Calsensin, a novel calcium-binding protein expressed in a subset of peripheral leech neurons fasciculating in a single axon tract

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Calsensin: A novel calcium-binding protein expressed in a subset of peripheral leech neurons fasciculating in a single axon tract

by

Kristen Kay Briggs

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INTRODUCTION

To ensure a fully functional organism, developing neurons must make precise connections with their targets (Dodd and Jessell, 1988). The growth cones of early neurons pioneer specific axon pathways, establishing the route that the axons of later developing neurons will follow (Dodd and Jessell, 1988). The growth cones of these later neurons are able to distinguish between axon pathways and choose the correct path to their targets by interacting with guidance cues that are located on the membranes of neighboring cells or axons, on the extracellular matrix, or soluble factors, through homophilic and heterophilic (ligand-receptor) interactions (Dodd and Jessell, 1988; Jessell, 1988). Some of these interactions represent specific growth-promoting signals, whereas others exert inhibitory effects on the growth cones (Dodd and Jessell, 1988; Jessell, 1988). The result of these interactions depends on the particular combinations of signals received by a specific growth cone, and on the hierarchy of the various signals (Dodd and Jessell, 1988; Jessell, 1988; Eisen et al., 1989). Different types of neurons could differ in their receptors, and therefore their susceptibility to various guidance cues (Dodd and Jessell, 1988).

One of the great challenges faced by developmental neurobiologists is to identify the guidance cues that are used by developing neurons and determine how those extracellular signals are converted into the complex behaviors involved in growth cone navigation. The antigen recognized by the Ian 3-6 monoclonal antibody has been hypothesized to be a potential mediator of guidance and/or fasciculation in the leech nervous system due to its localization to a small subpopulation of peripheral neurons whose axons form a single tract within the central nervous system (CNS) of all hirudinid leeches (Briggs et al., 1993). The goal of this research was to try to gain insight into the functional properties of the Ian 3-6 antigen by conducting a molecular and biochemical characterization of both the gene sequence that encodes it and its protein product.

The Ian 3-6 monoclonal antibody was used to isolate a clone encoding a novel protein, calsensin, which contains two EF-hand calcium-binding domains. In situ hybridizations with calsensin riboprobes, Western blots of Ian 3-6 monoclonal antibody immunoprecipitations, and immunohistochemistry with polyclonal antibodies generated against a partially-purified calsensin fusion protein confirmed that the sequence encoding the Ian 3-6 antigen had been identified. The calsensin calcium-binding domains are likely to function in vivo, as indicated by the demonstration that partially-purified calsensin fusion protein bound $^{45}$Ca$^{2+}$ in vitro. Furthermore, immunoaffinity purification with Ian 3-6
antibodies resulted in the copurification of a 200,000 Mr protein in two different leech species. These results suggest that calsensin may provide a link between neuronal recognition and the signal-transduction events leading to specific pathway selection by binding to and regulating this 200,000 Mr protein.

This chapter will provide an introduction to the well characterized guidance and signalling mechanisms used by developing neurons, the importance of intracellular calcium in these processes, and a possible role for EF-hand calcium-binding proteins, like calsensin, in mediating these calcium signals. In addition, some background into the leech nervous system, how it has been used as a model system for identifying and studying the guidance mechanisms used by peripheral sensory neurons, and the possible role of the lan 3-6 antigen in these processes, will be presented.

**Molecular Mechanisms Involved in Axon Guidance**

The events that occur early in growth cone guidance, before neurons become functionally active, have been shown in many cases to rely on multiple molecular mechanisms to form the initial pattern of connections with their targets (Dodd et al., 1988; Bastiani et al., 1987; Caudy and Bentley, 1986a, 1986b; Berlot and Goodman, 1984). These mechanisms are mediated by a variety of molecules and include differential adhesion, guidepost cells, chemotropism and differential inhibition (Dodd and Jessell, 1988).

**Differential adhesion**

A large number of adhesive molecules have been identified and characterized. These adhesion molecules, present on the neuron cell surface, interact with adhesion molecules on neighboring cell surfaces and in the extracellular matrix in either a homophilic or heterophilic manner (Dodd and Jessell, 1988). Most of the identified adhesion molecules are members of three conserved gene families (Dodd and Jessell, 1988): the integrins, the cadherins, and the members of the immunoglobulin superfamily, although there are additional gene families involved as well (Elkins et al., 1991; Yang et al., 1991; Pulido et al., 1992).

The members of the integrin family are transmembrane glycoproteins that are widely distributed throughout the nervous system (Hynes, 1992). They form α,β heterodimers, and are receptors for the extracellular matrix glycoproteins laminin, fibronectin, vitronectin, and collagen (Hynes, 1992). Although about 20 integrins have been identified in vertebrates, there are 14 known α subunits and 8 known β subunits, and some of these subunits are known to be alternatively spliced, thus the number of possible functional integrin receptors is
very large (Hynes, 1992). Antibodies to the β1 integrin subunit have been shown to inhibit neurite outgrowth on laminin, fibronectin, collagens, and complex extracellular matrices (Tomaselli et al., 1986; Hall et al., 1987). In addition, there is evidence that the growth cones of retinal ganglion cells switch from pathfinding to target recognition by inactivating their α6β1 receptor once they reach the optic tectum (Neugebauer and Reichardt, 1991). Furthermore, the cytoplasmic portion of the integrin receptors may be linked to the actin cytoskeleton via the cytoplasmic proteins α-actinin, talin, and vinculin (Hynes, 1992; Gumbiner, 1993), and these interactions may alter the strength of adhesion between ligands in the extracellular matrix and their integrin receptors (Gumbiner, 1993).

The cadherins make up the second main family of adhesion molecules. Most vertebrate cadherins identified are transmembrane glycoproteins that form homodimers in a calcium-dependent manner (Geiger and Ayalon, 1992). N-cadherin has been identified in the chicken, human, mouse, rat, and bovine nervous systems (Geiger and Ayalon, 1992). In addition, a truncated cadherin (T-cadherin) which is linked to the plasma membrane via a glycosphingolipid, and a retinal (R-cadherin) cadherin, have been identified in many neuronal cells (Ranscht et al., 1991). T-cadherin has been shown to promote cell adhesion in transfected cells in a calcium-dependent manner (Geiger and Ayalon, 1992). Furthermore, the adhesive properties of the cadherins are dependent on their interaction with the cytoskeletal catenin proteins (Geiger and Ayalon, 1992). Cadherins have been implicated in neurite outgrowth and extension on different substrates such as myotubes, Schwann cells, and astrocytes (Bixby et al., 1987 and 1988; Tomaselli et al., 1988).

The members of the immunoglobulin superfamily make up the third main group of cell adhesion molecules. These proteins typically have two to six C2-type immunoglobulin (IgG) domains that are often followed by repeated fibronectin type III motifs (FNIII) (Hortsch and Goodman, 1991), and both transmembrane and glycosphingolipid (GPI)-linked members of this superfamily have been identified (Hortsch and Goodman, 1991).

One of these adhesion molecules, N-CAM, has a very widespread distribution in the nervous system on both neural epithelium and neuronal cells, and mediates its adhesive interactions in a homophilic manner (Dodd and Jessell, 1988). Because of its uniform distribution throughout the developing nervous system, N-CAM is thought to be involved in the general adhesiveness between the growth cone and its environment rather than giving directional cues to the extending growth cones (Jessell, 1988; Dodd and Jessell, 1988). During the period of axon extension in development, the high polysialic acid form of N-
CAM is expressed, whereas later in development, when the growth cones have reached their targets, the low polysialic acid form of N-CAM predominates (Rutishauser et al., 1988; Tang et al., 1992). These regulated changes in the level of glycosylation on the N-CAM molecule could act to modulate axon extension in the developing organism (Dodd and Jessell, 1988; Doherty et al., 1990; Tang et al., 1992). Antibodies directed against N-CAM have been shown to disrupt the normal pattern of axon outgrowth and fascicle formation both in vitro and in vivo (Bixby et al., 1987; Doherty et al., 1989, 1990), and N-CAM knockout mice have smaller olfactory bulbs and show deficits in spatial learning (Cremer et al., 1994). Since the areas of the brain that maintain high degrees of post-developmental plasticity are affected by deletion of the N-CAM gene products, these results implicate N-CAM in the formation and regeneration of neuronal connections (Cremer et al., 1994).

Another immunoglobulin superfamily member, L1, is present mainly on fasciculated axons during development (Fushiki et al., 1986; Rathjen et al., 1984; Stallcup et al., 1985). Fab fragments to the L1 antigen inhibit the fasciculation of neurites in vitro (Fischer et al., 1986), and it appears to stimulate neurite outgrowth by both homophilic binding and also through heterophilic interactions with axonin-1(TAG-1), another immunoglobulin superfamily member (Lemmon et al., 1989; Kadmon et al., 1990; Chang et al., 1990; Stoeckli et al., 1991; Kuhn et al., 1991). Recent experiments have suggested that neurite outgrowth induced by homophilic binding of the cell adhesion molecules N-cadherin, L1, and N-CAM in vitro in rat day 1-8 cerebellar neurons, E17 hippocampal neurons, adult dorsal root ganglion neurons, and PC12 cells is mediated by the induction of the fibroblast growth factor receptor tyrosine kinase, which is upstream of a pertussis toxin-sensitive G protein that leads to the opening of L- and N-type calcium channels (Doherty et al., 1991; Doherty et al., 1994a and b; Doherty et al., 1995). Protein tyrosine phosphorylation has also been implicated as part of the integrin-mediated adhesion signal, but its neurite outgrowth-promoting ability on laminin, fibronectin, and collagen are independent of L- and N-type calcium channels, pertussis toxin-sensitive G proteins, and the fibroblast growth factor receptor tyrosine kinase, at least in the cells listed above (Gumbiner, 1993; Doherty and Walsh, 1994).

Neural surface molecules of the immunoglobulin superfamily with restricted distributions to specific axon pathways, or regions of axon pathways like TAG-1 (Furley et al., 1990), contactin/F3/Fil (Ransch, 1988; Brummendorf et al., 1989; Gennarini et al., 1989), SCI/DMGRASP (Pollerberg and Mack, 1994), fasciclin II (Grenningloh et al., 1990, 1991; Lin et al., 1994), fasciclin III (Snow et al., 1989), neurofascin (Rathjen et al., 1992),
Gicerin (Taira et al., 1994), Dtrk (Pulido et al., 1992), and Ian 3-2 (Johansen et al., 1992; Huang, unpublished observation) have been identified (Jessell, 1988; Dodd and Jessell, 1988; Bixby and Harris, 1991; Hortsch and Goodman, 1991; Goodman and Shatz, 1993). These molecules have been proposed to provide more specific cues to growth cones, largely due to their confined patterns of expression within the nervous system (Dodd and Jessell, 1988; Hortsch and Goodman, 1991).

In addition to the three main families of cell adhesion molecules, there are a number of axonal proteins with proposed outgrowth or guidance functions in the developing nervous system. Two receptor-like tyrosine phosphatases have been identified in Drosophila with discrete expression patterns on subsets of developing axons in the central nervous system (Yang et al., 1991), and there is evidence that another guidance molecule isolated from Drosophila, fasciclin I, may use tyrosine phosphorylation as part of its signalling mechanism in vivo (Elkins et al., 1990). In addition, an epidermal growth factor (EGF) repeat-containing protein, giant lens, has been implicated in axon guidance in the Drosophila visual system (Kretzschmar et al., 1992) as have the Ian 4-2 and Ian 3-6 antigens in the leech (Johansen et al., 1992; Briggs et al., 1993).

The final known group of molecules which mediate cell adhesion are the substrate adhesion molecules located in the extracellular matrix. Restrictin and neurite outgrowth factor (NOF) from the chick have defined expression patterns within the central nervous system, and are ligands for the immunoglobulin superfamily members F11 and Gicerin respectively (Hayashi and Miki, 1985; Rathjen et al., 1992). As already mentioned, the extracellular matrix proteins laminin, fibronectin and collagen are ligands for the integrin receptors and all can promote growth from several different types of neurons (Bixby and Harris, 1991). In addition, the interaction of integrins with laminin and fibronectin has been proposed to have a role in neural cell adhesion (Hynes et al., 1992).

Laminin is a very potent promoter of neurite outgrowth from a variety of neural cells from both the peripheral and central nervous systems (Kleinman et al., 1990). It is a very large heterotrimer composed of three subunits A, B1, and B2, and is part of the basal lamina in both the central and the peripheral nervous systems (Kleinman et al., 1990). Antibody perturbation studies suggest that the response of neuronal cells to laminin involves its integrin receptors (Tomaselli et al, 1988).

