Calsensin: A Novel Calcium-binding Protein Expressed in a Subset of Peripheral Leech Neurons Fasciculating in a Single Axon Tract

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Abstract. The mAb lan3-6 recognizes a cytosolic antigen which is selectively expressed in the growth cones and axons of a small subset of peripheral sensory neurons fasciculating in a single tract common to all hirudinid leeches. We have used this antibody to clone a novel EF-hand calcium-binding protein, calsensin, by screening an expression vector library. A full-length clone of 1.1 kb identified by the antibody was isolated and sequenced. In situ hybridizations with calsensin probes and antibody staining using new polyclonal antisera generated against calsensin sequence demonstrate that calsensin indeed corresponds to the lan3-6 antigen. Calsensin consists of 83 residues with a calculated molecular mass of 9.1 kD that contains two helix-loop-helix domains. The calcium-binding domains are likely to be functional in vivo since a fusion protein derived from the calsensin clone binds $^{45}Ca^{2+}$ in vitro. Immunoaffinity purification experiments with the lan3-6 antibody shows that a large 200,000 M_r protein selectively copurifies with calsensin in two different leech species. These results suggest that calsensin may be functioning as a trigger protein which interacts with the larger protein. These data are consistent with the hypothesis that calsensin may mediate calcium-dependent signal transduction events in the growth cones and axons of this small group of sensory neurons which fasiculate in a single axon tract.

D URING development the formation and maintenance of specific nerve pathways are dependent on the interactions of growth cones and axons with surface molecules expressed on other particular cells (Jessell, 1988; Goodman and Shatz, 1993). Recent experiments have shown that some of these interactions may be mediated through signal transduction events which induce changes in intracellular $[Ca^{2+}]_i$ leading to biological responses. For example, antibodies which bind to L1 and N-CAM, mimicking their natural ligands, activate second messenger systems that result in increased $[Ca^{2+}]_i$ (Schuch et al., 1989), and the neurite growth inhibitor NI-35–induced collapse of neuronal growth cones is in part mediated by release of Ca^{2+} from intracellular stores (Bandtlow et al., 1993). Thus, molecules which are modulated by or are modulating $[Ca^{2+}]_i$ and are selectively present in growth cones and axons are strong candidates for being molecules which play a functional role in growth cone guidance and/or selective fasciculation.

In a previous study we proposed, based on a partial cDNA clone and on immunoaffinity purification experiments (Briggs et al., 1993), that the mAb lan3-6 (Zipser and McKay, 1981) may recognize a large Ca^{2+}-binding protein which is selectively expressed in the growth cones and axons of a small subset of peripheral sensory neurons fasciculating in a single axon tract in hirudinid leeches. However, by obtaining a full-length clone and molecularly characterizing the lan3-6 antigen we now report that the actual antigen is a separate small 9-kD protein with which a larger 200,000 M_r protein selectively copurifies. Analysis of the deduced amino acid sequence shows that the 9-kD antigen defines a novel EF-hand Ca^{2+}-binding protein (Persechini et al., 1989; Strynadka and James, 1989) which has two helix-loop-helix (HLH)[1] domains that bind $^{45}Ca^{2+}$ in vitro. On the basis of these features we have named the lan3-6 antigen, calsensin. Our data are consistent with the hypothesis that calsensin and the 200,000 M_r protein may form a complex mediating Ca^{2+}-dependent signal transduction events in the growth cones and axons of this subset of peripheral sensory neurons.

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.

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1. *Abbreviations used in this paper:* aa, amino acids; CNS, central nervous system; HLH, helix-loop-helix; nt, nucleotide.

Dissections. Dissections of nervous tissue and embryos were performed in leech saline solutions with the following composition: 110 mM NaCl, 4 mM KCl, 2 mM CaCl$_2$, 10 mM glucose, and 10 mM 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. These 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 ~10–20 embryos in each cocoon and these sibling embryos develop synchronously within a few percent of development.

Immunocytochemistry

The mAb lan3-6 that is of the IgG$_{2a}$ subtype (Zipser and McKay, 1981; Briggs et al., 1993) or newly raised polyclonal antisera (see below) were used in these studies. The lan3-6 antibody was raised against *Haemopis* central nervous system (CNS) but cross-reacts with *Haemopis*, *Macrobdella*, and *Hirudo* CNS (Briggs et al., 1993).

Dissected *Macrobdella* and *Haemopis* embryos were fixed overnight at 4°C in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, incubated overnight at room temperature directly in hybridoma supernatant containing 0.4% Triton X-100 or 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 (1:200 dilution; Bio-Rad Labs., Richmond, CA). After washing in PBS the HRP-conjugated antibody complex was visualized by reaction in DAB (0.03%) and H$_2$O$_2$ (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 Calsensin cDNAs

The mAb lan3-6 was used to probe an oligo dT-primed λgt11d cDNA library prepared from size-selected (>400 nucleotide [nt]) *Haemopis marmorata* mRNA. The library was screened essentially according to the procedures of Sambrook et al. (1989). A total of 10^6 plaques were screened at a density of 30,000 plaque-forming units/150-mm 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 Inc., La Jolla, CA) using standard protocols (Sambrook et al., 1989).

