to obtain the full sequence of the cDNAs for the antigen the same cDNA library was rescreened using $^{32}$P-labeled fragments of the originally identified clones. The fragments were radiolabeled using random priming according to the manufacturer's procedure (Prime-a-Gene kit, Promega) and the library screened using standard procedures (Sambrook et al., 1989). Additional 3' sequences were obtained using the Access RT-PCR system (Promega) according to the manufacturer's protocols. DNA sequencing was performed at the Iowa State University DNA Sequencing and Synthesis Facility. Leech filamin sequence was compared with known and predicted sequences using the National Center for Biotechnology Information BLAST e-mail server. The sequence was further analyzed using SMART (Simple Modular Architecture Research Tool; http://smart.embl-heidelberg.de/) to predict the domain organization of the protein.

**Northern and Western blot analysis**

PolyA+ mRNA was purified from *Haemopis* nerve cords (large muscle cells are present in the connectives) using the FastTrack kit (Invitrogen), and 20 μg of polyA+ mRNA was fractionated on 1.2% agarose formaldehyde gels, transferred to nitrocellulose and hybridized with the addition of dextran sulfate (10%) according to standard protocols (Sambrook et al., 1989). Leech filamin specific probes were generated by purifying cDNA subclone fragments from bp 735-2099 of the leech filamin transcript using GeneClean (Bio 101) and synthesizing random primer $^{32}$P-labeled probe using the Prime-A-Gene kit (Promega) according to manufacturer's
instructions. High stringency hybridization and washing conditions were employed (Sambrook et al., 1989).

Protein extracts were prepared from *Haemopis* muscle and nerve cords and homogenized in lysis buffer (0.2 M NaCl, 2 mM CaCl₂, 0.2% NP-40, 0.2% Triton-X-100, 20 mM Tris pH 7.4). SDS-PAGE was performed according to standard procedures (Laemmli, 1970). Electroblot transfer was performed as in Towbin et al. (1979). For these experiments we used the Bio-Rad Mini PROTEAN II system, electroblotting to 0.2 μm nitrocellulose, and using anti-mouse HRP-conjugated secondary antibody (Bio-Rad) (1:3000) for visualization of primary antibody diluted in Blotto for immunoblot analysis. The signal was detected with the ECL chemiluminescence kit (Amersham) and digitized using Photoshop software (Adobe) and an Arcus II scanner (AGFA).

**Phylogenetic analysis**

Alignments used to produce maximum parsimony trees were generated with the Clustalw version 1.7 program and initially encompassed the entire filamin sequences. However, in the final analysis any gaps in the resulting alignments were removed by deleting residues corresponding to the gaps. Trees were constructed by maximum parsimony using the PAUP computer program version 4.0b (Swofford, 1993) on a Power Macintosh G4. All trees were generated by heuristic searches and bootstrap values in percent of 1000 replications are indicated on the bootstrap majority rule consensus tree.
RESULTS

The Lan3-14/Laz10-1 antigen is a muscle specific member of the filamin family of actin binding proteins

The Lan3-14 and Laz10-1 antibodies cross-react in both hirudinid and glossiphoniid leeches and have identical staining patterns of all muscle tissue. This is illustrated in Fig. 1(A) and (B) where embryonic *Haementeria* circular and longitudinal muscles forming an orthogonal grid have been labeled with the two mAbs, respectively. Furthermore, both antibodies recognize a major band of approximately 400 kDa on immunoblots [Fig. 1(C)], suggesting they recognize the same antigen. To verify this hypothesis and to determine the molecular identity of the Lan3-14 and Laz10-1 antigen(s) we screened 1x10⁶ plaques of a leech random primed expression vector library with the Laz10-1 mAb. This screen identified several partial and overlapping cDNA clones which in retesting also were positive for the Lan3-14 mAb (data not shown). Taken together with the immunoblot results these findings provide strong evidence that Laz10-1 and Lan3-14 recognize the same protein. Subsequently, the cDNA library was screened with radiolabeled nucleotide probes generated from the cDNA clones and RT-PCR was performed on *Hirudo* CNS extracts. In this way overlapping cDNA sequences were obtained that are likely to encompass the entire coding sequence since the predicted sequence has a 5' ATG start codon downstream from an in-frame TAA stop codon. The predicted sequence (GenBank: AY382663) is for a protein containing 3836 residues [Fig. 2(A)] with a calculated molecular mass of 409,496 Da. Several lines of
evidence suggest that the obtained cDNAs correspond to the antigen recognized by the Lan3-14 and Laz10-1 mAbs:

1. All clones identified in the original screen that were analyzed were recognized by both antibodies and proved to be derived from the same gene, making non-specific cross-reactivity with an unrelated gene product unlikely.

2. The predicted molecular mass of the coding region of the complete cDNA of 409 kDa is close to the relative molecular mass of the Laz10-1/Lan3-14 antigen of 400 kDa as estimated by SDS-PAGE [Fig. 1(C)].