Fibronectin is secreted by fibroblasts and other mesenchymal cells. It is composed of two disulfide-linked subunits, and in addition to its neurite outgrowth-promoting activity, it
has been shown to be involved in neural crest cell migration and in the regeneration of peripheral axons (Bixby and Harris, 1991).

**Guidepost cells**

In contrast to the cell adhesion molecules, which were largely identified and initially characterized biochemically, the guidepost cells were first identified *in vivo* in the developing grasshopper limb bud (Taghert et al., 1982; Bentley and Caudy, 1983).

Preaxogenic neurons in the epithelium of the developing grasshopper limb bud help to guide the growth cones of the neurons that pioneer the tract from the periphery to the central nervous system (Bentley and Caudy, 1983). If the guidepost cells are laser-ablated, the pioneer neuron growth cones often wander off in inappropriate directions (Bentley and Caudy, 1983). The growth cones of the pioneer neurons have been shown to form gap junctions with the guidepost neurons (Taghert et al., 1982), and this interaction causes them to change their trajectories. It appears that calcium ions are transferred through these gap junctions from the pioneer neurons to the guidepost cells, which results in a reduction in calcium in the growth cones and neurites of the pioneer neurons at points of contact with the guidepost cells (Bentley et al., 1991). Whether this decrease in calcium plays a role in the guidance of these neurons is unknown at this time (Bentley et al., 1991).

**Chemotropism**

In addition to the more static guidance mechanisms of cell adhesion and guidepost cells which are largely contact dependent, developing growth cones may be guided by gradients of diffusible factors that are released from target cells.

Diffusible neurotropic molecules have been identified in the maxillary epithelium (MAX factor), the floor plate (netrins), and in the pons (PONS factor) (Bixby and Harris, 1991; Goodman and Shatz, 1993; Kennedy et al., 1994; Serafini et al., 1994). Max factor is secreted by the rat maxillary whisker epithelium, and has been shown to attract the sensory neurons in the trigeminal ganglion (Lumsden and Davies 1983, 1986). In addition, chemotropism is involved in directing the projections of collateral branches from the cortical neurons in response to a factor released by the pons (O'Leary et al., 1990). The evidence for this came from experiments using fetal rats that were X-irradiated during the generation and migration of the basilar pontine neurons (target cells for collateral branches from descending axons of layer 5 cortical pyramidal neurons). The basilar pons in these rats was either absent or greatly reduced, and often misplaced, leading to ectopic islands of pontine neurons
(O'Leary et al., 1990). After X-irradiation, corticospinal axons no longer projected collaterals to the location of the missing basilar pons, but instead, projected collaterals to the ectopic islands of pontine neurons indicating that the pontine neurons are able to attract the cortical neurons projections in vivo (O'Leary et al., 1990).

In another example, commissural axons are attracted to the floor plate in response to netrins (Kennedy et al., 1994; Serafini et al., 1994). The floor plates of several vertebrates have been shown to provide chemotropic guidance signals for the growth cones of commissural interneurons in vitro (Tessier-Lavigne et al., 1988; Placzek et al., 1990). When a portion of neural tube is rotated in chick (Yaginuma and Oppenheim, 1991), or a notochord or floor plate grafted to an ectopic location (Yamada et al., 1991), commissural axons alter their trajectory and home in on the ectopic floor plate. In the case of the Danforth short-tail mutation, which has portions of the floor plate missing, many commissural axons are misrouted and take aberrant pathways (Bovolenta and Dodd, 1991). Two novel secreted proteins, netrin 1 and netrin 2, were purified from chick brains based on their ability to cause neurite outgrowth in vitro (Serafini et al., 1994). They share 72% identity to each other, but are expressed differently in vivo (Kennedy et al., 1994). Netrin 1 is expressed by the floor plate cells, whereas netrin 2 is expressed in the ventral two-thirds of the spinal cord (Kennedy et al., 1994). Both purified proteins were shown to promote the outgrowth of commissural interneurons in vitro (Kennedy et al., 1994).

**Differential inhibition**

In addition to the attractive guidance cues discussed thus far, there are also repulsive guidance cues that restrict the migration of their targets, and may therefore provide barriers to promiscuous target innervation. Inhibitory factors have been identified in the posterior sclerotome tissues, in the posterior tectum, on oligodendrocytes, and from brain (Bixby and Harris, 1991; Goodman and Shatz, 1993).

Neural crest cells migrate out the dorsal neural tube to form the ganglia of the peripheral nervous system. Later, motor axons grow out from the spinal cord. It was observed that the crest cells and motor fibers were only found in anterior sclerotomes, suggesting that the posterior sclerotomes contain molecules that repel both neural crest cells and motor fibers (Keynes and Stern, 1984). Two membrane-bound proteins have been isolated from posterior somites with molecular weights of 48 and 55 kd (Davies et al., 1990). These proteins cause collapse of dorsal root ganglion neurons in vitro, and antibodies generated against these proteins block this collapsing activity (Davies et al., 1990).
The projections of retinal ganglion neurons to their targets in the optic tectum have provided additional insight into differential inhibition. It has been shown that temporal retinal ganglion cell growth cones collapse if they encounter nasal retinal ganglion cell axons at a 90° angle \textit{in vitro} (Cox et al., 1990). When one side of a temporal retinal ganglion cell growth cone contacts a nasal retinal ganglion axon, the contacting side of the growth cone collapses (or fails to extend) locally, while the non-contacting side keeps advancing. This has been proposed to provide a mechanism for temporal retinal ganglion cell growth cones to turn away from nasal retinal ganglion axons \textit{in vivo} (Cox et al., 1990).

Furthermore, a posterior tectal protein that inhibits temporal retinal ganglion cell growth cones has been purified and analyzed (Stahl et al., 1990). The temporal retinal ganglion cell fibers project to the anterior optic tectum, while nasal retinal ganglion cell fibers project to the posterior optic tectum. Temporal and nasal retinal ganglion neurons were tested for outgrowth on strips of anterior and posterior tectal membranes (Walter et al., 1987a). It was found that temporal axons preferred to extend on anterior tectal membrane strips (Walter et al., 1987a). This preference was destroyed by heat and phosphoinositide-specific phospholipase C (i.e. attached via glycolipid anchor), suggesting that the posterior tectal membranes contain a membrane-associated protein-based inhibitory factor, that prevents temporal retinal ganglion axons from extending across it (Walter et al., 1987b). A 33 kd membrane-linked glycoprotein was isolated from the posterior tectum. This protein is present until E15 when establishment of the retinal terminal field is complete (Stahl et al., 1990). The purified protein is active in the stripe assay, and a monoclonal antibody generated against the purified protein stains the tectum in an anterior to posterior gradient (Stahl et al., 1990). Therefore, the combination of inhibitory activities present on nasal retinal axons and the posterior tectum, may help to keep the temporal retinal axons on track to the anterior optic tectum by providing barriers to their outgrowth.

Several observations led to the hypothesized existence of oligodendrocyte and central nervous system myelin-associated inhibitors (Kapfhammer and Raper, 1987; Raper and Kapfhammer, 1990). It was observed that the growth cones of central neurons retract when they contact the growth cones of peripheral neurons, that central neurons can regenerate damaged neurites in a peripheral environment, but not within the central nervous system, and that oligodendrocytes inhibit neurites of a variety of neurons, neuroblastoma cells, PC12 cells, and 3T3 fibroblasts \textit{in vitro} (Schwab and Caroni, 1988; Schwab et al., 1993). The fact that the oligodendrocytes need to touch the neurite to inhibit suggested that this inhibitory factor is not secreted, but probably membrane-associated (Caroni and Schwab, 1988). Two
spinal cord myelin proteins were isolated with molecular weights of 35 kd and 250 kd (complex contains the 35 kd protein), and are referred to as NI-35 and NI-250 (Caroni and Schwab, 1988). It has been shown that antibodies to these two proteins neutralize their effect on dorsal root ganglion neurites (Caroni and Schwab, 1988; Bandtlow et al., 1990). These proteins are first expressed in the brain postnatally as the oligodendrocytes start to synthesis myelin (Schwab et al., 1993), and Schwann cells, which promote axon regeneration, do not express these two proteins (Schwab et al., 1993). Intracellular calcium and a pertussis toxin-sensitive G protein have been implicated in the regulation of NI-35-induced collapse of rat dorsal root ganglion neurons in vitro (Schmidt et al., 1991; Bandtlow et al., 1992; Igarashi et al., 1993). These proteins probably function to delineate boundaries during late phases of central nervous system development, and later suppress side-branch formation in white matter, providing a narrow spatial restriction in gray matter (Schwab et al., 1993).

Collapsin is a 100 kd chick brain collapsing factor that is similar to the grasshopper and Drosophila Semaphorins, and a homolog of the human Semaphorin (Luo et al., 1993; Kolodkin et al., 1992; Kolodkin et al., 1993). The grasshopper Semaphorin, G-Sema1, is expressed on subsets of axons and an epithelial stripe in limb bud where it guides two sensory growth cones (Til neurons) as they turn when they reach the Sema1 boundary, and then cross (Kolodkin et al., 1992). Antibodies against the G-Sema1 protein caused the Til growth cones to be more branched and defasciculated, and also to contact the Cxi neurons more proximally (Kolodkin et al., 1992). Two Semaphorin genes have been identified in Drosophila (Kolodkin et al., 1993). D-Sema1 encodes a transmembrane protein that does not participate in homophilic binding in vitro (Kolodkin et al., 1993), whereas the proteins encoded by D-Sema2, H-SemaIII, and G-Sema1 are secreted (Kolodkin et al., 1993). D-Sema1 and D-Sema2 are expressed by subsets of neurons and muscles (Kolodkin et al., 1993), and D-Sema2 is essential (Kolodkin et al., 1993).

Calcium is Involved in Axon Growth and Guidance

Many molecules which affect the navigation of extending growth cones have been identified, but the signalling mechanisms that they use are still largely unknown. However, a unifying theme in the signal transduction pathways used by the various guidance molecules studied thus far, is that a rise in intracellular calcium either through release from intracellular stores, or influx through L- and N-type calcium channels is essential.

Studies of growth cone behavior suggest that an optimal range of intracellular Ca$^{2+}$ between 100-300nM is a basic element in the control of neuronal development, and that any
perturbation from this range results in cessation of growth cone motility (Kater and Mills, 1991). The control of intracellular Ca\(^{2+}\) levels may be an element of signal transduction used by both growth-promoting and inhibitory proteins (Gundersen and Barrett; 1980; Schuch et al., 1989, Doherty et al., 1991; Williams et al., 1992). For instance, neuronal differentiation of PC12 cells and neurite outgrowth from a variety of rat neurons cultured on either N-cadherin-, N-CAM-, or L1-expressing 3T3 monolayers, could be blocked by antibodies to the fibroblast growth factor receptor, by pertussis toxin, and by L- and N-type calcium channel blockers, suggesting that the fibroblast growth factor, a pertussis toxin-sensitive G protein, and calcium play essential roles in the signalling pathway involved in N-cadherin, N-CAM, and L1-mediated neuronal differentiation (Schuch et al., 1989; Doherty et al., 1991; Williams et al., 1992; Doherty et al., 1994a and b; Doherty et al., 1995). In addition, when rat dorsal root ganglion neurons were treated with NI-35-containing liposomes, a rapid transient rise in intracellular calcium in the growth cones and neurites preceded growth cone collapse (Schmidt et al., 1991; Bandtlow et al., 1992), and calcium channel blockers and pertussis toxin prevented this collapse, suggesting that as in the cell adhesion molecules N-cadherin, N-CAM, and L1, a pertussis toxin-sensitive G protein and calcium may be involved in the regulation of NI-35-induced collapse. Furthermore, increases in intracellular Ca\(^{2+}\) levels may be necessary for the turning behaviour of growth cones (Gunderson and Barrett; 1980; McCaig; 1989), and there is also evidence that it may be essential for fasciculation of neurites since clamping intracellular calcium at 50-75nM in rat sympathetic neurons prevented their fasciculation in vitro (Tolkovsky et al., 1990).

Regulation of the stability of the cytoskeleton may be the major function of calcium in growth cones. The neurite retraction caused by calcium-ionophore A23187-induced increases in intracellular calcium levels in cultured chick dorsal root ganglion neurons could be blocked or reversed by phalloidin, a drug that stabilizes actin filaments, or taxol, a drug that stabilizes microtubules (Lankford and Letourneau, 1989). In addition, collapsin has been shown to cause a reduction in F-actin in the leading edge of growth cones in vitro (Luo et al., 1993). Finally, many of the growth promoting molecules are associated with cytoskeletal components (Gumbiner et al., 1993).