For further screening of phage libraries, a 238 nt NH$_2$-terminal EcoRI fragment from the isolated calsensin cDNA clone was gene-cleaned (Bio101), and ^{32}P-radiolabeled using random priming according to the manufacturer's procedure (Prime-A-Gene kit; Promega Corp., Madison, WI). This radiolabeled fragment was used to probe a second oligo dT-primed λgt11d 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 NH$_2$-terminal sequence were obtained using a Clontech 5'-Ampli-FINDER 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 (United States Biochem. Corp., Cleveland, OH) and ^{35}S-dATP (Amersham Corp., Arlington Heights, IL) in the termination reactions. Templates were primed with either commercially available sequencing primers (M13 universal, reverse, T7, and T3 primers; Promega and United States Biochem. Corp.) 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 Group Program Manual for the GCG Package, Version 7, April 1991, 575 Science Drive, Madison, WI) suite of

programs (Devereaux 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.

Northern Analysis

Total leech RNA was isolated using a modification of a plant genomic DNA prep (Shure et al., 1983) as follows: 3–4 g of leech was ground in a tissue homogenizer with dry ice to a fine powder. Leech powder was mixed with 6 ml urea extraction buffer (8 M urea/0.35 M NaCl/0.05 M Tris-HCl [pH 7.5]/0.02 M EDTA/2% sarcosyl) and 6 ml of phenol/chloroform (1:1) was added. The mixture was vigorously shaken, incubated 15 min at room temperature, and shaken vigorously again before low-speed centrifugation. The aqueous phase was extracted, and precipitated with 0.17 vol 4.4 M ammonium acetate, pH 5.2, and 1 vol isopropanol, mixed well, and centrifuged at 10K for 10 min at 4°C in an SS34 rotor (DuPont, Bannockburn, IL). The pellet was dissolved in 500 μl 10 mM Tris/1 mM EDTA, pH 8.0, phenol/chloroform extracted, reprecipitated in isopropanol as above, and the nucleic acid pellet washed with 70% ethanol. Nucleic acids were dissolved in oligo-dT binding buffer, and poly(A)$^+$ mRNA was isolated using an oligo-dT column (Stratagene) according to standard protocols (Sambrook et al., 1989). 20 mg of poly(A)$^+$ mRNA was fractionated on 1.2% agarose formaldehyde gels, transferred to nitrocellulose, and hybridized to a [^{32}P]dCTP-labeled 238 nt EcoRI NH$_2$-terminal calsensin fragment according to standard methods (Ausubel et al., 1987) with the addition of dextran sulfate (10%). 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 NH$_2$-terminal 238 nt 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 RNA labeling kit and protocols (Boehringer Mannheim Corp., Indianapolis, IN). 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 0.1 M phosphate buffer, pH 7.4. However, the probe concentration was reduced to 0.1 mg/ml, hybridizations were at 45°C, and the antidigoxygenin antibody was used at a concentration of 1:3,000. After labeling the embryos were dehydrated in ethanol, cleared in xylene, and mounted in Depex.

Preparation of Calsensin Fusion Protein and Generation of Calsensin Antisera

The NH$_2$-terminal 238 nt 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 COOH-terminal end. In addition the fusion protein contains 10 residues of sequence upstream of the starting methionine and 23 residues of COOH-terminal translated vector sequence. The fusion protein was expressed in DH5αF'IQ cells and partially purified over a Softlink™ monomeric avidin column (Promega) using the manufacturer's recommended protocols.

The partially-purified calsensin fusion protein was used to generate polyclonal antibodies in rabbits. Two rabbits (Frigg and Hel) were injected with from 200 to 400 μg of fusion protein, and then boosted at 21-d intervals as described in Harlow and Lane (1988). After the second boost serum samples were collected seven and 10 d 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 were titrated from undiluted to a 1:5,000 dilution in Blotto (0.5% Carnation nonfat 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 0.2 μm nitrocellulose, and using HRP-conjugated secondary antibody (1:3,000) for visualization of primary antibody diluted 1:2,000 in Blotto in immunoblot analysis. The signal was developed with DAB (0.1 mg/ml) and H_2O_2 (0.03%) and enhanced with 0.008% $NiCl_2$. In some experiments to test for the specificity of the polyclonal antisera, 15 μg of the calsensin 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.

Immunoaffinity Purification. Immunoprecipitations were performed by incubating Protein A–Sepharose CL-4B beads which were covalently cross-linked (see below) to the lan3-6 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 (20 mM Tris-HCl, 200 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 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 three times in TBS, and once in PBS before SDS-PAGE and Western blotting.

To assay for proteins interacting with calsensin, *Haemopis* and *Macrobdella* nerve cords were homogenized in extraction buffer and the homogenates precleared by incubation with 10 mg protein A–Sepharose CL-4B beads (Sigma) for 2 h at 4°C on a shaking platform. After preclearing the homogenates were incubated with 500 mg protein A–Sepharose CL-4B beads which were crosslinked to lan3-6 monoclonal antibody following the procedures of Harlow and Lane (1988). The only alteration to these protocols was that 0.1 M sodium borate, pH 8.2, was used for washing instead of 0.2 M sodium borate, pH 9. After incubation and washing the beads were resuspended in 4 ml of 0.1 M sodium borate, pH 8.2, and packed into a column. The column was washed with 20 vol of 0.1 M sodium borate, pH 8.2, and by 10 vol of 10 mM sodium phosphate, pH 6.8, before elution with one volume of 0.1 M glycine, pH 2.5. 10 0.4-ml fractions of the eluate were collected into tubes containing 80 μl of 1 M sodium phosphate, pH 8, for neutralization. Fractions 1-2, 3-5, and 6-10 were pooled, dialyzed against 10 mM Tris-HCl, pH 7, and precipitated with absolute ethanol. The resulting protein pellets were resuspended in 20 ml homogenization buffer and analyzed by SDS-PAGE and silver staining of the gel.

