Five Members of a Novel Ca\textsuperscript{2+}-binding Protein (CABP) Subfamily with Similarity to Calmodulin*

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Among organisms as diverse as yeast and human, changes in the intracellular Ca\textsuperscript{2+} ion concentration initiate an array of signaling pathways. Ca\textsuperscript{2+} ions function as a diffusible signal that exerts its effect directly or through Ca\textsuperscript{2+}-binding proteins on plasma membrane and intracellular channels, intracellular proteins involved in membrane trafficking, and a broad range of enzymes, including kinases, phosphatases, and adenyl cyclases. Ca\textsuperscript{2+}-binding proteins sense changes in [Ca\textsuperscript{2+}] through either 130-amino acid (aa)\textsuperscript{i} structural elements called C2 domains, 29-aa EF-hand motifs, or through acidic regions of proteins or protein-lipid interfaces. In a growing number of eukaryotic signaling proteins, C2 and EF-hand motifs are present as either a single copy or clustered in multiple copies (1).

The largest group of Ca\textsuperscript{2+}-binding proteins belongs to the calmodulin (CaM) superfamily. They are structurally related and comprise four EF-hand motifs, some of which (one or two) may be nonfunctional in Ca\textsuperscript{2+} coordination (2). Neuronal Ca\textsuperscript{2+}-binding proteins (NCPB) are a subset of the EF-hand-containing proteins, whose function is largely unknown. The sequence similarity among members of the NCPB family varies from \(~25%\) between CaM and visinin to \(~60%\) between GCAP1 and GCAP3 (3). NCPBs are acidic and similar in length. CaM and CaM-like proteins are the shortest (149–150 aa; molecular mass, 16,837 Da); other members of this family are \(~200\) aa long (molecular mass, \(~23,000\) Da) (2).

NCPBs also display a variety of interesting structural features. Multifunctional CaM contains a pair of N-terminal (EF-hand 1 and EF-hand 2) and C-terminal EF-hand (EF-hand 3 and EF-hand 4) motifs (4). The "dumbbell shape" of CaM undergoes a major conformational change upon Ca\textsuperscript{2+} coordination. These conformational changes result in reorientation of the two N- and C-terminal domains with respect to each other and a rearrangement of \(\alpha\)-helices in the N-terminal domain that makes the hydrophobic target peptide binding site more accessible (5). The flexible region of the central \(\alpha\)-helix bends and unwinds upon binding of CaM to many target proteins (6). Myristoylated recoverin, hippocalin, frequenin, GCAPs, and...
other Ca\textsuperscript{2+}-binding proteins compromise a separate subset of NCBPs (2). Binding of Ca\textsuperscript{2+} to recoverin induces more subtle changes in its compact structure, including the unclamping and extrusion of the myristoyl group. This so-called “myristoyl switch” may be unique to recoverin; other myristoylated NCBPs, like GCAP2, appear to have their hydrophobic group exposed to solvent permanently (7, 8). The transition is also accompanied by a 45° rotation of the N-terminal domain relative to the C-terminal domain, as well as the exposure of many hydrophobic residues (9). The main chain folds of neurocalcin and GCAP2 are similar to Ca\textsuperscript{2+}-bound recoverin, except for structural differences near the N terminus (residues 2–18) and the binding of Ca\textsuperscript{2+} to EF-4 (8, 10).

Seidenbecher et al. (11) cloned the cDNA of a neuron-specific Ca\textsuperscript{2+}-binding protein, which displays ~70% similarity with CaM within two C-terminal EF-hand motifs and high expression levels in the cerebral cortex, hippocampus, and cerebellum. This protein, named “caldendrin,” is an uncommon member of the CaM superfamily possessing a predicted molecular mass of 33 kDa. The ultra-structural localization in dendrites and the postsynaptic density were interpreted as evidence of an association with the somatodendritic cytoskeleton. Yamaguchi et al. (12) reported the cloning of a 70-aa-long form of caldendrin, termed “calbrain,” containing two putative EF-hand motifs. No reference to the caldendrin studies was provided, and it is difficult to evaluate whether the authors considered caldendrin as a novel gene or a spliced form of caldendrin. In situ hybridization studies revealed abundant expression of mRNA containing calbrain sequence in the hippocampus, in the habenular area of the epithalamus, and in the cerebellum. Calbrain antagonized CaM in stimulation of CaM kinase II. These two studies raised an important question about the relationship between caldendrin and calbrain.