This calcium signal may be partly transduced into cytoskeletal rearrangements by EF-hand-containing calcium-binding proteins (Kretsinger, 1975). In fact, many of these proteins have been shown to interact with the cytoskeleton in a calcium-dependent manner. In addition, a few have been proposed to function in neurite outgrowth and the signal transduction events associated with vision and olfaction.
EF-Hand Calcium-Binding Proteins and Signal Transduction in the Nervous System

Parvalbumin was the first EF-hand family member to be crystalized (Moews and Kretsinger, 1975) (Figure 2). It has three calcium-binding domains which were designated, from the N- to the C-terminus, AB, CD, and EF (Moews and Kretsinger, 1975). Thus, the EF-hand family of calcium-binding proteins was named after the C-terminal calcium-binding domain of parvalbumin (Moews and Kretsinger, 1975). The members of this family are intracellular proteins that reversibly bind Ca²⁺ ions, and thereby modulate the actions of other proteins or enzymes (Persechini et al., 1989). EF-hand family members have a common 29 amino acid calcium-binding motif which consists of two α-helices (E and F) that flank a calcium-binding loop of 12 residues (Persechini et al., 1989) (Figure 1). The functionally

Figure 1. The canonical EF-hand domain consists of 29 amino acids in a helix-loop-helix structure. The first α-helix consists of amino acids 1-11, followed by the calcium-binding loop at positions 10-21, and the second α-helix extends from 19-29. If bound to calcium, the calcium ion is coordinated by six amino acids. The residues designated X, Y, Z, -X, and -Z are usually Asp (D), Asn (N), Ser (S), Thr (T), Glu (E), or Gln (Q) because these amino acids have oxygen-containing side chains. The oxygen at position -Y comes from the main chain. There is usually an Asp at position 10, a Glu at position 21, and Gly at position 15 to permit the bend in the calcium-binding loop. The amino acids designated by n's usually have hydrophobic side chains (I, L, V, F, W, C, or M), and along with the residue at position 17, interact with the homologous residues on another EF-hand to form a stable hydrophobic core. Modified from Montcrief et al., 1990.

Important amino acids have been inferred from the crystal structures of parvalbumin (Moews and Kretsinger, 1975), ICaBP (Szebenyi et al., 1988), troponin C (Herzberg and James, 1985), and calmodulin (Babu et al., 1985; Strynadka and James, 1989) (Figure 2). The two α-helices often have glutamate at positions 1 and 21, and hydrophobic amino acids at...
positions 2, 5, 6, 9, 22, 25, and 26 (Persechini et al., 1989) (Figure 1). Calcium is
coordinated by the side chains of five amino acids X, Y, Z, -X, and -Z (Persechini et al., 1989) (positions 10, 12, 14, 18, and 21 in Figure 1). The aspartate at position 10 and the
 glutamate at position 21 are nearly invariant, and the other calcium coordinating positions are
usually occupied by aspartate, asparagine, serine, threonine, glutamine, or glutamate
(Persechini et al., 1989) (Figure 1). A peptide carbonyl atom coordinates calcium at -Y
(position 16), so nearly any amino acid can be found in this position (Persechini et al., 1989)

![Figure 2. Stereoscopic views of the crystal structures of (A) turkey skeletal muscle troponin
C, (B) bovine brain calmodulin, (C) carp parvalbumin, and (D) bovine ICaBP. Troponin C
and calmodulin have a similar dumb-bell shaped structure with each pair of EF-hands
connected by a central α-helix. In contrast, the structures of parvalbumin and ICaBP are
globular. Modified from Strynadka and James, 1989.](image)

(Figure 1). Finally, glycine is usually found at position 15 to permit the bend in the calcium-
binding loop (Persechini et al., 1989) (Figure 1).

Members of the EF-hand family of calcium-binding proteins have from one to eight
EF-hand domains, although not all of the domains necessarily bind calcium (Persechini et al.,
1989). Functional EF-hand domains are usually found in pairs connected by a flexible α-helical linker region (Persechini and Kretsinger, 1989; Moncrief et al., 1990) (Figure 2). This linker region allows the hydrophobic residues of the EF-hand pairs to enfold an α-helix of its target (Persechini et al., 1989; Strynadka and James, 1989) (Figure 3). When calcium binds to the EF-hand domains of calmodulin, the hydrophobic residues in these domains are exposed, and the two paired EF-hand domains from each end of the molecule embrace the α-helical target peptide, thereby stabilizing the structure (Torok and Whitaker, 1994) (Figure 3).

Figure 3. Calmodulin embracing skeletal myosin light chain kinase (MLCK) as determined by using heteronuclear multidimensional NMR in solution. (A) a ribbon diagram depicting the calcium-bound calmodulin before interacting with MLCK. The *TA is the TA fluorophore attached to the α-helix in the unbound form, and in a loop region when calmodulin is bound to the MLCK peptide target. (B) the structure of the calcium-calmodulin bound to the peptide calmodulin-binding domain of MLCK. Modified from Torok and Whitaker, 1994.

To date, more than thirty subfamilies encompassing over 230 proteins have been analyzed (Moncrief et al., 1990; Nakayama et al., 1992; Nakayama and Kretsinger, 1993; Berchtold, 1993). In the nervous system, members of the calmodulin, calpain, calcineurin B, S100, diacylglycerol kinase, glycerol-3-phosphate dehydrogenase, calbindin-D28k, parvalbumin, visinin, frequenin (Pongs et al., 1993), and rdgC subfamilies have been
identified (Baimbridge et al., 1989; Moncrief et al., 1990; Nakayama et al., 1992). Members of the calmodulin, calpain, calcineurin, S100, diacylglycerol kinase, and glycerol-3-phosphate dehydrogenase subfamilies are ubiquitous in the nervous system. The remaining proteins are present in subsets of neurons in the peripheral and central nervous systems, but the physiological role of many of these calcium-binding proteins in the nervous system is unknown.

Calmodulin has been found in all cells of all eucaryotes studied thus far (Moncrief et al., 1990). It is encoded by a single gene in Dictyostelium, Chlamydomonas, Achlya, Saccharomyces, and Drosophila, whereas vertebrates have several copies as well as several pseudogenes (Moncrief et al., 1990; Nakayama et al., 1992; Nakayama and Kretsinger, 1993; Berchtold, 1993). There are three calmodulin genes in the mammals that have been studied, and in some cases these genes are differentially expressed in various tissues (Caceres et al., 1983). In the rat, CaMI has been proposed to be the housekeeping gene expressed in all cells, while CaMII, which is preferentially expressed in the brain, and CaMIII, which is present in both brain and skeletal muscle, have been proposed to be differentially regulated in response to external stimuli (Caceres et al., 1983). The calmodulins of nearly all vertebrates have identical amino acid sequences, but there is a greater range of sequences among the plants, protists, and fungi. This may be because calmodulin has fewer targets in these organisms. The calmodulin gene of S. cerevisae shares only about 60% identity with the vertebrate calmodulin sequences, and binds only three calcium ions (Davis et al., 1986; Luan et al., 1987). It is essential for S. cerevisae cell growth, yet when the wild-type yeast calmodulin gene was replaced with a mutated calmodulin that could no longer bind to calcium, the yeast were able to grow nearly normally at 30°C (Davis et al., 1986; Geiser et al., 1991). Therefore, at least in yeast, the calcium-binding ability of calmodulin is not essential for growth. In mammals, calmodulin activates at least 20 different enzymes or structural proteins (Moncrief et al., 1990; Nakayama et al., 1992; Nakayama and Kretsinger, 1993; Berchtold, 1993). The targets do not necessarily share any homology, but many contain cationic, amphipathic α-helices that have been demonstrated or inferred to be the site of calmodulin binding (Torok and Whitaker, 1994)(Figure 3). The linker region of the central helix allows the two lobes of calmodulin to enfold the target helix (Persechini and Kretsinger, 1988) (Figure 3). This linker region can undergo various deletions, insertions, and substitutions and still retain its ability to activate several of these targets (Haiech and Watterson, 1988)). Recent evidence has implicated calmodulin in signal transduction events in the growth cone. For example, when calmodulin function was blocked by an antagonist or
overexpressed in vivo in a subset of neurons in Drosophila, growth cones often stalled, defasciculated, and made errors in trajectory (VanBerkum et al., 1995).

Calpains are calcium-dependent sulfhydryl proteases with four EF-hand domains that are found ubiquitously in tissues of higher animals (Melloni and Pontremoli, 1989). They are composed of two subunits, an 80 kd catalytic subunit, and 28 kd regulatory subunit (Melloni and Pontremoli, 1989). Each subunit contains two EF-hand calcium-binding domains (Melloni and Pontremoli, 1989). Its function in the brain isn't known, but in human neutrophils it is translocated to the plasma membrane cytoskeleton in response to increases in intracellular calcium where it is activated, and this activation is associated with a reorganization of the cytoskeleton (Melloni and Pontremoli, 1989).

Calcineurin is a type 2B calcium and calmodulin-dependent protein phosphatase (Moncrief et al., 1990; Nakayama et al., 1992; Nakayama and Kretsinger, 1993). The 61 kd A subunit contains a catalytic domain in the N-terminus, and a regulatory domain which includes the calmodulin, and calcineurin B binding domains, as well as an inhibitory region (Rasmussen et al., 1990; Moncrief et al., 1990; Nakayama et al., 1992; Nakayama and Kretsinger, 1993). The 15 kd calcineurin B subunit is essential for calcineurin activation in all organisms studied except A. nidulans, it is N-myristilated and contains, four EF-hand calcium-binding domains (Moncrief et al., 1990; Nakayama et al., 1992; Nakayama and Kretsinger, 1993). Calcineurin dephosphorylates a large number of substrates in vitro, but endogenous substrates are probably much more limited (Cheung, 1986). There is a correlation between a significant increase in the concentration of calcineurin in the brain and the formation of synaptic membranes and densities (Cheung, 1986). In addition, chromophore-assisted laser inactivation of calcineurin in cultured chick dorsal root ganglion neurons suggest that the distribution of calcineurin activity may regulate growth cone motility (Chang et al., 1994), perhaps through the calmodulin and calcium-dependent inactivation of calcium channels (Kandel et al., 1991).

The S100 family of calcium-binding proteins includes two groups of proteins, the S100s and calpactins, that are expressed in the brain (Moncrief et al., 1990; Nakayama et al., 1992; Nakayama and Kretsinger, 1993). These proteins are small (9-11 kd), acidic proteins with two EF-hand calcium-binding domains (Donato, 1988). The first EF-hand domain is unique and characteristic of this family, because it has two extra amino acids, designated 12b and 16b (Donato, 1988).

The S100 dimers αα (αα), αβ (αβ), and ββ (ββ) have been identified in the nervous system of mammals, as well as in cardiac tissue and adipocytes, and both disulfide-bound
and noncovalently-associated dimers have been described (Donato, 1988). Each dimer binds four calcium ions with a moderate affinity (Kd ~ 3μM), but this affinity increases to a physiologically relevant range in the presence of lipid vesicles suggesting that these proteins are membrane-associated (Zolese et al., 1988). The α and β subunits are freely interchangeable, but the dimers seem to be differentially distributed in various tissues (Donato, 1988). For instance, S100a and S100b are found in glial cells, S100a is the predominant form expressed in neurons (Isobe et al., 1984), and rat brain S100 is primarily S100b. S100 binds to unassembled tubulin and to glial fibrillary acidic protein, the protein subunit of glial filaments, and inhibits their polymerization in a calcium-dependent manner (Donato, 1988; Bianchi et al., 1993). It also binds to the cytoskeletal-associated calpain I heavy and light chains and inhibits their phosphorylation by pp60^src and p130^p5 kinases (Hagiwara et al., 1988). In addition, the intracellular concentration of S100b was seen to increase four-fold during C6 glioma differentiation, and a disulfide-linked S100b was shown to promote neurite extension from embryonic chick cerebral cortical neurons *in vitro* (Kligman and Marshak, 1985). These results suggest that S100b may help mediate glial differentiation and neurite outgrowth *in vivo* (Kligman and Marshak, 1985; Donato, 1988).

Calpain is also a member of the S100 family of EF-hand calcium-binding proteins (Moncrief et al., 1990; Nakayama et al., 1992; Nakayama and Kretsinger, 1993). Calpain is composed of a heterotetramer of two heavy and two light chains (Moncrief et al., 1990; Nakayama et al., 1992; Nakayama and Kretsinger, 1993). It has been found in the brain, spleen, and thymus, and in high concentrations in the kidney, intestines, and lungs (Moncrief et al., 1990; Nakayama et al., 1992; Nakayama and Kretsinger, 1993). Both heavy and light chains bind calcium, but the heavy chain is not an EF-hand calcium-binding protein, and its calcium-binding site has not been identified (Moncrief et al., 1990; Nakayama et al., 1992; Nakayama and Kretsinger, 1993). The light chain is an EF-hand protein, but the EF-hands are degenerate and therefore it doesn't bind calcium with high affinity (Moncrief et al., 1990; Nakayama et al., 1992; Nakayama and Kretsinger, 1993). The calpain tetramer has been shown to interact with phospholipid, actin and nonerythroid spectrin in a calcium-dependent manner (Moncrief et al., 1990; Nakayama et al., 1992; Nakayama and Kretsinger, 1993).