$^{45}Ca^{2+}$-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 0.2 μm nitrocellulose. The membrane was washed 2 × 10 min in a buffer containing 60 mM KCl, 5 mM $MgCl_2$ and 10 mM imidazole-HCl (pH 6.8) before incubation for 10 min with 1 mCi/ml $^{45}CaCl_2$ in the same buffer. The nitrocellulose membrane was washed with either distilled water or with 200 μM $CaCl_2$ for 2 × 5 min and autoradiographed using Kodak XAR-5 x-ray film. In some experiments the binding of $^{45}Ca^{2+}$ to bovine γ-globulin (Sigma), protein extracts from DH5αF'IQ cells containing vector without calsensin insert, and purified bovine brain calmodulin, the generous gift from the laboratory of Dr. D. Graves (Dept. of Biochemistry and Biophysics, Iowa State University), were assayed as controls.

Results

Cloning and Characterization of cDNA Clones Encoding Calsensin

The mAb lan3-6, which specifically recognizes a small subset of peripheral neurons that extend their axons and growth cones into a single axonal tract in leech CNS (Fig. 1), was used to screen a λgtlld cDNA expression vector library constructed from total leech poly(A)$^+$RNA. Of ~1
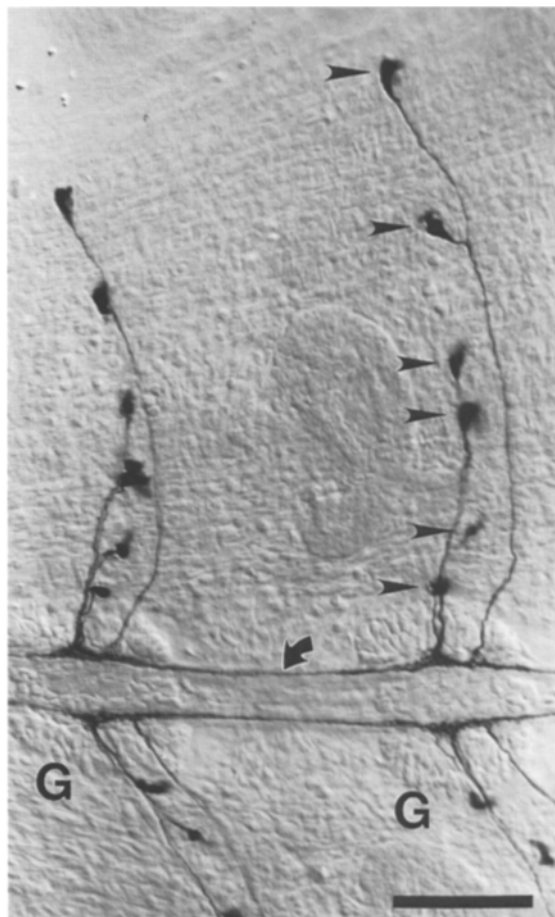
Figure 1. Calsensin localization to a subset of peripheral sensory neurons in an E10 *Macrobdella* leech embryo. The sensory neurons (*arrowheads*) extend their growth cones and axons into the ganglia (*G*) and connectives of the CNS where they fasciculate in a single axon tract (*curved arrow*). The preparation, which shows two hemisegments, was labeled with the lan3-6 antibody. Bar, 50 μm.

× 10^6 plaques screened, one was antibody positive. This clone contained a 820 nt insert and was plaque-purified, subcloned, and sequenced. To obtain additional independent clones, an NH$_2$-terminal 238 nt EcoRI fragment from this clone was radiolabeled and used to probe a different *Haemopis* leech cDNA library as well as a genomic library. From these libraries two independent partial cDNA clones of 777 nt and 754 nt and a 15-kb genomic clone were isolated and analyzed. The sequence of these clones were identical to the first within the coding region, with no introns present in the genomic sequence. The calsensin sequence assembled from these and ampliFINDER-RACE extension data contains a 249-nt open reading frame with 408 nt of 3′ and 153 nt of 5′ noncoding sequence (Fig. 2 A). The 3′ noncoding sequence contains a potential polyadenylation signal AATAAA upstream from the putative polyadenylation site (Fig. 2 A). The open reading frame contains a 5′ ATG initiation codon, which has a favorable A$_{-3}$/G$_{+4}$ context for initiation (Kozak, 1986) just downstream from an in frame TGA stop-codon (Fig. 2 A). The presence of this in frame stop-codon was confirmed by 5′-ampliFINDER-RACE extension using a specially de-

A

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                              GTGGAGCATTCCCACAAACTT   21
CTCTATCAAACTTTAGTTTGCAGAAATCTCTCAATAAAATTCCAATTGTTAATCTCGCCG   81
ATTAACTAGATTAATCTTTCAATCCTCGCTTCTATTTGACAACTGTCAATATCGCTCAAG  141
TCCTTCAAGAAAATGGCCTGCAAAGTCAAAGCCGAACTCGAAGCAGCCTTCAAGAAATTG  201
             M  A  C  K  V  K  A  E  L  E  A  A  F  K  K  L   16