Here, we report characterization of five novel neuronal Ca\textsuperscript{2+}-binding proteins: CaBP1, CaBP2, CaBP3, CaBP4, and CaBP5. These proteins display a new combination of functional EF-hand motifs and myristoylation. CaBP1, although structurally related to CaM, has a disabled EF2-hand motif and contains a consensus sequence for myristoylation. A large portion of the CaBP1 sequence is homologous to previously published caldendrin (11) and calbrain (12). We believe that caldendrin contains an extra sequence at the N terminus as a result of alternative splicing, although calbrain represents only a partial sequence. CaM-related to CaM, has a disabled EF2-hand motif and contains a consensus sequence for myristoylation. A large portion of the CaBP1 sequence is homologous to previously published caldendrin (11) and calbrain (12). We believe that caldendrin contains an extra sequence at the N terminus as a result of alternative splicing, although calbrain represents only a partial sequence.
Molecular Biochemicals) for 35 cycles with primers K65 (5'-GATG-GGCCAATCTGCTGACGTCG-3') and K7 (5'-CGCGCTCTACGACGGGAC-ATCATC-3') for CaBP1 (94°C for 30 s, 60°C for 30 s, 65°C for 1.5 min); primers K69 (5'-CATATGTGTCGACGACCGATAC-3') and K72 (5'-CATACCGACATCATCNGC-3') for CaBP2 (94°C for 30 s, 60°C for 30 s, 65°C for 1.5 min); primers K60 (5'-CATATGGCTGGTCTGCCG- ATCATC-3') and K30 (5'-CTCCCCATCTCATCCATTGGCCA-3') for CaBP5 (94°C for 30 s, 60°C for 1.5 min); and primers G3PDH-5p (5'-GAAGG- GCTAATGACCAAGTGCTT-3') and G3PDH-R (5'-TACGCGTAATGGTTGATCAGG-3') for mouse G3PDH (94°C for 30 s, 60°C for 1.5 min).

**Chromosomal Localization of CaBP2 and CaBP3/5 by Fluorescence in Situ Hybridization**

The localization of CaBP2 and CaBP3/5 was carried out by Genome Systems, Inc. using fluorescence in situ hybridization. DNA from CaBP clones were labeled with digoxigenin dUTP by nick translation. Labeled genomic clones from the identified loci were co-hybridized with CaBP clones.

**Preparation of Anti-CaBP Polyclonal Antibodies**

Rabbit anti-CaBP polyclonal antibodies were raised in New Zealand White rabbits by subcutaneous immunization with ~50 μg/50 μl of the antigen solution mixed with an equal volume of Freund's adjuvant (Cocalico Biologicals, Inc., Reamstown, PA). Animals were boosted at 1-2-week intervals with 25 μg of antigen solution mixed with an equal volume of Freund's adjuvant. UW72, UW73, and UW75 were raised against bacterially expressed CaBP1 (aa 25-227), UW92 against bacterially expressed CaBP2, and UW99 against bacterially expressed CaBP5.

**Affinity Chromatographies**

**Purification of Native Retinal CaBPs**—A retinal extract containing CaBPs was prepared by homogenizing 50 bovine retinas in 50 ml of water, containing 2 mM benzamidine and 20 μg/ml leupeptin. The extract, separated from retinal particulates by centrifugation (48,000 × g for 50 min) at 4°C, was then added to a final concentration of 15% Triton X-100. Calcium or magnesium ions were added to yield a final concentration of 10 mM CaCl2 or MgCl2, and the sample was loaded on a C4 column (W-Porex 5 C4, 4.6 × 150 mm, Phenomenex) equilibrated with 30% CH3CN in 0.1% trifluoroacetic acid. CaBPs were eluted with a linear gradient of CH3CN (30-60%) in 0.1% trifluoroacetic acid at a flow rate of 0.5 ml/min (0.5 ml fractions were collected). Final purification was obtained on a C8 column (Vydac 2990TP, 2.1 × 250 mm) equilibrated with 30% CH3CN in 5 mM BTP, pH 7.5. CaBPs were eluted with a linear gradient of CH3CN (30-60%) in 0.1% trifluoroacetic acid at a flow rate of 0.2 ml/min (0.2 ml fractions were collected). The separation of different forms of CaBPs was obtained on SDS-PAGE.