Calbindin-D28k, and the very similar, calretinin are highly-conserved six-domain EF-hand proteins (Rogers, 1987; Gross et al., 1993). Four of the EF-hand domains (1, 3, 4, and 5) are predicted to bind calcium (Gross et al., 1993). These proteins are encoded by single genes that appear to have arisen by duplication and translocation (Berchtold, 1993). The synthesis of calbindin-D28k in avian intestines is dependent on vitamin D-derived hormones,
but brain calbindin-D28k is not regulated in this way (Moncrief et al., 1990). Calbindin-D28k and calretinin are abundant, have specific distributions to subsets of neurons within the peripheral and central nervous systems, but their functions are unknown (Baimbridge et al., 1992).

Parvalbumin is also limited to distinct subsets of neurons in the nervous system (Baimbridge et al., 1992). There are two types of parvalbumin, α and β, in vertebrates (Moncrief et al., 1990; Nakayama et al., 1992; Nakayama and Kretsinger, 1993). They are inferred to function as kinetic buffers of calcium in skeletal muscle by acting as carriers for calcium from troponin C to the sarcoplasmic reticulum, increasing the rate of muscle relaxation (Ushio et al., 1994). Recent evidence suggests that, in addition to acting as passive carriers of calcium, they may also activate calcium uptake by the sarcoplasmic reticulum by binding a 130 kd sarcoplasmic reticulum protein (Ushio et al., 1994). Parvalbumins are also present in brain, bone and several endocrine tissues in mammals; however, no function has been inferred for parvalbumin in these tissues (Epstein et al., 1986). The α and β parvalbumins are encoded by single genes, but seem to be differentially regulated in mammalian skeletal muscle and brain (Berchtold, 1993). Muscle parvalbumin mRNA increases twenty-fold from day 4 to day 20 postnatal, while brain parvalbumin stays constant during this period (Epstein et al., 1985).

The visinin subfamily of EF-hand calcium-binding proteins includes the visinins (Yamagata et al., 1990), recoverins (Dizhoor et al., 1991), GCAPs (Palczewski et al., 1994), Vilip (Braunewell et al., 1994), and RD25 (Nemoto et al., 1993). These are small calcium-binding proteins that have three EF-hand domains that may function in visual and odorant signal transduction (Yamagata et al., 1990; Dizhoor et al., 1991; Nemoto et al., 1993; Braunewell et al., 1994; Palczewski et al., 1994). Visinin is an avian retinal cone-specific protein suggested to be involved in phototransduction in the cone cells (Yamagata et al., 1990). In the bovine retina a guanylate cyclase is activated by apo-recoverin, thereby resynthesizing cyclic GMP which reopens cation channels that have been closed by photoexcitation-induced hydrolysis of cGMP (Dizhoor et al., 1991). This is the first example of the apo-protein being the activating form.

Another EF-hand calcium-binding protein present in the nervous system is frequenin (Pongs et al., 1993). This protein has four EF-hand domains, and is thought to facilitate synaptic transmission in Drosophila. Finally, the rdgC gene in Drosophila is necessary for the prevention of light-induced retinal degeneration, and has been found to encode a
serine/threonine protein phosphatase with five EF-hand Ca\(^{2+}\)-binding domains (Steele et al., 1992).

EF-hand family members are functionally grouped into two classes. Proteins which interact with and regulate other proteins upon calcium binding, such as calmodulin, are referred to as trigger proteins (Levine and Dalgarno, 1983; Baimbridge et al., 1992). On the other hand, the buffer proteins, such as ICaBP, have no identifiable targets, and are thought to maintain calcium-homeostasis within the cell by controlling the level of free calcium (Levine and Dalgarno, 1983; Baimbridge et al, 1992; Heizmann and Braun, 1992).

These proteins have also been implicated in various neurodegenerative disorders (Heizmann and Braun, 1992). For instance, populations of hippocampal neurons that express a high level of parvalbumin and calbindin-D28k seem to be relatively resistant to damage induced by seizures, suggesting that these calcium-binding proteins could perform a protective role in these neurons. (Heizmann and Braun, 1992). In contrast, the gene which encodes S100b is located on chromosome 21 in humans, so patients with Downs syndrome (trisomy 21) overexpress S100b. The levels of S100 during development are regulated by nerve growth factor, so the overexpression of S100 could disrupt its regulation resulting in aberrant development (Heizmann and Braun, 1992).

The Leech Nervous System is a Model System for Studying Axonal Guidance

To further dissect the signalling 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 outgrowth and fascicle selection of the peripheral sensory neurons.

The leech has a very simple segmentally-repeated nervous system that is reasonably accessible to perturbation throughout development (Muller et al., 1981; Jellies and Kristan, 1988; Zipser et al., 1989). Another 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), so that the molecules involved in axon guidance and pathway selection would be expected to persist throughout the life of the leech.

The central nervous system consists of a head brain (four fused ganglia), a tail brain (7 fused ganglia), and 21 segmental body ganglia which contain approximately 400 bilaterally symmetric neurons (Muller et al., 1981; Sawyer, 1986) (Figure 4). Most central neurons are unipolar, extend their process into, and make synaptic connections within, the
central neuropile (Sawyer, 1986). Many of the individual central neurons can be identified by their size, shape, ganglionic position, electrical properties, synaptic connections, and function (Sawyer, 1986). Six glial packets, each formed by a single large glial cell, ensheath the central neurons (Sawyer, 1986). This ensheathment takes place after the formation of the connectives (Sawyer, 1986). The body ganglia are joined together by connectives which contain approximately 2600-3200 axons that arise from both central and peripheral neurons, and are divided into two lateral and one smaller medial axon bundles (Muller et al., 1981; Sawyer, 1986) (Figure 4). The peripheral sensory neurons extend their axons into the CNS through the four large nerve roots at each body ganglia (Muller et al., 1981) (Figure 5). These roots are further divided into the anterior-anterior nerve, the medial-anterior nerve, the posterior-posterior nerve, and the dorsal-posterior nerve (Sawyer, 1986). Each nerve root of *Hirudo medicinalis* was found to contain an average of 2351 axons with most travelling in fascicles from the periphery (Sawyer, 1986). The dorsal-posterior nerve appears to be pioneered by the ipsilateral dorsal P cell (Johansen et al., 1994; Jellies et al., 1994), and the medial-anterior nerve may be pioneered by both the ipsilateral ventral P cell and the sensory neurons located in the S3 sensillum (Jellies et al., 1994). The origins of the anterior-anterior and posterior-posterior nerves are not yet known.

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**Figure 4.** Body plan of the leech *Hirudo medicinalis*. (Above) the segments VII-XXVII which contain the 21 mid-body ganglia. The ganglia are located in the center of each segment. (Below) dissection of the leech *Hirudo medicinalis* showing the ventral nerve cord. Adapted from Sawyer, 1986.
The tegument of *Hirudo medicinalis* contains specialized neuroepithelial sensory cells (Sawyer, 1986). There are cells which contain intracellular light-sensitive membranes that act as photoreceptors, and bipolar cells that have apical cilia and function in chemoreception and detection of water movement (Sawyer, 1986). The majority of the photoreceptive cells are located in five pairs of eyes in segments II-VI (Sawyer, 1986).

Another tegumentary sense organ, composed of aggregations of sensory cells, are the sensillae (Sawyer, 1986). They have very stereotypical positions on the central annulus of each of the body segments and are composed primarily of ciliated sensory cells that function in the detection of nearby water movements (Sawyer, 1986) (Figure 6). They also contain relatively few photoreceptive cells that are identical to those of the leech eye (Sawyer, 1986).

The development of the central and peripheral nervous systems occurs at E7-8 stage,

Figure 5. A cross-section through the leech *Hirudo medicinalis*. This figure shows the ventral nerve cord in the ventral haemocoelomic channel (VHC). The nerve cord consists of ganglia (G) linked together by connectives (C) with the large nerve roots leaving each hemisegment for the periphery. The nerve roots shown in this diagram are the anterior nerve root (AN), the posterior-posterior nerve (PN), and the dorsal-posterior nerve (DN). The four major types of muscle are shown: circular (CM), diagonal (DM), dorso-ventro (DVM), and longitudinal (LM). Other structures include: Annulus (A), crop caecum (CC), crop (CR), cuticle (CU), epidermis (E), dorsal haemocoelomic channel (DHC), and the lateral haemocoelomic channel (LHC). Taken from Nicholls and Van Essen, 1974.
and proceeds in a rostral-caudal progression (Stent et al., 1982). Each posterior segment is approximately 2.5 hrs. later in development than the preceding anterior segment, so that each leech embryo provides a 2-3 day profile of development (Stent et al., 1982). The first peripheral neurons to differentiate are the sensillar neurons (Johansen et al., 1992). The sensillae are designated S1-S7, with S1 being the most ventral in each hemisegment (Sawyer, 1986) (Figure 6). The afferents from S1-S5 enter the central nervous system through the medial-anterior nerve (Sawyer, 1986; Briggs et al., 1993), while the afferents from S6-S7

Figure 6. The sensory sensilla of one hemisegment. There are seven groups of sensory neurons that make up the S1-S7 sensilla. The afferents from S1-S5 enter the ganglionic central nervous system through the medial-anterior nerve, while the S6 and S7 afferents enter through the dorsal-posterior nerve. When the axons from the sensillar neurons reach the ganglion, they bifurcate, and segregate into initially three, and later four axon tracts. This figure was taken from Johansen et al., 1994.
enter the CNS through the dorsal-posterior nerve (Sawyer, 1986; Briggs et al., 1993) (Figure 6). The first sensillar neurons to differentiate are located in the S3 sensillum at E7-8, followed by the sensory neurons in S6 and S7 (Johansen et al., 1992; Briggs et al., 1993). Then the neurons in sensillae S1, S2, S4 and S5 differentiate (Johansen et al., 1992; Briggs et al., 1993). When the growth cones of the sensillar neurons reach the ganglionic CNS, they bifurcate into rostral and caudal branches, and extend along one of the four fascicles formed by the peripheral sensory neurons in each of the lateral connectives (Johansen et al., 1992; Briggs et al., 1993; Johansen et al., 1994; Jellies et al., 1994) (Figure 6). The sensory neurons that make up each sensillum appear to be functionally heterogeneous, since each sensillum contains neurons whose axons travel along each of the four different central fascicles formed by the peripheral sensory neurons (Jellies et al., 1994). Once the fascicles have formed in the lateral connective, they are ensheathed by a single glial cell in each of the connectives (Sawyer, 1986). Another population of extrasensillar peripheral neurons differentiate around E16-E25 (Johansen et al., 1992; Briggs et al., 1993). The growth cones of these neurons enter the central nervous system and fasciculate with the four fascicles formed earlier by the sensillar neurons (Johansen et al., 1992; Briggs et al., 1993) (Figure 6).

In an effort to identify molecular markers which may be used to generate the precise connections between neurons, a large number of nervous system-specific monoclonal antibodies were generated against adult *Haemopis marmorata* nervous system proteins (Zipser and McKay, 1981). About 40 were found to define small groups of neurons and/or subsets of axons in the nervous system (Zipser and McKay, 1981). For the present study, discussion will be limited to three of these monoclonal antibodies lan 3-2, lan 4-2, and lan 3-6 that have been proposed to be involved in the formation of specific axon tracts within the leech central nervous system (Zipser and McKay, 1981; Johansen et al., 1992; Briggs et al., 1993).

**Putative Guidance Mechanisms in the Leech**

The segregation of the peripheral sensory sensillar neurons into four distinct fascicles within the leech central nervous system (Johansen et al., 1992), and experiments that showed that following nerve root crushing, regenerating peripheral axons were able to reestablish their correct axon pathways, suggested that precise molecular guidance cues are used by the developing leech nervous system (Peinado et al., 1987). There is strong evidence for the presence of factors which may mediate axon guidance and fascicle formation by differential adhesion (Johansen et al., 1992; Jellies and Johansen, 1994). In addition, a specific cell has
been identified that is necessary for the formation of the sex nerve that projects from the anterior root of ganglion VI (Jellies and Kristan, 1988).