3. Northern blot analysis of total leech poly (A)+ RNA with probe from NH2-terminal sequence (encompassing calponin domain 2 and ABP repeat 1-3) labels a single band of 14.5 kb [Fig. 2(B)] in good agreement with the expected size for the cDNA.

The complete sequence of the Lan3-14/Laz10-1 antigen is shown in Fig. 2(A) and the inferred protein product contains all the defining features of a protein of the filamin-family of actin binding proteins (Stossel et al., 2001; van der Flier and Sonnenberg, 2001) wherefore the Lan3-14/Laz10-1 antigen in the following will be referred to as leech filamin. It has two NH2-terminal calponin homology domains [Fig. 3(A)] that contain sequence motifs shared with many actin-filament binding proteins. In addition, it has 35 repeated sequences of ~96 amino acids (filamin/ABP repeats) interrupted by "hinge" segments [Fig. 3(A)]. These repeats are known to form antiparallel β-sheet domains that partially overlap so as to generate a rod (Stossel et al., 2001; van der Flier and Sonnenberg, 2001). The COOH-terminal ABP-repeat is thought to form a dimerization domain (Stossel et al., 2001; van der Flier and Sonnenberg, 2001). Figure 3(A) compares leech filamin with other filamins in the data bases. All the filamins share the two actin binding domains in addition to
various numbers of the filamin/ABP repeats. Leech filamin is so far the largest filamin-family member to be identified. The evolutionary relationship between filamins is illustrated in Fig. 3(B). Leech filamin is most closely related to nematode and diptera filamins.

**Dorsoventral muscle development and formation of the DP nerve**

In leech there are four major segmental nerves per hemisegment (AA, MA, DP, and PP) that contain axons from both peripheral and central neurons (Jellies and Johansen, 1995). Three of these (AA, MA, and PP) are pioneered by peripheral neurons differentiating within the body wall (Fig. 4) (Jellies et al., 1996; Huang et al., 1998). However, the fourth nerve (DP) follows a completely different trajectory and is aligned with the dorsoventral flattener muscles [Fig. 5(A,B)]. The DP nerve is pioneered by the axon of the dorsal P-cell (Pd) the soma of which is located in the CNS (Kuwada, 1985; Jellies et al., 1994; 1996). After the Pd axon reaches the area of the dorsal body wall, axons from the dorsal sensilla, S6 and S7 (Fig. 4) start extending toward the CNS fasciculating with the Pd axon and establishing the nerve (Jellies and Johansen, 1995). However, previous studies with CNS ablations have indicated that the Pd neuron may not be obligatory for nerve formation suggesting that the dorsoventral flattener muscles may serve as a sufficient substrate for establishing the DP pathway (Jellies et al., 1995; 2000). For these reasons we used the muscle specific leech filamin mAb Laz10-1 to explore the dynamics of dorsoventral muscle development as it relates to DP nerve formation.

The dorsoventral flattener muscles are a segmentally iterated array of loosely associated myofibrils forming a bilateral veil extending between the dorsal and
ventral body wall [Fig. 5(A)]. As the name implies these muscles serve to flatten the leech as occurs for example during swimming. Figure 5(B) shows a stereomicrograph from a *Hirudo* E11 embryo where the muscles are labeled by Laz10-1 (red) and the projections from the CNS are labeled by an antibody to acetylated tubulin (ACT) (Jellies et al., 1995). The DP nerve extends across the germinal plate along the loose constellation of dorsoventral muscle fibrils. In contrast, axons forming the three other nerves extend circumferentially along the future body wall muscle layers. The confocal images in Figure 6 illustrate the initial development of the dorsoventral flattener muscles. Three consecutive segments are shown double labeled with Laz10-1 and ACT antibody. Because leech embryogenesis occurs in a rostro-caudal gradient the development of adjacent segments is approximately 3-4 h apart (Jellies and Kristan, 1991). The muscle develops from three discrete muscle anlagen (a, b, and c) with the most anterior to develop first. As the middle anlage differentiates the Pd axon extends along its surface toward the dorsal edge of the germinal plate. As development progresses more and more myofibrils are added in alignment with each bundle [Fig. 5(B)].

Interestingly, immunoreactivity to the L1-family CAM, Tractin (Huang et al., 1997), appears to be coincident with the localization of the middle group of dorsoventral muscle anlagen (Fig. 7) but not with the other two (Fig. 8). Figure 7 shows confocal images from three consecutive hemisegments of a *Hirudo* E9 embryo double labeled with Tractin (red) and ACT (green) antibody. Tractin immunoreactivity has hitherto only been reported for neuronal somata and axons (Huang et al., 1997; 1998) and this is the first documentation of localization outside the nervous system. The Pd axon (in yellow since it is labeled by both Tractin and
ACT antibody) clearly navigates along a Tractin antibody labeled surface in the same confocal plan as the axon. Furthermore, the Tractin antibody labeling of the pathway is in a pattern that closely corresponds to the position and morphology of the middle dorsoventral muscle anlagen (Fig. 8), suggesting that they are the source of the Tractin expression. We have not been able to verify this directly in double labeling experiments since the Tractin and muscle antibodies applied are all of the IgG1 subtype. Also it is formally possible that the labeling could represent secreted Tractin as the Laz6-56 mAb is to the extracellular domain of Tractin (Xu et al., 2003) or that some other unidentified cell type may be involved. However, this appears unlikely since as development progresses all myofibrils added to the middle dorsoventral muscle bundle are Tractin antibody positive. At these later stages the muscle cells can unequivocally be identified as such. The earliest detectable Tractin immunoreactivity on the middle muscle anlagen with the Laz6-56 antibody is just prior to peripheral axon extension from the CNS [Fig. 9(A,B), yellow arrow] and as the Pd axons grow out toward the dorsal midline the level of muscle associated Tractin expression gradually increases [Fig. 9(A,B), white arrows]. However, the Tractin immunoreactivity of the dorsoventral flattener muscles is transient and subsides after E11 (data not shown).