**Microsequencing of Peptides Derived from CaBPs**

Electrotransfer of proteins from SDS-polyacrylamide gels onto an Immobilon-PSQ transfer membrane (Millipore, Bedford, MA) was performed in 10 mM BTP, pH 8.4, containing 10% methanol at 90 V for 1 h at 4°C. After transfer, the membrane was stained for 10 min with 0.1% Coomassie Brilliant Blue R-250 in 45% methanol, 7% acetic acid and then destained in 45% methanol, 7% acetic acid for approximately 10 min. The excised bands were in situ tryptic digested using 0.2 μg of sequencing grade trypsin (Promega) in 1% (w/v) Zwittergent 3-16 (Calbiochem) in 0.1% ammonium bicarbonate for 2 h at 37°C (13) and fractionated by reverse phase HPLC (14) using a 0.8-μm Vydac C18 column. Selected peak fractions were analyzed by a combination of delayed extraction matrix-assisted laser-desorption/ionization reflectron time-of-flight mass spectrometry (MALDI re-TOF MS; REFLEX III, Bruker-Franzen, Bremen, Germany) and automated Edman sequencing (model 477A, PE Applied Biosystems, Foster City, CA). (15). Peptide monoisotopic masses were summed from the identified residues using ProComp version 1.2 software (obtained from Dr. P. C. Andrews, University of Michigan, Ann Arbor, MI).

**Expression of CaBPs in Escherichia coli**

The coding sequences for the long and short h-CaBP1 were amplified from human retina cDNA library by PCR using K5 (5'-CATATGGCAATCTGCTGACGTCG-3') and K7 (5'-CGCGCTCTACGACGGGACATCATC-3') through 35 cycles at 94°C for 30 s, 65°C for 1.5 min. The resulting sequence was amplified from a cDNA library with primers K3 (5'-CATATGGAACAAACTCTCCAAGCCGAC-3'), which placed an Ndel site on the ATG and K6 (5'-TACGACGTTGATGATGATGATGATGCGCAGACATCATCCCGAAAAC3'), which adds a His tag at the C terminus of the protein. The PCRs were cycled at 94°C for 30 s, 56°C for 30 s, and 68°C for 2 min. The coding sequence for m-CaBP5 was amplified from the mouse retina cDNA library with primers K42 (5'-TACGACGTTGATGATGATGATGATGCGCAGACATCATCCCGAAAAC3') and K14 (5'-TACGACGTTGATGATGATGATGATGCGCAGACATCATCCCGAAAAC3') and then destained in 45% methanol, 7% acetic acid for approximately 10 min. The reactions were cycled 35 times through 94°C for 30 s, 60°C for 30 s, and 68°C for 1.5 min. The short form of m-CaBP5 (without 1-4 aa) was amplified with primers K60 (5'-CATATGGTCTGCTGCTGCTGATACTC-3') and K16 (5'-TACGACGTTGATGATGATGATGATGCGCAGACATCATCCCGAAAAC3') and added to K17 (5'-TACGACGTTGATGATGATGATGATGCGCAGACATCATCCCGAAAAC3'), which adds a His tag at the C terminus of the protein. The reactions were cycled 35 times through 94°C for 30 s, 60°C for 30 s, and 68°C for 1.5 min. The PCR product for each CaBP was cloned in the pCRII-TOPO vector and sequenced by dyeexeterodinator sequencing (ABI-Prism, Perkin-Elmer). The coding sequences were cloned as fragments inside BamHI to the pET-3b vector (Novagen). CaBPs were expressed in BL21 bacteria after induction with 0.2 mM isopropyl-β-D-thiogalactoside.
human retina cDNA library with primers K56 (5'-AGGCTCTCTCAGGAGG-3') and K94 (5'-GGGCGGATCCTCCGACC-3'). The PCR were cycled 35 times at 94 °C for 30 s, 55 °C for 30 s, and 68 °C for 2 min. Both PCR products (0.5 and 0.7 kb) were cloned in pCR-TOPO vector and sequenced by dyeexeterminator sequencing (ABI-Prism, Perkin-Elmer). Both the short form (h-CaBP1) and the long form (Lh-CaBP1) of CaBP1 were cloned as a fragment EcoRI-EcoRI in pEG-FPF-1 opened XhoI-EcoRI.