GACGCCAATGGGGACGGTTATGTCACTGCTTTAGAGTTGCAGACCTTCATGGTGACCCTG  261
 D  A  N  G  D  G  Y  V  T  A  L  E  L  Q  T  F  M  V  T  L   36

GATGCTTACAAGGCCCTGAGCAAGGACAAGGTCAAAGAGGCTTCTGCGAAACTCATCAAG  321
 D  A  Y  K  A  L  S  K  D  K  V  K  E  A  S  A  K  L  I  K   56

ATGGCGGACAAGAATAGTGATGGGAAGATTAGCAAAGAGGAATTCTTGAATGCCAATGCT  381
 M  A  D  K  N  S  D  G  K  I  S  K  E  E  F  L  N  A  N  A   76

GAACTGTTGTGCCAGTTGAAGTGATACCATAAGATCCTCTTGTACGCATTTATGCATGAC  441
 E  L  K  C  Q  L  *                                         83

ACAAGAAGACAACAAGAGGATCCTTTCGTACATTTGGCATCTTTATTGACATTCCATTTT  501
TCGAACAAGTAGTTTTATTTCTAGTAGCCACTTTGTGTCCATCCAATTAGCATTAATTAA  561
CATAAATTATCATTGCTATAAATAGCAGCCTCTGCAAGTGATATCGAATTTAGAATCAAG  621
TCTGCAAACCAAAAATATCACAATTTATTTCCTTAATGATGATCAGCAAATATTAATAAC  681
ACTAGCAATAATTTATAACTAAAATTTGTTGATTGGATTATAACACCATAATATGGATAT  741
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AAAAAAAAA                                                    810
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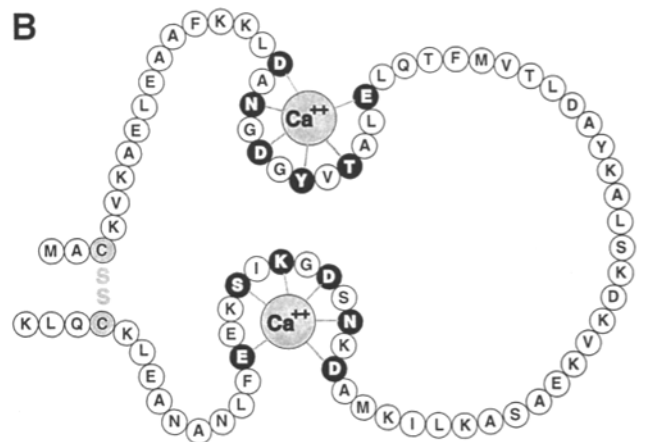
B

Figure 2. Calsensin sequence and diagram. (*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 nt 154 and the stop codon is located at nt 403 and is indicated by an asterisk. The polyadenylation signal consensus sequence lies between residues 755 and 762 and is shown in bold as is the in-frame upstream stop codon at nt 118. The two underlined regions denote the two calcium-binding loops. The predicted calsensin protein consists of 83 aa 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 aa sequence. 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 NH_2- and COOH-terminally situated cysteines.

signed primer and isolated *Haemopis* cDNA as a template. This indicates that the full-length coding region for calsensin has been identified. The predicted amino acid sequence of the open reading frame extends over 83 amino acids (aa) and has a calculated molecular mass of 9,117 D and a predicted isoelectric point of 8.6.

Northern blot analysis was performed by probing 20 μg of total leech poly(A)+RNA with a random-primed [^{32}P]dCTP-labeled probe synthesized from the NH_2-terminal 238-nt EcoRI fragment. The results show that under high stringency conditions a single band is detected of ~1.1 kb (Fig. 3 *A*). Furthermore, on Southern blots of genomic DNA the radiolabeled EcoRI fragment hybridizes to single EcoRI, SacI, and HindIII fragments in a pattern that directly correlates with the restriction map of the calsensin genomic clone (data not shown). These data make it likely that calsensin is encoded by a single gene that does not give rise to alternatively spliced forms.

To verify that the calsensin cDNAs isolated correspond to the lan3-6 antigen we performed in situ hybridizations with digoxygenin-labeled calsensin RNA probes and compared their labeling with that of the lan3-6 antibody in E10 *Haemopis* embryos (Fig. 3, *B* and *C*). In all hirudinid leeches examined the lan3-6 antibody labels an identical subset of sensillar and peripheral sensory neurons (Briggs et al., 1993); however, in *Haemopis* as well as in *Hirudo* a small number of central neurons are also labeled during development (Macagno et al., 1983). Thus, the calsensin distribution in the CNS of the *Macrobdella* embryo in Fig. 1 is different from that of the *Haemopis* embryo illustrated in Fig. 3 *C*. However, a comparison of the calsensin mRNA expression with that of the lan3-6 staining in Fig. 3, *B* and *C*, respectively, clearly shows that the pattern of labeling by the two probes of peripheral and central neurons is identical in *Haemopis* embryos. This provides strong evidence that calsensin indeed corresponds to the lan3-6 antigen.