The Tractin antibody labeling of the middle group of dorsoventral flattener muscles that serve as a substrate for DP nerve formation suggests that muscle-associated Tractin may serve a functional role in establishing or maintaining the nerve. We therefore attempted to perturb DP-nerve formation by injecting purified Tractin antibody into the germinal plate of E8 Hirudo embryos (see Methods).
However, the injections of Laz6-56 as well as a mixture of Laz6-56 and mAb 1H4 did not produce any observable phenotypes.

DISCUSSION

In this article we have provided evidence that the muscle specific mAbs, Laz10-1 and Lan3-14, both recognize a leech member of the filamin-family of actin binding proteins. Muscle filamins are generally enriched at the Z-lines and myotendinous junctions although they are also present at lower levels at the plasma membrane in association with the cortical actin cytoskeleton (van der Flier and Sonnenberg, 2001). At the plasma membrane filamins may help to organize the localization of transmembrane receptors and signaling molecules through interactions of these proteins with the various filamin ABP-repeat domains (van der Flier and Sonnenberg, 2001). Leech filamin is so far the largest filamin characterized with 35 ABP-repeat domains as compared to 24 for human and other vertebrate filamin family members. The presence of additional ABP-repeat domains in leech filamin may represent an extended capacity for protein-protein interactions with other molecules.

Previous studies in leech showed that leech filamin is expressed in both adult muscle cells as well as in embryonic muscle precursor cells (Thorey and Zipser, 1991). Expression of leech filamin is therefore an ideal marker for following muscle development. Here we have used the leech filamin specific antibody Laz10-1 to demonstrate that the segmentally iterated dorsoventral flattener muscles develop from three distinct anlagen a, b, and c. The dorsoventral flattener muscles are of
particular interest in the context of axon guidance as they may serve as a guide for the formation of the DP nerve. It has long been suggested that the DP nerve is pioneered by the Pd cell axon using the dorsoventral muscle anlagen as a substrate and that the Pd cell axon in turn would serve as a guide for later developing central and peripheral axons (Kuwada 1985; Jellies et al., 1994; 1996). Numerous studies in both invertebrates and vertebrates have described pathfinding strategies for nerve formation which rely on pioneer neurons establishing initial scaffolds of axon tracts that are then utilized by later extending axons as substrates for directed migration (Macagno, 1978; Bentley and Keshishian, 1982; Ho and Goodman, 1983; Raper et al., 1983; Ghosh et al., 1990; McConnell et al., 1994). This kind of strategy is particularly well suited to ensure the formation of common nerve pathways between afferent and efferent projections. However, experiments in Hirudo in which the CNS was removed before extension of the Pd axon into the periphery show that peripheral neurons in the absence of the Pd axon still are capable of forming a fasciculated nerve aligned with the middle bundle of dorsoventral flattener muscles (Jellies et al., 1995; 2000). The observation that only the middle of the three dorsoventral muscle anlagen serves as a substrate for nerve formation suggests that these muscles are molecularly distinct. Interestingly, we found that only the middle bundle is marked by immunoreactivity to the L1-family CAM Tractin. Thus, an attractive hypothesis is that Tractin expression may be correlated with creating a pathway that is permissive for axon extension and nerve formation.

To test this hypothesis we injected purified antibodies to two of the extracellular domains of Tractin into the germinal plate of the living embryo in order to perturb nerve formation. However, no apparent phenotypes were observed. This
could be because we do not have function blocking antibodies to Tractin in hand and/or that one or more other molecules differentially expressed by these muscle cells can provide redundant function. Previous studies in leech have shown that all neurons express the CAMs Tractin and LeechCAM and that both these molecules are differentially glycosylated in different populations of neurons in a similar pattern (Huang et al., 1997; Jie et al., 1999; 2000). Furthermore, in vivo antibody perturbations of specific glycomodifications of these CAMs demonstrate that they can selectively regulate extension of neurites and filopodia (Huang et al., 1997). These experiments indicate that Tractin and LeechCAM may provide functional redundancy and that post-translational modifications such as differential glycosylation may govern some of the functional properties as they relate to axon guidance and nerve formation. Similar molecular mechanisms that we have not been able to detect with our present probes may be responsible for regulating nerve/muscle interactions of the middle dorsoventral muscle bundle. The transient expression of Tractin in only this muscle bundle at the right developmental stages where these interactions occur strongly suggests that Tractin may play such a functional role.