The antigen recognized by the Ian 3-2 monoclonal antibody is present in all of the peripheral neurons, and the four axon tracts formed by their afferents (Johansen et al., 1992). Fab fragments of the Ian 3-2 monoclonal antibody were able to perturb this tract formation in cultured leech germinal plates (Zipser et al., 1989), therefore it was hypothesized to be involved in this process in vivo. In support of this hypothesis, partial purification of this antigen, followed by peptide sequencing, has revealed that it is likely to be a member of the immunoglobulin superfamily (Huang, unpublished). These data strongly suggest that the Ian 3-2 antigen may be an adhesion molecule involved in fascicle formation in the leech.

Another proposed fasciculation antigen is recognized by the Ian 4-2 monoclonal antibody (Zipser and McKay, 1981; Johansen et al., 1992). This antigen is present in a subpopulation of the peripheral neurons whose axons fasciculate into the most lateral of the four axon tracts (Johansen et al., 1992). In addition, it was found to be immunologically related to the Ian 3-2 antigen (Johansen et al., 1992). It has been proposed that either the two antigens are coimmunoprecipitated because they interact in vivo, or the peripheral neurons share a common protein core with a subset expressing both the Ian 4-2 and Ian 3-2 epitopes (Johansen et al., 1992).

More definitive evidence for the existence of a hierarchy of neuronal guidance molecules came from experiments involving surgical removal of part of the developing leech central nervous system (Johansen et al., 1994). When part of the central nervous system of a leech embryo was removed, the growth cones of the peripheral neurons failed to migrate to the location of the central nervous system and form the four distinct axon fascicles. Instead they fasciculated together and migrated to the nephridiopore where they formed circular knots of axons (Johansen et al., 1994). The guidance cues used by the growth cones of peripheral neurons for segregation into the four stereotypical axon tracts are probably provided by the central nervous system, since the growth cones of the rostral and caudal projections of the peripheral neurons meet in the connectives in register as if they were following a preformed tract (Briggs et al., 1993; Johansen et al., 1994) (See Figure 9). These results taken together indicate that all of the peripheral neurons express a molecule, which when the central nervous system-derived cues are removed, will allow the neurons to fasciculate to one another, and migrate to the nephridiopore in each segment (Briggs et al., 1993; Johansen et al., 1994). In addition, subsets of peripheral neurons may express molecules that can interact with the central nervous system-derived cues to mediate the
selective fasciculation into four separate tracts in the central nervous system (Johansen et al., 1994).

An important potential candidate for mediating fascicle selection and/or maintenance is the antigen recognized by the lan 3-6 monoclonal antibody. This antigen has been proposed to mediate selective fasciculation due to its restricted expression to a subset of peripheral neurons that selectively fasciculate into a single tract within the leech central nervous system (Briggs et al., 1993).

The Lan 3-6 Antigen

The lan 3-6 antigen was first identified by an antibody raised against antigens from the *Haemopis* central nervous system (Zipser and McKay, 1981). In this leech species, the antibody labels a small subset of peripheral neurons whose axons selectively fasciculate into the central axon tract formed by the peripheral neurons in the connective (Figure 7). The first lan 3-6 labeled neurons to differentiate are in the S3 sensillum (Briggs et al., 1993) (Figure 8A and B). The growth cones from these neurons extend rostrally and caudally into the lateral connectives, where they meet perfectly without any realignment (Briggs et al., 1993) (Figure 9A and B). This suggests that the pathway followed by these growth cones was pioneered previously perhaps by central neurons (Briggs et al., 1993). This very restricted distribution to a small population of peripheral neurons who select and fasciculate with a single axon tract within the central nervous system suggests that this antigen may be involved in this process and therefore warranted further study (Briggs et al., 1993). However, the lan 3-6 monoclonal antibody does not recognize denatured antigen on Western blots, making its biochemical analysis difficult, and the epitope recognized by the antibody is intracellular, since nonpermeabilized ganglia and connective are not labeled by the antibody (J. Johansen, unpublished observation), therefore antibody perturbation experiments are not possible. In order to facilitate the molecular and early biochemical characterization of the lan 3-6 antigen, the lan 3-6 monoclonal antibody was used to isolate the sequence encoding the lan 3-6 antigen.
Figure 7. The Ian 3-6 monoclonal antibody labels a single axon tract in each hemisegment. (A) A ganglion from an E11 embryo labeled by the Ian 3-6 monoclonal antibody. The labeled axons form two bilaterally symmetric fascicles in each ganglion. Scale bar, 25 μm. (B) A cross section of a ganglion labeled with the Ian 3-6 monoclonal antibody. The arrows point to the single Ian 3-6-positive tract in the neuropil (np) of each hemisegment. Cross sections of neuronal somata are labeled n. Scale bars, 10 μm. From Briggs et al., 1993.
MATERIALS AND METHODS

Experimental Preparations

Leech species

For the present experiments we used two different leech species, namely the hirudinid leeches *Haemopis marmorata* and *Macrobdella decora*. The leeches were either captured in the wild or purchased from commercial sources.

Dissections

Dissections of nervous tissue and embryos were performed in leech saline solutions with the following composition (in nM): 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 muscle contractions.

Embryos

*Macrobdella* and *Haemopis* embryos were obtained from leeches captured gravid in the wild. The leeches were placed in boxes with moist peat moss in which they lay their cocoons. Cocoons were maintained at 24°C and embryos were staged according to the criteria described by Fernandez and Stent (1982). There are about 10-20 embryos in each cocoon and these sibling embryos develop synchronously within a few percent of development.

Immunocytochemistry

The monoclonal antibody (mAb) lan 3-6 which is of the IgG2a subtype (Zipser and McKay, 1981; Briggs et al., 1993) or newly raised polyclonal antisera (see below) were used in these studies. The lan 3-6 antibody was raised against *Haemopis* central nervous system (CNS) but cross-reacts with *Haemopis, Macrobdella*, and *Hirudo* central nervous system (Briggs et al., 1993).

Dissected *Macrobdella* and *Haemopis* embryos were fixed overnight at 4°C in 4% paraformaldehyde in 0.1M phosphate buffer, pH 7.4, incubated overnight at room temperature directly in hybridoma supernatant containing 0.4% Triton X-100 or in polyclonal antisera diluted 1:800 in PBS with 0.4% Triton X-100, washed in PBS with 0.4% Triton X-100, and incubated with HRP-conjugated goat anti-mouse or anti-rabbit antibody (Bio-Rad, 1:200 dilution). After washing in PBS the HRP-conjugated antibody complex was visualized...
by reaction in DAB (0.03%) and H₂O₂ (0.01%) for 10 min. The final preparations were dehydrated in alcohol, cleared in xylene, and embedded as whole-mounts in Depex mountant.

**Identification and Cloning of the Calsensin Sequence**

The mAb Ian 3-6 was used to probe an oligo dT-primed λgtlId cDNA library prepared from size-selected (>400nt) *Haemopis marmorata* mRNA. The library was screened essentially according to the procedures of Sambrook et al (1989). A total of 10⁶ plaques were screened at a density of 30,000 plaque forming units/150mm plate. One cDNA clone was identified as positive after a second round of antibody screening. The phage DNA from this positive cDNA clone was isolated and subcloned into pBluescript II KS +/- vectors (Stratagene) using standard protocols (Sambrook et al, 1989).

For further screening of phage libraries, a 238 nucleotide N-terminal EcoRI fragment from the isolated calsensin cDNA clone was gene-cleaned (Bio 101), and ³²P-radiolabeled using random priming according to the manufacturer's procedure (Prime-A-Gene kit, Promega). This radiolabeled fragment was used to probe a second oligo dT-primed λgtlId cDNA library prepared from size-selected (>400 nt) *Haemopis marmorata* mRNA as well as a *Haemopis* genomic library. The libraries were screened using standard procedures (Sambrook et al, 1989). Two independent cDNA clones and one genomic clone were isolated and subcloned into pBluescript II KS +/- vectors (Stratagene). Additional N-terminal sequence was obtained using a Clontech 5'-AmpIiFINDER RACE kit and protocols. The RACE products were subcloned into pBluescript II KS+/- vectors using standard protocols (Sambrook et al, 1989).

**Nucleotide Sequencing and Computer Analysis**

Nucleotide sequencing of isolated single- or double-strand templates was either conducted at the Iowa State University Nucleic Acid Facility using an Applied Biosystems 373A DNA Sequencer or performed manually by the dideoxynucleotide sequencing procedure using the Sequenase kit (USBiochemicals) and ³⁵S-dATP (Amersham) in the termination reactions. Templates were primed with either commercially available sequencing primers (M13 universal, reverse, T7, and T3 primers; Promega and USBiochemicals) or by specific primers synthesized at the Iowa State University Nucleic Acid Facility based on internal predicted calsensin sequence. DNA extension reactions were resolved on 6% gradient gels and analyzed using autoradiography.
The nucleotide and predicted amino acid sequences were analyzed using the GCG (Genetics Computer Groups Program Manual for the GCG Package, Version 7, April 1991, 575 Science Drive, Madison, Wisconsin, USA 53711) suite of programs (Devereux et al, 1984). The calsensin sequence was compared with known and predicted proteins in the SwissProt and Genbank databases using the FASTA and TFASTA programs within the GCG package. In addition, a BLAST search was performed using the NCBI BLAST e-mail server (Altschul et al, 1990) comparing the Calsensin sequence with SwissProt, PIR, and GenPept databases. Finally, the program TopPredII was used to predict the topography of the calsensin protein (Claros and Heijne, 1994).

**Northern and Southern Analysis**

Total leech RNA was isolated using a modification of a plant genomic DNA prep (Shure et al, 1983) as follows: 3-4g of leech was ground in a coffee grinder with dry ice to a fine powder. Leech powder was mixed with 6ml urea extraction buffer (8M urea/0.35M NaCl/0.05M Tris-HCl (pH 7.5)/0.02M EDTA/2% sarcosyl) and 6ml of phenol:chloroform (1:1) was added. The mixture was vigorously shaken, incubated 15 minutes at room temperature, and shaken vigorously again prior to low-speed centrifugation. the aqueous phase was extracted, and precipitated with 0.17 vol 4.4M ammonium acetate, pH 5.2 and 1 volume isopropanol, mixed well, and centrifuged at 10K for 10 minutes at 4°C in an SS34 rotor (Sorvall). The pellet was dissolved in 500ml 10mM Tris/1mM EDTA, pH 8.0, phenol:chloroform extracted, re-precipitated in isopropanol as above, and the nucleic acid pellet washed with 70% ethanol. For the Northern blots, nucleic acids were dissolved in oligo-dT binding buffer, and polyA+ mRNA was isolated using an oligo-dT column (Stratagene) according to standard protocols (Sambrook et al, 1989). 20μg of polyA+ mRNA was fractionated on 1.2% agarose formaldehyde gels, transferred to nitrocellulose, and hybridized to a [32P]dCTP-labeled 238 nucleotide EcoRI N-terminal calsensin fragment according to standard methods (Ausubel et al, 1987) with the addition of dextran sulfate (10%). For the Southern blots, the nucleic acids were dissolved in ddH2O, treated with RNase H, and 10 μg were digested with EcoRI, EcoRI/HindIII, and EcoRI/Saci using standard conditions. The DNA was fractionated on a 0.5% agarose/TBE gel, transferred to nitrocellulose, and hybridized to a [32P]dATP-labeled 238 nucleotide EcoRI N-terminal calsensin fragment according to standard methods (Sambrook et al., 1989). High stringency hybridization and washing conditions were employed (Ausubel et al, 1987), and the filters were exposed to X-OMAT XAR film (Kodak) at -80°C with an intensifying screen.
In situ Hybridization

The N-terminal 238 nucleotide EcoRI calsensin fragment was linearized with BamHI or HindIII using standard procedures (Sambrook et al., 1989). These linearized DNA molecules were used to generate sense- and antisense digoxygenin-labeled RNA probes using a Boehringer Mannheim RNA labeling kit and protocols. The antisense probe was generated using T7 RNA polymerase on the BamHI-linearized DNA. The sense probe was generated using T3 RNA polymerase on the HindIII-linearized DNA. The method of Tautz and Pfeifle (1989) was used to hybridize, wash, and stain Haemopis E10 embryos which were previously fixed in 4% paraformaldehyde in 0.1M phosphate buffer, pH 7.4. However, the probe concentration was reduced to 0.1µg/ml, hybridizations were at 45°C, and the antidigoxygenin antibody was used at a concentration of 1:3000. After labeling the embryos were dehydrated in ethanol, cleared in xylene, and mounted in Depex.