Calsensin Is a Novel EF-hand Ca²⁺-binding Protein

The complete sequence of calsensin is shown in Fig. 2 *A*. The inferred protein product contains two regions which conform to the HLH-structure of the EF-hand family of calcium-binding domains (Strynadka and James, 1989). Fig. 2 *B* shows a putative diagrammatic representation of calsensin based on the structure of bovine ICaBP (Szebenyi and Moffat, 1986) which, like calsensin, is a small 9-kD calcium-binding protein with two EF-hand domains. The structural features of the loops as well as the calcium-chelating residues, which are highlighted in black (Fig. 2 *B*), conform to the consensus sequence for functional calcium-binding loops as reviewed in Strynadka and James (1989). An interesting feature of calsensin is the presence of two cysteine residues at the NH_2- and COOH-terminal ends at positions 3 and 80, respectively, suggesting that under some conditions the conformation of the protein may involve the formation of disulfide bridges.

Fig. 4 shows the sequence comparison of calsensin with four other EF-hand calcium-binding proteins, three of which contain the most homologous sequences in the data bank to calsensin. The fourth protein aligned is bovine ICaBP which is the most closely related calcium-binding protein to calsensin in terms of size, both being 9-kD proteins with two HLH-domains, although in terms of sequence homology they are quite divergent only sharing 25% of the residues. Furthermore, ICaBP has an anomalous Ca²⁺-binding loop I that contains two extra residues (Szebenyi and Moffat, 1986; Strynadka and James, 1989) (Fig. 4). Calsensin is most closely related to the two HLH domains (39% homology) of I-plastin (Lin et al., 1994), a 68-kD calcium-dependent actin-bundling protein found in human intestine, to protozoan calcineurin B (unpublished, accession number U04380) which is the calcium-binding part of the calcineurin complex which interacts with calmodulin (Persechini et al., 1989), and to *Drosophila* cal-
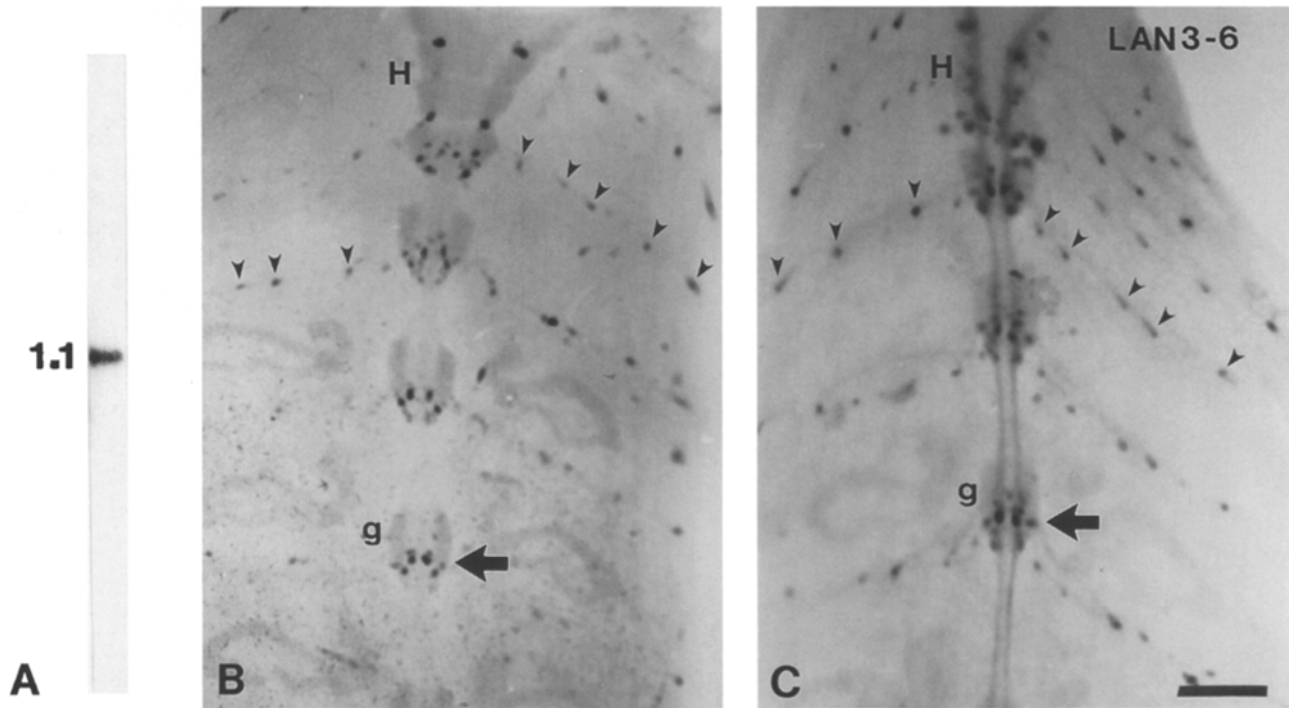
Figure 3. Northern blot and in situ hybridization analysis of calsensin transcripts. (*A*) Northern blot analysis of calsensin mRNA. 20 μg of total leech poly(A)$^+$ mRNA was fractionated on a 1.2% agarose formaldehyde denaturing gel, transferred to nitrocellulose, and probed under high stringency conditions using random primer-labeled calsensin sequences. A single band of approximately 1.1 kb was detected. (*B*) In situ hybridization with digoxygenin-labeled calsensin RNA probe to an E10 *Haemopis* 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 *Haemopis* embryo labeled with lan3-6 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. Bar, 75 μm.

modulin (Yamanaka et al., 1987). However, these proteins are much larger than calsensin and/or have double the number of EF-hand domains. Thus, while calsensin shares obvious sequence motifs with these EF-hand domain containing calcium-binding proteins it does not appear to be a homologue or orthologue of any known calcium-binding protein in the data bank. This suggests that calsensin represents a novel small calcium-binding protein which is expressed selectively in the nervous system.