A direct guidance function for nerve formation by a muscle cell in leech has been previously described in the case of the axonal runway cell (ARC) (Jellies and Kristan, 1988b). The ARC is a single cell that is positive for the muscle specific Lan3-14 mAb and is required for the formation of the stereotyped "sex nerve" that projects from the anterior root of ganglion 6 to the male reproductive structures in the adjacent anterior segment (Jellies and Kristan, 1988b). When the ARC was killed either by physical disruption with a microelectrode or by photoablation after filling it
with lucifer yellow, the "sex nerve" always failed to form whereas all other nerves formed normally (Jellies and Kristan, 1988b). The dorsoventral flattener muscles form at much earlier developmental stages than the ARC and the "sex nerve" and for technical reasons we have not yet been able to perform similar experiments with the dorsoventral muscle anlagen. Nonetheless, our findings provide further evidence that muscle cells similar to the ARC and the middle dorsoventral muscle anlage may function as a suitable substrate for early axonal outgrowth and nerve formation in leech and that this function may be associated with differential expression of cell adhesion molecules. In Manduca, normal migration of neurons in the enteric nervous system along specific visceral muscle bands are regulated by transient expression of the CAM fasciclin II by the muscle cells during the migratory period (Copenhaver et al., 1996; Wright et al., 1999; Wright and Copenhaver, 2000). Thus, the transient expression of CAMs by muscle cells may be a general mechanism for governing directed axon outgrowth and neuronal migration.

ACKNOWLEDGMENTS

We wish to thank Dr. Paul Kapke at the Iowa State University Hybridoma Facility for help with maintaining the monoclonal antibody lines. This work was supported by NIH grant NS 28857 (JJo), by NSF grant 9724064 (JJe), by Fung and Stadler Graduate Fellowship Awards (Y-ZX, DW), and by NSF training grant DIR 9113595 undergraduate fellowships (LP, KB).
REFERENCES


FIGURE LEGENDS

Figure 1 Immunoreactivity of the Lan3-14 and Laz10-1 mAbs. (A) Labeling of body wall muscles in a Haementeria embryo by Laz10-1. (B) Labeling of body wall muscles in a Haementeria embryo by Lan3-14. The labeling in (A) and (B) is visualized using TRITC-conjugated secondary antibody. (C) Immunoblots of SDS-PAGE fractionated Haemopis muscle proteins were labeled with Lan3-14 and Laz10-1 antibody, respectively. Both antibodies recognize an identical approximately 400 kDa band. The migration of molecular weight markers is indicated in grey.

Figure 2 (A) The complete predicted amino acid sequence of leech filamin. Leech filamin is a 3836 residue protein with a calculated molecular mass of 409,496 Da. (B) Northern blot analysis of leech filamin mRNA. A single band of approximately 14.5 kb was detected.

Figure 3 (A) Domain structure of leech filamin compared to the most closely related filamins from other organisms. Black boxes indicate the two actin-binding calponin homology domains. White boxes indicate filamin/ABP-repeat domains. (B) Phylogenetic relationship of leech filamin with other filamins. The consensus
maximum parsimony tree was derived from an alignment with all gaps removed. The tree is unrooted and is depicted with the associated bootstrap support values from 1000 iterations.

**Figure 4** The DP nerve forms independently of the peripheral nervous system. The micrograph in (A) is a composite of confocal images from an E8 *Hirudo* embryo double labeled with the Laz6-56 (in red) and ACT (in green) antibodies. Laz6-56 labels all neurons whereas the ACT antibody at this stage labels only the projections of central neurons. The Laz6-56 and ACT antibody labeling in (A) are merged from two different confocal planes that are shown separately in (B) and (C), respectively. The AA, MA, and PP nerves are prefigured by the differentiation and axon extension of peripheral neurons (HO1, HO2, and HO4-HO6 are stretch receptor neurons; S3 and S5-S7 indicate the position of four of the seven groups of sensillar sensory neurons). In contrast, the DP nerve is pioneered by central neurons and is the only nerve with peripheral extensions from centrally located neurons at this stage of development. Anterior is to the left.

**Figure 5** (A) Diagram of the leech nervous system and muscle layers. The CNS is ventrally located and three of the four peripheral nerves (AA, MA, and PP) extend circumferentially in close apposition to the inner body wall and longitudinal muscle layer. In contrast, the DP nerve (green) extends dorsally along the dorsoventral flattener muscles (red). The figure is modified from Nicholls and Van Essen (1974). (B) Stereo pair of a double labeling of a E10 *Hirudo* embryo with Laz10-1 (red) and ACT (green) antibody. The DP nerve extends along the
dorsoventral muscle (DV) fibers to the future dorsal region of the bodywall. Beneath the dorsoventral muscles are the longitudinal and circular muscle layers to which the remaining nerves project. The stippled lines indicate the approximate segmental borders. Anterior is up.