Preparation of Calsensin Fusion Protein

The N-terminal 238 nucleotide EcoRI fragment from one of the calsensin cDNA clones was subcloned into the Promega Pinpoint Xa2 vector as a HindIII-BamHI cassette. The resulting fusion protein codes for residues 1-72 of calsensin which encompasses both calcium-binding loops but truncates the extreme C-terminal end. In addition, the fusion protein contains 10 residues of sequence upstream of the starting methionine and 23 residues of C-terminal translated vector sequence. The fusion protein was expressed in DH5αF'IQ cells and partially purified over a Soft-link™ monomeric avidin column (Promega) using the manufacturer's recommended protocols.

Generation of Calsensin Antisera

The partially-purified calsensin fusion protein was used to generate polyclonal antibodies in rabbits. Two rabbits (Frigg and Hel) were injected with from 200µg to 400µg of fusion protein, and then boosted at 21 day intervals as described in Harlow and Lane (1988). After the second boost serum samples were collected seven and ten days after injection (Harlow and Lane, 1988). The sera was analyzed for specificity by comparing the staining obtained with the antisera and the preimmune sera on nitrocellulose filters spotted with nerve cord homogenates from Haemopis, partially-purified fusion protein, and the expressed Xa2 vector. The sera was titrated from undiluted to a 1:5000 dilution in Blotto (0.5% Carnation non-fat dry milk in TBS).
Biochemical Analysis

SDS-PAGE and Western blotting

SDS-PAGE was performed according to standard procedures (Laemmli, 1970) except that acrylamide in some experiments was used at 12% and bis-acrylamide at 0.13%. Electroblot transfer was performed as in Towbin et al. (1979). For these experiments we used the Bio-Rad mini-gel system, electroblotting to 0.2μm nitrocellulose, and using HRP-conjugated secondary antibody (1:3000) for visualization of primary antibody diluted 1:2000 in Blotto in immunoblot analysis. The signal was developed with 3,3' diaminobenzidine (0.1mg/ml) and H2O2 (0.03%) and enhanced with 0.008% NiCl2. In some experiments to test for the specificity of the polyclonal antiserum, 15μg of the calponsin fusion protein was added to the diluted antisera before staining. Gels were fixed and silver stained using the Bio-Rad Silver Stain Kit as per the manufacturer's instructions.

Subcellular fractionation

The proteins from 87 Haemopis and 101 Macrobdella nerve cords were homogenized in a buffer containing 250mM sucrose, 5mM Tris-Cl pH 7.5, and protease inhibitors. The subcellular fractionation was performed according to Rickwood, 1984. The homogenate was centrifuged at 1000g, 4°C for 10 minutes to remove nuclei, plasma membrane sheets, heavy mitochondria, and unbroken cells. The resulting supernatant fraction (S1) was centrifuged at 3000g, 4°C, for 10 minutes to remove heavy mitochondria and plasma membrane fragments. The subsequent supernatant (S2) was centrifuged 10,000g, 4°C, for 20 minutes to remove mitochondria, lysosomes, peroxisomes, golgi, and some rough endoplasmic reticulum. Finally, the resulting supernatant (S3) was centrifuged at 100,000g, 4°C, 1 hr to separate the pelleted fraction containing membrane vesicles from the endoplasmic reticulum, golgi, and plasma membrane, from the supernatant containing the soluble components of the cytoplasm. The P1 fraction was rehomogenized and centrifuged as above. The fractions were analyzed by SDS-PAGE and Western blotting as described previously.

Immunoaffinity purification

Immunoprecipitations were performed by incubating Protein A-Sepharose CL-4B beads which were covalently-crosslinked (see below) to the lan 3-6 monoclonal antibody with Haemopis nerve cord homogenates at 4°C overnight on a rotator after preincubation and preclearing of the homogenate with the uncoupled beads. Protein from dissected nerve cords were homogenized in extraction buffer (20mM Tris-HCl, 200mM NaCl, 1mM CaCl2, 1mM
MgCl$_2$, 0.2% NP-40, 0.2% Triton X-100, pH 7.4) containing protease inhibitors and the resulting homogenate cleared by brief centrifugation. The immunoprecipitates were washed 3 times in TBS, and once in PBS before SDS-PAGE and Western blotting.

To assay for proteins interacting with calsensin, *Haemopis* and *Macrobdda* nerve cords were homogenized in extraction buffer and homogenates precleared by incubation with 10mg Protein A-Sepharose CL-4B beads (Sigma) for 2 h at 4°C on a shaking platform. After preclearing the homogenates were incubated with 500mg Protein A-Sepharose CL-4B beads which were crosslinked to the lan 3-6 monoclonal antibody following the procedures of Harlow and Lane (1988). The only alteration to these protocols was the 0.1M sodium borate, pH 8.2 was used for washing instead of 0.2M sodium borate, pH 9. Following incubation and washing the beads were resuspended in 4ml of 0.1M sodium borate, pH 8.2 and packed into a column. The column was washed with 20 volumes of 0.1M sodium borate, pH 8.2 and by 10 volumes of 10mM sodium phosphate, pH 6.8, before elution with one volume of 0.1M glycine, pH 2.5. Ten 0.4ml fractions of the eluate were collected into tubes containing 80ml of 1M sodium phosphate, pH 8 for neutralization. Fractions 1-2, 3-5, and 6-10 were pooled, dialyzed against 10mM Tris-HCl, pH 7, and precipitated with absolute ethanol. The resulting protein pellets were resuspended in 20ml homogenization buffer and analyzed by SDS-PAGE and silver staining of the gel.

In addition, these experiments were repeated using *Haemopis* proteins from 300 nerve cords that had been precleared through an anti-IgG column, before preclearing with protein A. An additional wash with 5 column volumes of 0.5M NaCl, 50mM Tris-Cl, pH 8 was added before the elution with 0.1M glycine, pH 2.5. The fractions corresponding to the eluted peaks were collected, tested for immunoreactivity with the lan 3-6 monoclonal antibody, pooled, dialyzed against PBS with protease inhibitors, and concentrated using centricon-3 concentrators (Amicon).

**Calcium-Binding Assays**

The binding of $^{45}$Ca$^{2+}$ to the calsensin fusion protein was assayed as described by Maruyama et al (1984). The partially-purified calsensin fusion protein was resolved on a 15% SDS-polyacrylamide gel, and transferred to 0.2μm nitrocellulose. The membrane was washed 2 x 10 minutes in a buffer containing 60mM KCl, 5mM MgCl$_2$, and 10mM imidazole-HCl (pH 6.8) before incubation for 10 minutes with 1μCi/ml $^{45}$CaCl$_2$ in the same buffer. The nitrocellulose membrane was washed with either distilled water or with 200μM CaCl$_2$ for 2 x 5 minutes and autoradiographed using Kodak XAR-5 X-ray film. In some
experiments the binding of $^{45}$Ca$^{2+}$ to bovine $\gamma$-globulin (Sigma), protein extracts from DH5αFIQ cells containing the vector without calsensin insert, and purified bovine brain calmodulin, the generous gift from the laboratory of Dr. D. Graves, were assayed as controls.
RESULTS

Identification and Isolation of the Sequence Encoding Calsensin

The sequence encoding the Ian 3-6 antigen was isolated in order to begin to elucidate its functional properties. The Ian 3-6 monoclonal antibody was used to probe a cDNA expression library, the B library, that was prepared from total Haemopis poly(A)+ RNA. A single 807 bp cDNA clone was isolated and analyzed (Figure 10). A 15kb genomic clone and two independent cDNA clones, 777 bp and 754bp, were isolated by screening a Haemopis genomic library, as well as a different Haemopis cDNA library, the E library, with the radiolabeled N-terminal 238bp EcoRI fragment from the B library cDNA clone (Figures 11 and 12A). These clones were identical to the first within the coding regions, and no

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Figure 10. Calsensin B allele sequence. The nucleotide and expected amino acid sequence of the calsensin B allele. The predicted initiation codon begins at nucleotide 35 and the stop codon is located at position 272 and is shown with an asterisk. The two underlined amino acid regions correspond to the calcium-binding loops of the EF-hands. The underlined nucleotides indicate base changes from the calsensin E allele.
introns were detected within the coding region of the genomic sequence. Since these leeches were collected from wild populations, the polymorphisms seen in both the putative N- and C-termini of the cDNA clones are likely to be due to natural polymorphisms present in the leech genome. An additional 52bp of N-terminal sequence was obtained using 5'-Amplifinder RACE extension with a gene-specific primer and *Haemopis* cDNA as a template (Figure 12A). These sequences were compared and collated to form the final calsensin sequence from the E library allele (Figure 12A). The sequence encoding the calsensin E allele contains a 249bp open reading frame, a N-terminal untranslated region of 153bp, and 408bp of C-

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AACAGCAACTGATGGAAACCAGCCATCGCCATCTTTGCTGCACGCCGAAGAG  53
Q QLMETSHRLAARR  17
CACATGGCTGAATATGCGGATTTTCATATGGGAGTTGGGCGACGACTCTGAGACC  113
HMAEYRPPFPYGDWWRRLLEP  37
GTGACTGATCGTACCAGGATTGCCCAGTCGGCGAGATCCTCGTCAGG  173
VSIIPDFGQVQNSVSISSLK  57
TCCTCAGAAAATGGGCCGCAAGGTCAAAAAGCAGCAGCCTTCAAGAATTG  233
SFKKMKACKVKAELEAFFKKL  77
GACCACAGGAGGAGTCTATTAATGCAGACCTTCTATGCTGACCCTTG  293
DANGDGYVTALELQTFMVT  97
GTGCTTACAGGCCCTGACAGGAAGCTCAAGGCTTTGCGAAACTGACTCAAG  353
DAYKALKSDKVKKEASAKLIK  117
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MADKNSDGKISKEBFLNANA  137
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ELKCKQLK  144
ACAGAAGACAGCAGAACAGGATCTTTTCGATACATTGGCATTTATGACATTTTTATC  533
TCGAAACAATGTTTTTTATTTCTGCTCCCTCCTCTACTTTTCTCCAAATGACTTTAATG  593
CATAAAATCTCATCTTTAAATACAGCCCTGCAAGTGTTACATCTTGGACATGCTT  653
TGCCAAACAAAAATATCACAATTTATACATCCGGACATTCATATTTTTTTTCTGAC  713
TTTAAAATTTATCTCGGCTTCTTT  744
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Figure 11. The nucleotide and inferred amino acid sequence of the calsensin genomic clone. The predicted initiation codon begins at nucleotide 190 and the stop codon begins at nucleotide 435. The underlined amino acids represent the calcium-binding domains of the calsensin EF-hands. The highlighted amino acids in the N-terminus are from an insertion of a portion of the *E. coli* Lac Z gene.
Figure 12. Calsensin sequence and structure. (A) Nucleotide and predicted amino acid sequence of calsensin. The entire cDNA sequence corresponding to the 1.1 kb calsensin transcript is shown with its predicted amino acid sequence below. The presumed initiation codon begins at nucleotide 154 and the stop codon is located at nucleotide 403 and is indicated by an asterisk. The polyadenylation signal consensus sequence lies between nucleotides 755 and 762 and is shown in bold as is the in-frame upstream stop codon at nucleotide 118. The two underlined regions denote the two calcium-binding loops. The predicted calsensin protein consists of 83 amino acids with an estimated molecular mass of 9.1 kd. These sequence data are available from EMBL/Genbank/DDBJ under accession number U22066. (B) Diagram of the calsensin amino acid structure. The calcium-binding coordinating residues of the loops are shown in black. The diagram indicates the potential formation of a disulfide bridge in light gray by the two N- and C-terminally situated cysteines.
terminal untranslated sequence. The open reading frame contains a potential 5' ATG initiation codon which fits the consensus A-3/G+4 for ribosome binding and initiation (Kozak, 1986), and is downstream of an inframe stop codon (Figure 12A). This indicates that the full coding sequence for calsensin has been isolated. The C-terminal untranslated sequence contains a potential polyA signal upstream from the polyadenylation site. The deduced sequence is 83 amino acids, predicted to encode a protein of 9,117 daltons, with an isoelectric point of 8.6. Finally, topographical analysis of calsensin indicates that it is an intracellular protein (Figure 13).

![Figure 13](image_url)

**Localization of Calsensin mRNA**

It was important to verify that the clones isolated encoded the same antigen that was recognized by the Ian 3-6 monoclonal antibody, and determine whether multiple forms of calsensin exist within the nervous system of the leech. To this end, Northern blots of 20μg poly(A)+ RNA from *Haemopis* were hybridized under conditions of high stringency with the radiolabeled N-terminal 238 nucleotide EcoRI fragment from the calsensin B library cDNA clone. This calsensin probe detected a single band of approximately 1.1 kb (Figure 14A).