Calsensin Is a Functional Calcium-binding Protein

To assay whether calsensin is a functional calcium-binding protein we constructed a fusion protein derived from the
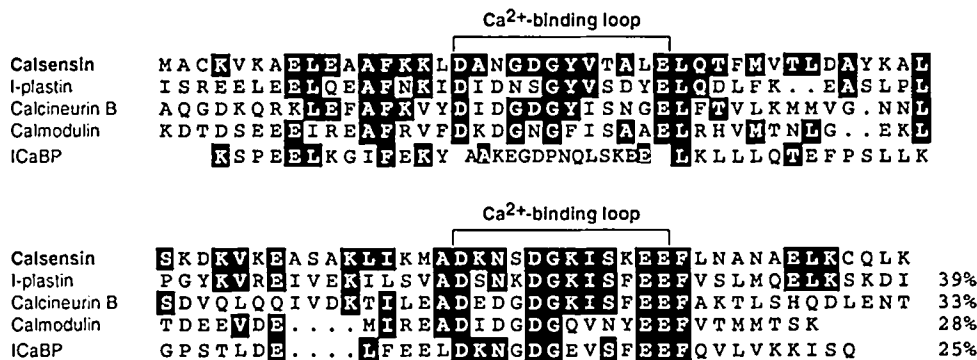
Figure 4. Sequence comparison of calsensin with other calcium-binding EF-hand proteins. The entire predicted sequence of calsensin is aligned with the most homologous sequence domains of human I-plastin (Lin et al., 1994), protozoan calcineurin B (unpublished, accession number U04380), *Drosophila* calmodulin (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.
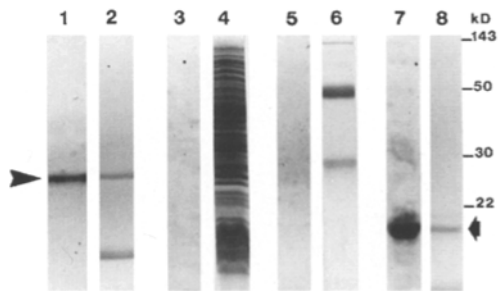
Figure 5. Calsensin binds $^{45}Ca^{2+}$ in vitro. Lane *1* shows $^{45}Ca^{2+}$ binding to partially purified calsensin fusion-protein (*arrowhead*) which was fractionated by SDS-PAGE and transferred to nitrocellulose. Lanes *3*, *5*, and *7* are control lanes treated in a similar way to lane 1 except protein extracts from DH5αF'IQ cells containing vector protein without insert (lane *3*), γ-globulin (lane *5*), and purified calmodulin (lane *7*, *arrow*) were assayed. The binding was visualized by autoradiography. Lanes *2*, *4*, *6*, and *8* show a sample of the proteins blotted in lanes *1*, *3*, *5*, and *7*, respectively, which were run on a parallel gel and stained with Coomassie blue. The lower band in lane *2* which does not bind $^{45}Ca^{2+}$ is a native biotinylated *E. coli* protein which copurifies with the calsensin fusion protein during avidin affinity chromatography.

calsensin clone using the PinPoint vector system that adds a short biotinylated sequence allowing for avidin affinity column purification of the fusion protein. The construct codes for residues 1-72 of calsensin, which encompasses both calcium-binding loops but truncates the COOH-terminal end. This fusion protein was partially purified over a Soft-link™ monomeric avidin column and transferred to nitrocellulose paper after SDS-PAGE. Then following the method of Maruyama et al. (1984), calcium-binding of calsensin was assayed by incubation with $^{45}Ca^{2+}$ and subsequent autoradiography. Fig. 5 shows that the calsensin fusion protein selectively binds $^{45}Ca^{2+}$ under these conditions in vitro. Control experiments demonstrated that the binding could be competed off with cold Ca^{2+} (data not shown), that the vector protein without calsensin insert in DH5αF'IQ cells as well as γ-globulin do not bind $^{45}Ca^{2+}$, and that purified calmodulin binds calcium in this assay (Fig. 5). Thus, these data indicate that calsensin is a functional calcium-binding protein that binds $^{45}Ca^{2+}$ in vitro.