**Figure 6** Developmental sequence of the dorsoventral muscle anlagen. Confocal images from three consecutive hemisegments are shown in (A), (B), and (C) from an E8 *Hirudo* embryo double labeled with Laz10-1 (red) and ACT (green) antibody. The three dorsoventral muscle anlage (a, b, and c) develop in a rostrocaudal progression. The most rostral segment is shown in (A) and the most caudal segment in (C). Central axons forming the DP nerve extend in the same confocal plane along the middle anlagen. Anterior is to the left and dorsal is up.

**Figure 7** The cell adhesion molecule Tractin is expressed along the path of the forming DP nerve. (A), (B), and (C) show confocal images from three consecutive hemisegments from an E8 *Hirudo* embryo double labeled with Tractin (red) and ACT (green) antibody. The most rostral segment is shown in (A) and the most caudal segment in (C). The DP nerve appears yellow, as it is positive for both Tractin and ACT antibody. The peripheral Tractin labeling is indicated by arrows. Anterior is to the left and dorsal is up.

**Figure 8** Tractin expression is restricted to the position of the middle dorsoventral muscle anlage. The micrographs show confocal images of hemisegments from E8 *Hirudo* embryos at the same developmental stage double
labeled with Tractin/ACT antibody in (A) and with muscle/ACT antibody in (B). Although all three dorsoventral muscle anlagen (a, b, and c) have differentiated at this stage (B) only the middle anlage, b, is associated with Tractin expression as indicated by the arrows in (A). Anterior is to the left and dorsal is up.

**Figure 9** The earliest detectable muscle-associated Tractin immunoreactivity occurs just prior to DP nerve extension. The micrograph in (A) is a composite of confocal images from three posterior segments from an E8 *Hirudo* embryo double labeled with the Laz6-56 (in red) and ACT (in green) antibodies. (B) and (C) show the separate antibody labeling of Laz6-56 and ACT, respectively. The arrows in (A) and (B) indicate the position of peripheral Tractin immunoreactivity associated with the middle dorsoventral muscle anlagen. The yellow arrow indicates labeling prior to Pd axon extension from the CNS. Anterior is at the top.

Fig. 5
Fig. 6

Fig. 7
EF-hand calcium-binding proteins have been implicated in a variety of pathological diseases including Alzheimer's, Down's syndrome and inflammatory disorders (Griffin et al., 1998). Calcium-binding proteins perform diverse cellular functions by interacting with a variety of target proteins. The affinity of calcium-binding proteins for their downstream targets has been shown to be modulated by calcium. Solving the structure of these proteins would provide insight into their functions.

Calsensin is a member of the two EF-hand calcium-binding protein family that includes the S100 and polcalcin subfamilies. Molecules like Calsensin that are expressed selectively in certain neurons are likely to function as signal transducers during growth cone fasciculation and guidance (Briggs et al., 1995). Calsensin can multimerize through the formation of intermolecular disulfide bridges. Disulfide linked dimerization of S100 proteins has been suggested to play a role in mitogenic and neurotrophic activities (Kligman and Marshak, 1985, Selinfreund et al., 1991, Winningham-Major, et al., 1989). We have used multidimensional NMR to solve the structure of calcium-loaded Calsensin. The structure of Calsensin reveals an antiparallel stacking of the two helices of each EF-hand with an axially anisotropic diffusion tensor. The relatively lower order of H3 as compared to other helices is due to the presence of low-amplitude motions and conformational exchange. The observed higher flexibility could be attributed to chemical exchange between
calcium-loaded and free states and/or closed and open conformations. The results of mobility shift assay along with the titration data suggest that Calsensin undergoes conformation change upon calcium-binding.

Using a GST-Calsensin overlay screen of the *Haemopis* expression library, we have identified putative interacting partners (refer to appendix). One of the potential candidates, Caldesmon, has been shown to be expressed in chick forebrain growth cones and interact with EF-hand calcium-binding proteins in a calcium-dependent manner (Alexanian, et al., 2001). Caldesmon has been shown to co-localize with the actin cytoskeleton and interact with microtubules in a Ca$^{2+}$-CaM dependent manner in the rat hippocampus (Agassandian, et al., 2000). Owing to its localization to neuronal growth cones and colocalization with actin and microtubule network in a calcium-dependent manner, it is an ideal candidate for providing a link between the calcium signal and cytoskeletal changes.