In addition, high-stringency Southern blots of *Haemopis* genomic DNA with the radiolabeled N-terminal EcoRI fragment of the B library cDNA clone resulted in
hybridization to a single EcoRI fragment, SacI fragment, and HindIII fragment (Figure 15). This hybridization pattern agrees with the restriction pattern of the calsensin genomic clone, and when taken together, these data make it likely that calsensin is encoded by a single gene in the leech which gives rise to a single calsensin transcript.

Figure 14. Northern blot and in situ hybridization analysis of calsensin transcripts. (A) Northern blot analysis of calsensin mRNA. 20μg of total Haemopsis polyA+ mRNA were fractionated on a 1.2% agarose formaldehyde denaturing gel, transferred to nitrocellulose, and probed under high stringency conditions using random primer-labeled calsensin sequences. A single band of approximately 1.1 kb was detected. (B) In situ hybridization with digoxigenin-labeled calsensin RNA probe to an E10 Haemopsis embryo. In addition to the peripheral sensory neurons (arrowheads), in this leech species, a few central neurons (arrow) in the ganglia (g) are also expressing calsensin (compare with C). (C) E10 Haemopsis embryo labeled with lan 3-6 monoclonal antibody of comparable segments to those in B. Note the identical staining pattern of peripheral (arrowheads) and central (arrow) neurons to that in B. Anterior and the head ganglion (H) is at the top of the figure. Scale bar, 75 μm.

To verify that the calsensin cDNAs isolated encode the lan 3-6 antigen, the hybridization pattern seen in Haemopsis E10 embryos with digoxigenin-labeled calsensin RNA probes was compared with the lan 3-6 monoclonal antibody labeling (Figures 14B and C). The calsensin antisense RNA probe labeled the same neurons in the embryos as that seen with the monoclonal antibody. No specific hybridization was seen with the lan 3-6 sense strand RNA probe or no probe controls. These data strongly indicate that calsensin is the lan 3-6 antigen.
Figure 15. Southern blot analysis of *Haemopis* genomic DNA. 10 μg of *Haemopis* genomic DNA was digested with EcoRI/Hind III, EcoRI, and EcoRI/Sac I and separated on a 0.5% agarose/TBE gel, transferred to nitrocellulose, and probed under conditions of high stringency using random primer-labeled calsensin sequences. Single bands of 2.5 kb in the EcoRI/HindIII digest (lane 1), and 4.5 kb in the EcoRI (lane 2) and EcoRI/SacI (lane 3) digests were identified.

**Calsensin is a New Member of the EF-Hand Family of Calcium-Binding Proteins**

The sequence encoding calsensin was analyzed to see if it could provide clues as to its function within the developing leech nervous system. The complete cDNA sequence of the calsensin E library allele is shown in Figure 12A. Searches of the SwissProt, Genbank, PIR, and GenPept databases revealed that calsensin shares significant similarity with a number of calcium-binding proteins of the EF-hand family (Kretsinger, 1980). Figure 16 shows the sequence comparison of calsensin with four other EF-hand calcium-binding proteins. Calsensin shares 39% identity with human I-plastin, a calcium-dependent actin-bundling protein present in intestines and kidneys (Lin et al., 1994), 33% identity with the protozoan *Naegleria gruberi* calcineurin B, the calcium-binding subunit of the calcium and calmodulin-dependent type 2B protein phosphatase calcineurin (unpublished, accession number...
Figure 16. Sequence comparison of calsensin with other calcium-binding EF-hand proteins. The entire predicted sequence of calsensin is aligned with the most homologous sequence domains of human I-plastin (Lin et al., 1994), *N. gruberi* calcineurin B (unpublished, accession number U04380), *Drosophila* calmodulin (Yamanaka et al., 1987), and bovine ICaBP (Szebenyi and Moffat, 1986). Shared amino acids of these proteins with calsensin are in white typeface outlined in black and the overall homology in percent is indicated at the end of the sequence alignment.

U04380), 28% to *Drosophila* calmodulin, and 25% to bovine ICaBP, an intestinal calcium-binding protein of the S100 subfamily that, like calsensin, is 9 kD and has two EF-hand domains (Szebenyi and Moffat, 1986). A possible diagrammatic representation of calsensin based on the structure of bovine ICaBP is shown in Figure 12B.

Calsensin has two cysteine residues at position 3 in the N-terminus and at position 80 in the C-terminus (Figure 12B). It is possible that calsensin may form disulfide bridges (Figure 12B), alternatively calsensin may, like the S100 proteins, form disulfide-linked dimers (Donato, 1986). The structure of the loops as well as the calcium-coordinating residues, which are highlighted in black (Figure 12B), conform to the consensus sequence for functional calcium-binding domains (Strynadka and James, 1989) (Figure 1). Figure 17 shows the two EF-hand domains from calsensin compared with the canonical EF-hand as proposed in Tufty and Kretsinger, 1975. Although calsensin shares homology with other EF-hand family members within the helix-loop-helix calcium-binding domains, it does not appear to be a homolog of any known calcium-binding protein in the data banks. This suggests that calsensin is a novel small calcium-binding protein which is expressed selectively in the nervous system of hirudinid leeches.
Figure 17. Comparison of calsensins EF-hands with the canonical EF-hand domain. Calsensin shares all of the conserved residues that indicate that the domain is functional. The Asp (D) and Glu (E) at residues 10 and 21 respectively are nearly invariant, as is the Gly (G) at position 15. Both of calsensins calcium-binding loops share these amino acids. In addition, the other calcium-coordinating residues (Y, Z, and -X) also have amino acids that have oxygen-containing side chains. Furthermore, many of the hydrophobic residues (n) are present, especially in the first EF-hand of calsensin.

Calsensin is a Functional Calcium-Binding Protein

The sequence that encodes the EF-hand domains of calsensin, when compared with those of other functional calcium-binding proteins, appear to encode functional calcium-binding domains (Figure 17). To verify that the calsensin protein could function as a calcium-binding protein, a calsensin fusion protein was constructed and used to assay the ability of calsensin to bind calcium in vitro. The fusion protein was constructed using the PinPoint vector system which adds a short biotinylated sequence that permits avidin affinity column purification of the fusion protein. The construct encodes the first 72 residues of calsensin, which includes both EF-hands calcium-binding domains, but truncates the C-terminus. This fusion protein was partially purified over a Soft-link™ monomeric avidin column, transferred to nitrocellulose after SDS-PAGE, and then the calcium-binding of calsensin was tested by incubation with 45CaCl2 and subsequent autoradiography (Maruyama et al., 1984). The calsensin fusion protein selectively binds 45Ca2+ using these assay conditions in vitro (Figure 18). In control experiments, the binding of 45Ca2+ could be competed off with cold CaCl2, the vector protein without calsensin insert in DH5αF′IQ cells.
Figure 18. Calsensin binds $^{45}\text{Ca}^{2+}$ in vitro. Lane 1 shows $^{45}\text{Ca}^{2+}$-binding to partially-purified calsensin fusion protein (arrow) which was fractionated by SDS-PAGE and transferred to nitrocellulose. The binding was visualized by autoradiography. Lanes 3, 5, and 7 were control experiments with the DH5αF1Q cells with the Pinpoint vector only, γ-globulin, and bovine brain calmodulin respectively. Lanes 2, 4, 6, and 8 show samples of the 1, 3, 5, and 7 lanes which were run on a parallel gel and stained with Coomassie blue. The lower band in lane 2 which does not bind $^{45}\text{Ca}^{2+}$ is a native biotinylated *E. coli* protein which copurifies with the calsensin fusion protein during avidin affinity chromatography.

and γ-globulin did not bind $^{45}\text{Ca}^{2+}$, and purified bovine brain calmodulin bound $^{45}\text{Ca}^{2+}$ (Figure 18). These experiments provide very strong evidence that calsensin functions as a calcium-binding protein in vitro.

**Generation of Polyclonal Antibodies Against Calsensin and Western Blotting Experiments**

As mentioned previously, the lan 3-6 monoclonal antibody does not recognize the denatured antigen on Western blots (Briggs et al., 1993). Therefore, a biochemical characterization of the antigen was not possible. The calsensin fusion protein was used to generate polyclonal antibodies against calsensin in rabbits, so that we could further characterize the calsensin protein. Two antisera were generated, Frigg and Hel, which recognize a single band, of 10,000 Mr on Western blots of leech central nervous system proteins, that was not present in the preimmune sera (Figure 19). In addition, the recognition
Figure 19. Polyclonal antiserum identifies calsensin as a 10,000 Mr protein on immunoblots of leech CNS extracts. Lane 1 shows labeling of a 10,000 Mr protein (arrow) from leech CNS extracts recognized by the Frigg antiserum which is not present in the preimmune serum (lane 2). The Frigg antiserum was raised against a calsensin fusion protein. A weak background band is present in both lane 1 and 2 (arrowhead). Lane 3 shows an immunoprecipitation of leech CNS extracts by the Ian 3-6 monoclonal antibody coupled to protein A-Sepharose. A 10,000 Mr protein is selectively recognized by the Frigg antiserum on the blot. Lane 4 demonstrates that preabsorption of Frigg antiserum with calsensin fusion protein abolishes staining by the Frigg antiserum of the 10,000 Mr protein. All lanes show immunoblots of *Haemopis* CNS proteins fractionated by 20% SDS-PAGE.

of the calsensin protein by either antisera is abolished by preincubating the antisera with the calsensin fusion protein (Figure 19). Furthermore, the 10,000 Mr protein recognized by the antisera is immunoprecipitated from leech central nervous system proteins by the original Ian 3-6 monoclonal antibody (Figure 19). Finally, the staining pattern seen with the Frigg antiserum in E10 *Haemopis, Macrobdella*, and *Hirudo* embryos is the same as that seen with the Ian 3-6 monoclonal antibody staining (Figure 21). The fact that both antisera recognize the native calsensin protein, and the predicted molecular weight of calsensin based on the amino acid sequence correlates well with the mobility of the native calsensin on SDS-PAGE, provides further evidence that calsensin is the Ian 3-6 antigen.
**Calsensin is Both Cytosolic and Membrane-Associated**

The EF-hand family of calcium-binding proteins are generally cytosolic proteins, but many are associated with the cytoskeleton and/or the intracellular face of the plasma membrane, and this membrane-localization can change upon calcium binding (Braunewell, 1994). In an effort to determine the location of calsensin in vivo, crude subcellular fractionations of *Haemopis* and *Macrobelda* nerve cord proteins, followed by SDS-PAGE and Western blotting were performed. The majority of immunoreactivity was seen in the cytosolic fraction, but immunoreactivity was also seen in all membrane fractions, suggesting that although predominantly cytosolic, a fraction of calsensin is associated with membrane (Figure 21).

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Figure 20. The Frigg polyclonal antisera recognizes the same neurons in *Haemopis* E10 embryos as the Ian 3-6 monoclonal antibody. A *Haemopis* E10 embryo labeled with the Frigg polyclonal antisera. The head region of the embryo is to the left in this figure. At this stage of development, subpopulations of both the peripheral sensillar neurons and central neurons are recognized by the Frigg polyclonal antisera and the Ian 3-6 monoclonal antibody.
A 200,000 M_r Protein Selectively Co-Immunopurifies with Calsensin

Immonoaffinity purification of *Haemopis* CNS extracts with the lan 3-6 monoclonal antibody covalently crosslinked to protein A-Sepharose beads yielded a protein of 200,000 M_r (Briggs et al., 1993). This protein was initially thought to be a likely candidate for the lan 3-6 antigen, although this could not be validated by Western blot analysis since the lan 3-6 monoclonal antibody does not recognize the denatured antigen (Briggs et al., 1993). The cloning of calsensin and the generation of polyclonal antibodies which have identified native calsensin on Western blots as a 10,000 M_r protein, make it clear that the immunopurified 200,000 M_r protein is not the lan 3-6 antigen. Calsensin was not detected in the earlier experiments due to its small size (Briggs et al., 1993). These data suggest that calsensin may be interacting with a larger protein that is selectively copurified using immunoaffinity chromatography with calsensin antibodies. Further immunoaffinity purification experiments

![Figure 21](image-url)

Figure 21. Crude subcellular fractionations reveal that calsensin is present in both membrane and cytosolic fractions. CNS proteins from *Haemopis* were subjected to a step-wise centrifugation to separate subcellular components. The fractions were subjected to SDS-PAGE on 20% gels, and Western blotting with the Frigg polyclonal antisera. The P2 fraction should contain proteins associated with mitochondria and plasma membrane fragments. The P3 lane should contain proteins associated with the mitochondria, lysosomes, peroxisomes, golgi and some rough endoplasmic reticulum. The P4 and P4' fractions should contain membrane vesicles from the golgi, endoplasmic reticulum, and plasma membrane. The S2 and S3 fractions are the cytoplasmic fractions generated after the 3,000 g and 10,000 g spins, and they were very dilute in comparison to the other fractions. Finally, the S4 fraction should contain the soluble components of the cytoplasm. *Haemopis* (H) total nerve cord homogenate was analyzed as a control for calsensin mobility. A 10,000 M_r protein was detected on Western blots in the P2, P3, P4, P4', and S4 fractions.
were conducted with the lan 3-6 antibody covalently coupled to protein A-Sepharose in order to investigate whether the large protein present in *Haemopis*, could also be immunopurified from *Macrobdella*, the leech species used for the developmental studies of selective fasciculation (Briggs et al., 1993). A prominent broad protein band of approximately 200,000 Mr was selectively affinity purified from both *Macrobdella* and *Haemopis* (Figure 22). Although the fractions collected containing the 200,000 Mr band also contained small amounts of eluted primary antibody (Figure 22), the 200,000 Mr bands shows no immunoreactivity using the calsensin polyclonal antiserum on Western blots. The calsensin protein is too small to be resolved in these gels, but can be resolved by 20% SDS-PAGE as demonstrated in Figures 19. To further confirm the specificity of the binding of this 200,000 Mr protein, proteins from 50 *Haemopis* nerve cords were immunoaffinity purified as before using a Bio-Rad Econocolumn system. The column was washed with 0.5M NaCl, 50mM Tris-Cl, pH 8.2 to remove proteins bound nonspecifically, and then eluted with 0.1M glycine.