Polyclonal Antibody Production to Calsensin

Since the mAb lan3-6 does not recognize the denatured antigen on immunoblots, a definitive biochemical characterization of the antigen had not been possible (Briggs et al., 1993). For these reasons and to characterize the calsensin protein in vivo, we used the calsensin fusion protein to generate new polyclonal antibodies to calsensin in rabbits. We obtained two antisera, Frigg and Hel, which recognize a single band not present in the preimmune sera migrating with a M_r of 10,000 on immunoblots of leech CNS proteins, as demonstrated for the Frigg antiserum in Fig. 6. Furthermore, a 10,000 M_r protein recognized by the Frigg antiserum is specifically immunoprecipitated from leech CNS proteins by the original mAb lan3-6 and this staining is abolished by preincubation of the antisera with calsensin fusion protein (Fig. 6). In addition, the Frigg antiserum (data not shown) labels E10 *Haemopis* embryos in
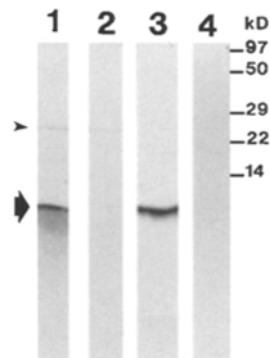
Figure 6. Polyclonal antiserum identifies calsensin as a 10,000 M_r protein on immunoblots of leech CNS extracts. Lane *1* shows labeling of a 10,000 M_r protein (*arrow*) from leech CNS extracts recognized by the Frigg antiserum which is not present in the preimmune serum (lane *2*). The Frigg antiserum 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 lan3-6 antibody coupled to protein A–Sepharose. A 10,000 M_r 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 M_r protein. Lanes *1*, *2*, and *4* show immunoblots of *Haemopis* CNS protein and lane *3* shows lan3-6 immunoprecipitated *Haemopis* CNS proteins fractionated by 20% SDS-PAGE.

a pattern indistinguishable from that of the mAb lan3-6 staining shown in Fig. 3 *C*. Thus both the Frigg and Hel antisera recognize the native calsensin protein and provide additional evidence that calsensin is the lan3-6 antigen. The close correspondence of the M_r for native calsensin with that predicted from the molecular analysis corroborates that we have identified the full-length sequence of calsensin.

A Large 200,000 M_r Protein Selectively Coimmunopurifies with Calsensin

In a previous study (Briggs et al., 1993) we reported that immunoaffinity purification of *Haemopis* CNS extracts with lan3-6 antibody bound to protein A–Sepharose beads yielded a protein of 200,000 M_r. This protein was hypothesized to be a possible candidate for the lan3-6 antigen, although as mentioned above, its identity could not be confirmed by immunoblot analysis using lan3-6 antibody since lan3-6 fails to recognize the denatured antigen (Briggs et al., 1993). However, with the cloning of calsensin and the production of new polyclonal antibodies that recognize native calsensin on immunoblots as a 10,000 M_r protein, the larger protein is clearly not the lan3-6 antigen. Due to the small size of calsensin it was not detected by standard SDS-PAGE and silver gel staining in the initial analysis. Thus these data raise the possibility that calsensin may be molecularly interacting with a larger protein which can be selectively copurified using immunoaffinity chromatography with calsensin antibodies. We therefore conducted further immunoaffinity purification experiments with the lan3-6 antibody coupled to protein A–Sepharose (Fig. 7) in order to ascertain that in addition to being present in *Haemopis*, the large protein also was present in *Macrobdella*, the leech species used in our developmental studies of selective fasciculation (Briggs et al., 1993). CNS extracts from both these leech species were applied to a lan3-6 antibody column which was washed with 0.1 M sodium borate, pH 8.2, before bound protein was eluted with glycine, pH 2.5, into 10 0.4-ml fractions in which the eluate was neutralized with sodium phosphate, pH 8. Fractions 1-2,
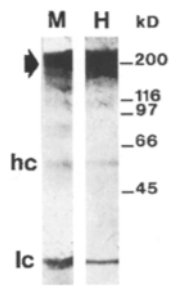
Figure 7. Lan3-6 immunoaffinity purification of leech CNS proteins. (*A*) A 200,000 M_r protein is selectively immunoaffinity purified from CNS extracts of both *Macrobdella* (*M*) and *Haemopis* (*H*) leeches by a lan3-6 protein A–Sepharose column. The figure shows pooled fractions which were silver stained after separation by 12% SDS-PAGE. In addition to the major 200,000 M_r 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.

3-5, and 6-10 were pooled, dialyzed against 10 mM Tris-HCl, pH 7, and precipitated with ethanol. After resuspension the eluted fractions were subjected to SDS-PAGE and the gels analyzed by silver staining. Fig. 7 shows that a prominent broad protein of approximately 200,000 M_r is being selectively affinity purified by this procedure in both *Macrobdella* and *Haemopis*. The fractions collected containing the 200,000 M_r band also contained small amounts of eluted primary antibody (Fig. 7). However, the pooled fractions 1-2 and 6-10, respectively, contained no proteins detectable by SDS-PAGE and silver staining of the gel. Immunoblot analysis of gels resolving the 200,000 M_r band shows no immunoreactivity using the newly generated calsensin antisera. Calsensin itself is too small to be retained in these gels but can be resolved by 20% SDS-PAGE as demonstrated in Fig. 6. Thus, a similar 200,000 M_r protein appears to interact with calsensin in both *Macrobdella* and *Haemopis*.