The leech homolog of Caldesmon interacts with Calsensin in a calcium dependent manner. Using two-dimensional NMR, we have identified the residues of Calsensin involved in binding Caldesmon. The residues involved in binding were mapped to the helices H1, H4 and also the hinge region. Most of the residues involved in binding are in the fourth helix, which has been shown to be important for target interactions in S100 proteins (Yap et al., 1999). Solving the structure of Calsensin has allowed us to elucidate the calcium-dependent interaction between Calsensin and Caldesmon at the structural level. The binding surface is formed by residues that are close together in the tertiary structure, but are far apart in the primary sequence.
The Laz10-1 and Lan3-14 monoclonal antibodies identify a 400 kDa antigen expressed in all muscle cells. We have cloned and identified this antigen to be a member of filamin family of actin-bundling proteins. Leech Filamin consists of two calponin homology domains which are thought to be involved in actin binding and 35 filamin/ABP-repeats. The ABP-repeats have been implicated in dimerization as well as protein-protein interactions. Using the Laz10-1 antibody, we have characterized the development of dorso-ventral flattener muscles and show that they develop as three discrete bundles. The middle bundle transiently expresses neuronal CAM Tractin concomitant with the formation of DP nerve. This suggests that the middle dorso-ventral muscle anlagen may provide the substrate for the axonal outgrowth and nerve formation.

Mutations in Filamin have been shown to cause neuronal migration defects leading to periventricular heterotopia (van der Flier and Sonnenberg, 2001). Filamin can regulate trafficking of furin thereby modulating the processing of CAMs, like L1 (Liu, et al., 1997). Filamin has been shown to interact with various molecules involved in axon guidance and signaling including presenilins, Rho GTPases, integrins, CaM kinases (Stossel, et al., 2001). Filamin might regulate axon guidance by acting as a bridge between the extracellular signals and actin cytoskeletal rearrangements.

FUTURE DIRECTIONS

Calcium-binding proteins have been extensively studied for years and there is a vast amount of literature available. However, the mechanisms by which these
proteins regulate various cellular processes are still unclear. The calcium-binding proteins via interactions with target proteins transduce calcium signals. By identifying the interacting partners, the various pathways that are modulated by these proteins can be dissected. Solving the structure of the calcium-binding proteins is the first step in understanding their function. By studying the biochemical and molecular interactions of calcium-binding proteins, we can understand the mechanism and specificity of target binding. The binding interaction between Calsensin and Caldesmon is sensitive to calcium and hence solving the structure of Calsensin/Caldesmon complex will provide insight into the conformational changes accompanying calcium as well as target binding. The other putative binding partners identified from the screen are promising as candidates for molecules involved in calcium signaling. It would be of interest to assay their interaction with Calsensin and also determine their localization in the nervous system.
APPENDIX. ADDITIONAL DATA

INTRODUCTION

Calcium-binding proteins which function as trigger proteins convert a calcium signal into downstream signal transduction events. To understand the function of calcium-binding proteins, it is essential to identify their interacting partners. This would help in dissecting the signaling pathway in which they are involved. Calsensin is expressed by a subset of peripheral neurons which fasciculate into a single axon tract. The molecular feature of Calsensin suggests that it might function as trigger protein, binding its targets in a calcium-dependent manner. A GST-Calsensin overlay screen of a Haemopis cDNA expression library was carried out. After multiple rounds of screening, we identified 7 different interacting clones. One of the identified clone was similar to calmodulin and the actin-binding protein, Caldesmon. Caldesmon has been shown to be expressed in the chick cortical neurons (Alexanian, et al., 2001). Owing to its possible function as a link between calcium signal and cytoskeletal rearrangement, we selected this clone for further analysis.

MATERIALS AND METHODS

Fusion proteins. The entire ORF of Calsensin was cloned into pGEX4T3 and Xa3 vectors to express as GST and pinpoint fusion proteins. The sequence of Caldesmon identified from the overlay screen was cloned in frame with pGEX4T3 and Xa3 vectors to generate GST- and biotinylated fusion proteins. The constructs
were transformed into BL21 cells, expressed, and purified according to manufacturer's protocols (Amersham Biosciences).

**GST-Calsensin pulldown and overlay assay.** To identify proteins interacting with Calsensin, GST-Calsensin and GST were bound to glutathione-agarose beads (Sigma). The beads were washed with IP buffer (25 mM Tris, pH 7.4, 75 mM NaCl, 2 mM CaCl$_2$, 0.4% Triton-X-100). The Haemopis nerve cords were homogenized in IP buffer and pre-cleared by incubation with glutathione-agarose beads. The GST-Calsensin and GST-only beads were incubated with nerve cord extract overnight at 4°C. The beads were washed 3 times for 10 min each with IP buffer. The proteins were eluted using SDS-PAGE loading buffer and separated by a 10% acrylamide gel. The proteins were detected by silver stain. For the overlay assay, the nerve cord extracts were separated on 7.5% or 15% SDS-PAGE gels and transferred onto nitrocellulose membrane. After blocking with 5% milk in TBS for 1 hour, the blot was incubated overnight with 5% blotto containing 10 µg/ml of GST-Calsensin or GST control. The blots were washed, incubated with lan 3-6 (anti-Calsensin) or anti-GST primary antibody, which was detected using anti-mouse HRP-conjugated secondary antibody (Bio-Rad) (1:3000). The signal was detected with the ECL chemiluminescence kit (Amersham) and digitized using Photoshop software (Adobe) and a flatbed scanner (Epson Expression 1680).