Figure 22. Lan 3-6 immunoaffinity purification of leech CNS proteins. (A) A 200,000 Mr protein is selectively immunoaffinity purified from CNS extracts of both *Haemopis* (H) and *Macrobdella* (M) leeches by a lan 3-6 monoclonal antibody-Protein A-Sepharose column. The figure shows pooled fractions which were silver stained after separation by 12% SDS-PAGE. In addition to the major 200,000 Mr protein, some heavy (hc) and light (lc) chains from the primary antibody were eluted. That these bands were derived from the primary antibody was determined by immunoblotting and staining with secondary antibody only (data not shown). The two lanes are from different gels which were photographically adjusted in size using the positions of the light and heavy antibody chains as a reference.
pH 2.5. The proteins eluted in two peaks. The fractions composing the first peak didn't hybridize to the Ian 3-6 monoclonal antibody and contained the broad 200,000 M_r protein. The fractions composing the second peak hybridized to the monoclonal antibody, and contained antibody which came off the column in the elutions. In addition, this second protein peak contained a 10,000 M_r protein that hybridized to the calsensin polyclonal antisera, and is therefore likely to be calsensin. These data show that a 200,000 M_r protein appears to specifically interact with calsensin in both *Macrobidea* and *Haemopsis*. 
DISCUSSION

Calsensin is a novel neuronal EF-hand calcium-binding protein in leech that was originally identified by the lan 3-6 monoclonal antibody (Zipser and McKay, 1981), and has been proposed to be involved in fascicle selection and/or maintenance by a subset of peripheral neurons because it defines a single axon fascicle that occupies a stereotyped position in each lateral connective, it is expressed in the growth cones at early stages of neurite outgrowth, and later differentiating lan 3-6-labeled peripheral neurons preferentially fasciculate with the calsensin fascicle within the developing leech central nervous system (Briggs et al., 1993).

Calsensin is the Lan 3-6 Antigen

To further characterize its antigen, the lan 3-6 monoclonal antibody was used to isolate cDNA and genomic clones encoding calsensin. This sequence was used for in situ hybridization and polyclonal antibody production. The staining pattern obtained by in situ hybridization of Haemopis embryos using calsensin riboprobes, looks exactly like the labeling seen with the lan 3-6 monoclonal antibody. In addition, the polyclonal antisera generated against a calsensin fusion protein labels Haemopis, Hirudo, and Macrobdella embryos in a pattern that is identical to that seen with the lan 3-6 monoclonal antibody. Furthermore, the lan 3-6 monoclonal antibody immunoprecipitates a protein which is recognized by the calsensin polyclonal antisera on Western blots. These data provide very strong evidence that calsensin is the antigen recognized by the lan 3-6 monoclonal antibody. Finally, the presence of an in-frame stop codon just upstream of the starting methionine, and the fact that the calsensin polyclonal antisera recognizes a 10,000 Mr protein on Western blots which correlates well with the molecular weight predicted by the calsensin sequence suggest that the full-length sequence encoding calsensin has been identified.

Calsensin is an EF-Hand Calcium-Binding Protein

Sequence analysis has revealed that calsensin defines a new subfamily in a large superfamily of EF-hand-containing calcium-binding proteins. The members of this family are intracellular calcium-regulated proteins that modulate the actions of other enzymes and structural proteins (Persechini et al., 1989). These proteins contain one to eight copies of a calcium-binding motif consisting of a helix-calcium-binding loop-helix structure that are usually found in functional pairs (Persechini et al., 1989). The EF-hand domains of calsensin
fit the consensus sequence for functional calcium-binding domains (Strynadka and James, 1989) in that both hands have the conserved aspartate at position 10 (X), the glycine at the bend in the calcium binding loop at position 15, the conserved glutamate at position 21 (-Z), and the other calcium coordinating residues have asparagine, serine, and threonine (Y, Z, and -X) (Strynadka and James, 1989). In addition, calcium-binding assays with a calsensin fusion protein confirmed that calsensin can bind $^{45}$Ca\textsuperscript{2+} \textit{in vitro}. Therefore, it is probable that calsensin acts as a functional calcium-binding protein \textit{in vivo}.

Calsensin shares the highest identity with I-plastin (39%), an EF-hand calcium-binding protein expressed in human intestines and kidney that has been shown to crosslink actin in the absence of calcium (Rosales, 1994). It is one of several proteins in a small subfamily of actin-crosslinking EF-hand containing calcium-binding proteins that include chicken fimbrin, human I-, T- and L-plastins, \textit{S. cerevisiae} Sac6, and \textit{Dictyostelium} ABP-120 (Bretscher and Weber, 1980; Bretscher, 1981; Glenney et al., 1981; Goldstein et al., 1985; Adams et al., 1989; Bresnick et al., 1990; de Arruda et al., 1990; Adams et al., 1991; Lin et al., 1994). These proteins have two N-terminal EF-hand calcium-binding domains followed by a pair of actin-binding domains that are similar to those found in \(\alpha\)-actinin, an EF-hand calcium-binding protein located in the Z-lines of skeletal muscle (Bretscher and Weber, 1980; Bretscher, 1981; Glenney et al., 1981; Goldstein et al., 1985; Adams et al., 1989; Bresnick et al., 1990; de Arruda et al., 1990; Adams et al., 1991; Lin et al., 1994). Chemical crosslinking studies with \(\alpha\)-actinin have suggested that the actin and the calcium-binding domains interact, and in the cases of L-, I-plastin, and probably fimbrin, calcium inhibits actin crosslinking (Goldstein et al., 1985; Lin et al., 1994; Rosales et al., 1994). I-plastin has been proposed to be the human homolog of chicken fimbrin since they share the highest identity and are both localized to the intestinal brush border microvilli (Bretscher and Weber, 1980; Glenney et al., 1981). Fimbrin is also found at the adhesion points between cells, and in the leading edges of motile cultured cells (Bretscher et al., 1980; Bretcher and Weber, 1980). I-plastin has been implicated in the release of calcium from intracellular stores that occurs in human neutrophils upon Fc receptor binding (Rosales et al., 1994). Thus, it provides an example of the importance of a calcium-binding protein in mediating the calcium signal transduced by an extracellular ligand.

Calsensin shares 33% identity with the calcineurin B subunit of the protozoan \textit{Naegleria gruberi}. The calcineurin B subunit contains four EF-hand calcium-binding domains and interacts with the calcineurin A subunit to form an active calmodulin-dependent type 2B protein phosphatase. Calsensin also shares about 28% identify with \textit{Drosophila}
calmodulin, and finally, bovine ICaBP was included in the alignment (25% identity), because it has a similar molecular weight, and has two EF-hand calcium-binding domains. Since calsensin has two cysteines at the N- and C-termini, respectively, it may be able to form intra- or intermolecular disulfide bonds. Formation of disulfide bonds would be expected to change the conformation of calsensin, and could regulate its interactions with its target molecules in vivo. The Calcium Vector Protein, CaVP, and the S100s (Donato, 1986; Szebenyi and Moffat, 1986; Kobayashi et al., 1987) are thought to form disulfide bridges in vivo (Donato, 1986; Kobayashi et al., 1987). CaVP is an 18 kd EF-hand calcium-binding protein found in Amphioxus muscle. It has four EF-hand calcium-binding domains, and interacts with a 36 kd protein in vivo (Kobayashi et al., 1987). The S100 dimers are found in both disulfide-linked and noncovalent associations, but the disulfide-linked S100b dimer has been found to promote neurite outgrowth from chick cerebral cortical neurons in vitro (Kligman and Marshak, 1988). Cysteine residues in the cytosol are usually found in a reduced state (Branden and Tooze, 1991), so it's unclear whether disulfide bonds are formed by calsensin in vivo.

Another interesting feature of calsensin is its intracellular localization. Although calsensin is found predominantly in the cytosol, preliminary crude experiments indicate that there is a significant portion that is membrane-associated. Other EF-hand members have been found to be similarly distributed (Donato, 1986; Braunewell et al., 1994). For example, the EF-hand calcium-binding protein Vilip, which associates with the cytoskeleton, has been shown to change its intracellular localization from cytosolic to membrane-associated upon calcium binding (Pongs et al., 1993; Braunewell et al, 1994).

EF-hand family members are functionally grouped into trigger proteins and buffer proteins. Trigger proteins, like calmodulin, interact with and regulate other proteins upon calcium binding (Levine and Dalgarno, 1983; Baimbridge et al., 1992), while the buffer proteins, like ICaBP, have no identifiable targets, and are thought to maintain calcium-homeostasis within the cell by controlling the level of free calcium (Levine and Dalgarno, 1983; Baimbridge et al, 1992; Heizmann and Braun, 1992). A 200,000 Mr protein selectively immunopurifies with calsensin from CNS homogenates of both Haemopis and Macrobdella (Briggs et al., 1993). This suggests that calsensin may function as a trigger protein which binds to and modulates this large protein in vivo.

There are a few EF-hand calcium-binding proteins that have a very restricted expression to certain tissues and types of neurons like parvalbumin, calbindin, and calretinin (Baimbridge et al., 1992), but the physiological role of these nervous system-localized
calcium-binding proteins is unknown (Baimbridge et al., 1992). In addition, a subfamily of small calcium-binding proteins with three EF-hand calcium-binding domains has recently been identified which may function in visual and odorant signal transduction (Palczewski et al., 1994; Dizhoor et al., 1991; Yamagata et al., 1990; Nemoto et al., 1993). Furthermore, Frequenin, a four EF-hand-containing calcium-binding protein has been shown to facilitate synaptic transmission when overexpressed in *Drosophila*, and the *rdgC* gene in *Drosophila* has been found to encode a serine/threonine protein phosphatase with five EF-hand Ca$^{2+}$-binding domains whose function is necessary to prevent light-induced retinal degeneration (Steele et al., 1992).

As discussed previously, intracellular calcium has been strongly implicated in the signalling pathways involved in neurite outgrowth, turning, fasciculation, and collapse. Thus, calcium-binding proteins present in growth cones and axons which modulate or are being modulated by intracellular calcium may affect the calcium-dependent processes of growth cone extension and pathway formation. In support of this, EF-hand calcium-binding proteins have been implicated in the signal transduction pathways associated with neurite outgrowth (Kligman and Marshak, 1985; Chang et al., 1994). The disulfide-linked S100b dimer has been shown to promote neurite outgrowth from chick cerebral cortical neurons *in vitro* (Kligman and Marshak, 1985). In addition, blocking or overexpressing *Drosophila* calmodulin *in vivo* caused aberrations in growth cone guidance and fasciculation (VanBerkum et al., 1995). Furthermore, experiments using chromophore-assisted laser inactivation of calcineurin in chick dorsal root ganglion neurons indicated that its distribution may regulate growth cone motility (Chang et al., 1994). Finally, the molecular features of calsensin, its association with the 200,000 Mr protein, and its very restricted expression to a subset of peripheral neurons that fasciculate into a single axon tract in the CNS of hirudinid leeches are consistent with the hypothesis that the putative calsensin protein complex may play a role in the formation and/or maintenance of these specific pathways *in vivo* (Briggs et al., 1993).
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