Discussion

This study reports the cloning and sequencing of cDNAs coding for calsensin, a novel neuronal EF-hand calcium-binding protein in leech originally identified by the mAb lan3-6. Several lines of evidence suggest that the calsensin cDNA corresponds to the antigen recognized by the lan3-6 antibody: (*a*) the labeling of leech embryos by in situ hybridization using calsensin sequence as probe exactly matches the lan3-6 antibody staining pattern, as does staining with polyclonal antisera raised against a calsensin fusion protein; (*b*) lan3-6 immunoprecipitates a protein which is specifically recognized by the newly generated calsensin antisera; (*c*) on immunoblots calsensin antibodies recognize a 10,000 M_r protein that correlates well with the predicted molecular mass of 9.1 kD based on the calsensin sequence. This data, together with the presence of an in-frame stop codon upstream of the initiating methionine, also suggests that the full-length sequence of calsensin has been identified.

Analysis of the calsensin sequence shows it belongs to a growing family of calcium-binding proteins that contain EF-hand domains. The members of this family, which include calmodulin and troponin C (Kretsinger 1980; Persechini et al., 1989), are characterized by containing a common calcium-binding motif composed of a loop of 12 contiguous residues with oxygen atoms involved in calcium-binding and two flanking α-helices that stabilize the complex. Functional EF-hand calcium-binding domains are always found in pairs (Persechini et al., 1989; Strynadka and James, 1989) and some members of the family have up to eight such domains, although not all of these may be functional (Persechini et al., 1989). Many EF-hand calcium-binding proteins are found exclusively in the nervous system (Baimbridge et al., 1992) where they often have a restricted expression to certain tissues and types of neurons. The function of many of the nervous system specific calcium-binding proteins is unknown (Baimbridge et al., 1992); however, they are generally classified into two separate functional groups, the trigger and the buffer proteins (Levine and Dalgarno, 1983; Baimbridge et al., 1992). The trigger proteins, such as calmodulin, change their conformation in response to calcium-binding and in turn can then interact with and modulate various enzymes and ion channels. The buffer proteins, such as parvalbumin and ICaBP, on the other hand, are believed to passively regulate the level of intracellular free calcium concentration. Some of these calcium-binding proteins are thought to be important for calcium-homeostasis in the nervous system and have been implicated in the pathology of various neurodegenerative disorders (Heizmann and Braun, 1992). Recently, a subfamily of small calcium-binding proteins with three EF-hand calcium-binding domains that may function in visual and odorant signal transduction have been described in retinal photoreceptors (Yamagata et al., 1990; Dizhoor et al., 1991; Palczewski et al., 1994) and in the rabbit olfactory epithelium (Nemoto et al., 1993).

The physiological role of calsensin is presently unknown. However, the two HLH-domains of calsensin conform to the consensus sequence for functional calcium-binding proteins (Strynadka and James, 1989) and calcium-binding assays using a calsensin fusion protein directly demonstrate that calsensin can bind $^{45}Ca^{2+}$ in vitro. Thus, calsensin is likely to be functioning as a calcium-binding protein in vivo as well. Furthermore, immunoaffinity purification experiments with the lan3-6 antibody show that a large 200,000 M_r protein selectively copurifies with calsensin from CNS extracts of both *Haemopis* and *Macrobdella* leeches. These results provide evidence that calsensin may be functioning as a trigger protein that interacts with and/or modulates the larger protein. In this context, it is interesting to note that calsensin has two cysteines at the NH_2- and COOH-terminal ends, respectively, which under certain conditions would have the capacity to form intra- or intermolecular disulfide bridges. This could potentially modulate the conformation of the molecule and be a requirement for interactions with other molecules. The calcium-binding protein CaVP in *Amphioxus* muscle is thought to possess such an internal disulfide bridge in vivo (Kobayashi et al., 1987). However, since cysteine residues generally are found in a reduced state in the cytosol (Branden and Tooze, 1991) we do not know whether this mechanism might be employed by calsensin.

The molecular features of calsensin and its association with the 200,000 M_r protein are consistent with the hypothesis that it may participate in a protein complex mediating calcium-dependent signal transduction events in growth cones and axons (Johansen et al., 1994; Jellies and

Johansen, 1995). It has become increasingly clear that the functions of many molecules involved in cell adhesion and fascicle formation, apart from being effected by their extracellular interactions, are also to a great extent determined by their associations with cytoplasmic proteins and cytoskeletal structures, associations which are often regulated by signal transduction processes and second messenger pathways (Schuch et al., 1989; Atashi et al., 1992; Hynes, 1992; Gumbiner, 1993). For example, the adhesive state of cadherin at the cell surface can be modulated in response to intracellular signals (Gumbiner, 1993) and both NCAM and N-cadherin participate in signal transduction events involving calcium which stimulate neural outgrowth (Doherty et al., 1991). Recently, experiments with chromophore-assisted laser inactivation of the intracellular calcium-binding protein calcineurin in cultured chick dorsal root ganglion neurons have suggested that the spatial distribution of calcineurin activity regulates growth cone motility (Chang et al., 1994). Thus, calcium-binding proteins present in growth cones and axons which modulate or are being modulated by $[Ca^{2+}]_i$ are strong candidates for being molecules that affect calcium-dependent processes of growth cone extension and pathway formation. It is therefore tempting to speculate that since calsensin has a very restricted expression common to all hirudinad leeches in a small subset of peripheral neurons which fasciculate together in a single tract during development, that the putative calsensin protein complex may play a role in the formation and/or maintenance of these specific axon fascicles. The cloning of calsensin and the immunoaffinity purification of its associated protein reported here should facilitate the generation of probes to test this hypothesis and to help in the determination of a physiological function for this novel calcium-binding protein.

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