**Overlay screening of expression library.** The oligo-dT primed Haemopis cDNA λgt11 expression library was plated at a density of 30,000 plaque-forming units/150 mm plate. The screening was performed essentially according to the
procedures in Sambrook et al., (1989) except for the additional step of incubating the filters with GST-Calsensin (10 μg/ml) before detecting with lan 3-6 antibody. Positive clones were tested by overlaying with GST-Calsensin, followed by immunoblotting with the lan 3-6 antibody. The plaques were purified using a CsCl step gradient, and the phage stocks digested with EcoRI and NotI. The digested fragments were cloned into KS+ and sequenced. Several partial cDNAs were identified in these screens. The sequences were compared with known and predicted sequences using the National Center for Biotechnology Information BLAST e-mail server.

**Calcium-dependent interaction assay.** To assay for the interaction between Calsensin and Caldesmon, we used the PinPoint fusion proteins coupled to streptavidin beads to pulldown the GST fusion proteins in the presence of EDTA and EGTA or in the presence of calcium. The pulldown experiments were performed as described above for the GST-Calsensin pulldown, but the signal was detected by immunoblotting using anti-biotin or anti-GST antibodies.

**Caldesmon NMR titration.** The effect of Caldesmon on the structure of Calsensin was studied by titrating 1mM 15N labeled sample with 4 mM unlabeled Caldesmon and monitoring the changes with 1H-15N HSQC experiments. The titrations were carried out by adding appropriate aliquots of Caldesmon to calcium-saturated Calsensin to obtain the desired concentration. The protein and/or salt precipitated upon addition were removed by centrifugation and the remaining sample was used for NMR experiments. To verify that Caldesmon binds Calsensin in a calcium-dependent manner, EDTA was added to obtain the calcium-depleted apo
form of Calsensin. Caldesmon was added to this sample, and titrated with increasing concentration of calcium. The changes in the structure were followed using HSQC experiments.

RESULTS AND DISCUSSION

**GST-Calsensin pulldown and overlay screen.** To determine the proteins interacting with Calsensin, a GST-Calsensin pulldown of the leech nerve cord extract was performed. The proteins that were separated on 10% SDS-PAGE gel and detected by silver staining. Proteins of different sizes (210 kD, 155 kD, 81 kD and 72 kD are the prominent bands) that selectively interact with GST-Calsensin but not with GST- alone control beads were identified (Fig. 1). To develop a screen for identifying interacting partners, overlay assays were performed. The leech nerve cord extract was separated by 7.5% and 15% SDS-PAGE, electroblotted and overlaid with either GST-Calsensin or GST as control. The proteins were detected by immunoblotting with lan 3-6 or anti-GST primary antibody. GST-Calsensin bound to numerous proteins which were not detected in the GST-only control lane (Fig. 2). The molecular sizes of the proteins are similar to those obtained in the pulldown experiment (200 kD, 145 kD/135 kD, and 68 kD/75 kD are the predominant bands).

**Possible interacting partners.** Using the overlay screen of the cDNA expression library, we have identified seven putative interacting partners of Calsensin. The clones obtained from the screening were cloned into KS+ and
sequenced. The sequences were compared with known and predicted sequences using NCBI BLAST server. The possible interacting proteins are:

1. Calmodulin and actin binding protein, Caldesmon
2. C-type lectin or IgE binding protein – 2 independent clones
3. Serine protease – 2 independent clones
4. Small proline rich protein
5. Laminin
6. Fibronectin/Fibrinogen binding protein
7. Nicotinic acetylcholine receptor beta subunit

Interaction assay for Calsensin and Caldesmon. Caldesmon was identified from an overlay screen as an interacting partner of Calsensin. To further, test this interaction, GST and PinPoint fusion proteins were expressed. The PinPoint fusion of Calsensin and Caldesmon were used to pull down Caldesmon- and Calsensin-GST fusion proteins, respectively. Caldesmon binds Calsensin only in the presence of calcium (Fig. 3). Removal of calcium by adding chelating agents, EDTA and EGTA abolishes this interaction. In the converse experiment, Calsensin interaction with Caldesmon is stronger in the presence of calcium than in its absence (Fig. 4). This suggests that the interaction between Calsensin and Caldesmon is calcium-dependent and that Calsensin might function as a trigger protein in the peripheral nervous system.

Caldesmon titrations. The binding of Caldesmon to Calsensin has been shown to be calcium-dependent in vitro. To map the residues of Calsensin involved
in binding Caldesmon, $^{15}$N chemical shift mapping was carried out. Calcium was depleted from the Calsensin sample by the addition of 8 mM EDTA. Caldesmon was added to the apo form of Calsensin. There were no chemical shift changes following this addition. This sample was titrated with increasing amounts of calcium. Caldesmon did not induce any change in chemical shift until both the calcium-binding sites were saturated. Upon increasing the calcium concentration to 64 mM CaCl$_2$, Caldesmon induced chemical shift changes of selected residues (Fig. 5). This suggests that calcium-dependent conformational change of Calsensin is required for Caldesmon binding. The residues involved in this interaction form a hydrophobic surface (Fig. 6) and they are close together in tertiary structure, although they are far apart in the primary sequence. The calcium-induced conformational change may expose the hydrophobic residues necessary for target binding.

FIGURE LEGENDS

FIGURE 1: GST-Calsensin pulldown of leech neuronal proteins. GST- and GST-Calsensin coupled glutathione beads were incubated with pre-cleared nerve cord extract. The proteins bound to the beads were separated on a 10% SDS-PAGE gel and detected by silver staining. Proteins of different sizes (210 kD, 155 kD, 81 kD, and 72 kD are the prominent bands) selectively interact with GST-Calsensin as compared to GST-control beads.

FIGURE 2: GST-Calsensin overlay of leech neuronal proteins. The leech nerve cord extract was separated by 7.5% or 15% SDS-PAGE (left and right gels, respectively),
electroblotted and overlaid with either GST-Calsensin or GST as control. The proteins were detected by immunoblotting. GST-Calsensin bound to numerous proteins, which were not detected in the GST-control. The sizes of the proteins are similar to those obtained in the pulldown experiment (200 kD, 145 kD/135 kD, and 68 kD/75 kD are the predominant bands).

FIGURE 3: Biotinylated-Calsensin pulldown of GST-Caldesmon. Purified biotinylated-Calsensin (lane 1, detected with anti-biotin antibody) coupled to streptavidin beads was incubated with GST-Caldesmon (lane 2) either in the presence of chelating agents (lane 3) or in the presence of calcium (lane 4). The proteins were separated on 12% gel and immunoblotted with anti-GST antibody (lanes 2-5). The interaction between Calsensin and Caldesmon is stronger in the presence of calcium than in the presence of EDTA and EGTA. The beads alone (lane 5) does not pull down GST-Caldesmon even in the presence of calcium.

FIGURE 4: Biotinylated-Caldesmon pulldown of Calsensin. Purified biotinylated-Caldesmon (lane 1, detected with anti-biotin antibody) coupled to streptavidin was incubated with Calsensin (lane 2) either in the presence of EDTA and EGTA (lane 3) or in the presence of calcium (lane 4). The proteins were separated on 16% gel and immunoblotted with lan 3-6 antibody (lane 2-5). Recombinant biotinylated-Caldesmon can pull down Calsensin in the presence of calcium, but not in its absence. The beads alone (lane 5) does not pull down Calsensin even in the presence of calcium.
FIGURE 5: Overlay of $^1$H-$^{15}$N HSQC $^{15}$N labeled 1 mM Calsensin in 50 mM Phosphate buffer containing 75 mM NaCl, 2 mM DTT, 0.02% sodium azide, 8 mM EDTA and 64 mM CaCl$_2$ without (blue) or with (red) 0.8 mM unlabeled Caldesmon. The residues that show a shift upon the addition of the binding partner are indicated by arrows. The residues L9, L72 and C80 undergo significant shifts, while a few other residues show moderate changes.

FIGURE 6: Binding surface of Calsensin. (A) Calsensin backbone structure with the sidechains involved in Caldesmon binding rendered in green and red. The sidechains in green show the maximum chemical shift changes while those in red undergo moderate shifts. (B) Surface plot of Calsensin showing the electrostatic potentials in blue (positive), red (negative) and white (neutral, hydrophobic). The binding surface for Caldesmon is colored in magenta.
Fig. 1

Fig. 2
Fig. 3

Fig. 4
Fig. 5
REFERENCES CITED


Palczewski, K., Subbaraya, I., Gorczyca, W. A., Helekar, B. S., Ruiz, C. C. Crabbs, J. W., Johnson, R. S., Walsh, K. A., Gray-Keller, M. P., Detwiler, P. B., and


ACKNOWLEDGEMENTS

I would like to first and foremost thank my major professors, Dr. Jørgen Johansen and Dr. Kristen Johansen, for their encouragement, guidance and most importantly patience. They have been excellent mentors and were ready to help when I needed advice on research as well as any other issues during my graduate study. I am grateful to Dr. Amy Andreotti for helping me through the NMR work and also allowing me to use her laboratory resources. I wish to thank Dr. Bruce Fulton for helping me with the NMR experiments.

I thank my committee members, Dr. Don Sakaguchi and Dr. Ted Huiatt for serving on my committee and providing valuable advice and support. I wish to thank Dr. Melissa Blacketer, Dr. Monica Sundd and Stephanie Lerach, for the valuable scientific discussions as well as their friendship. I would also like to recognize former members of the lab, Dr. Dong Wang, Dr. Yun Ji, Dr. Ying-Zhi Xu and excellent undergraduates Katie Bock and Liliana Ponguta for their contributions to the Filamin project. I value the help, advice and friendship provided by the past and present members of the lab.

I would like to thank my parents for their support and love, and helping me through all the ups and downs in life. Last but not the least, I would like to thank my love, Jay for his constant encouragement, support and understanding. He has not only assisted me in my scientific endeavors, but through his faith in my ability, given me confidence.