Expression of CaBP1-GFP Fusion Proteins in CHO Cells

Twenty micrograms of pcDNA3.1, pEGFP-1, pSh-CaBP1-GFP, or pLh-CaBP1-GFP were transiently transfected into 50–80% confluent CHO cells using co-precipitates of calcium phosphate and DNA (21). Three days after transfection, cells were plated on glass coverslips, placed in culture dishes, and incubated overnight at 37 °C. Four days after transfection, fluorescent transfected cells were analyzed with a Bio-Rad MRC-600 confocal microscope.

Immunocytochemistry

Adult C57BL/6 mice were anesthetized with sodium pentobarbital (Nembutal) delivered by intraperitoneal injection. Animals were perfused transcardially with 20 ml of 4% formaldehyde in 86 mM NaPO4, pH 7.3. Eyes were removed and incised at the limbus to facilitate rapid fixation of the retina. After 10 min in fixative, the anterior segments and lenses were dissected from the eyes, and the eye cups were returned to the fixative. Eyes were immersed in 4% formaldehyde in 86 mM NaPO4, pH 7.3, for 4 h on ice. Residual aldehydes were removed by washing eye cups repeatedly in 137 mM NaPO4, pH 7.3 (3 x 20 min). Retinal tissue was prepared for confocal immunofluorescence labeling according to Hala and Matsumoto (22). Briefly, eye cups or peeled retinas were embedded in disposable weigh-boats containing molten low gel temperature agarose (5%) (Sigma) in phosphate-buffered saline (PBS), pH 7.3. Eyes were removed and incised at the limbus to facilitate rapid fixation of the retina. After 10 min in fixative, the anterior segments and lenses were dissected from the eyes, and the eye cups were returned to the fixative. Eyes were immersed in 4% formaldehyde in 86 mM NaPO4, pH 7.3, for 4 h on ice. Residual aldehydes were removed by washing eye cups repeatedly in 137 mM NaPO4, pH 7.3 (3 x 20 min). Retinal tissue was prepared for confocal immunofluorescence labeling according to Hala and Matsumoto (22). Briefly, eye cups or peeled retinas were embedded in disposable weigh-boats containing molten low gel temperature agarose (5%) (Sigma) in phosphate-buffered saline containing 0.05% sodium azide at ~40 °C. Agarose was solidified on ice for 1 h. Agarose blocks containing retinal tissue were trimmed and attached to metal stubs with cyanoacrylate adhesive. A Leica vibrating microtome was used to cut 100-μm slices of tissue, which were transferred to phosphate-buffered saline prior to immunolabeling. To reduce nonspecific labeling, retinal sections were incubated for 1 h in ICC buffer (phosphate-buffered saline, 0.5% bovine serum albumin, 0.5% Triton X-100, 0.01% sodium azide, pH 7.3), containing 5% normal goat serum. Sera from rabbits used for CaBP antibody production were diluted in ICC buffer as follows: UW72/CaBP1, 1:200; UW98/CaBP5, 1:400. Sections were incubated in primary antibody for 12 h at 4 °C. A monoclonal antibody to PKC (MC5 clone, Amersham Pharmacia Bio-tech) was diluted 1:50 and used for double-labeling studies to identify rod bipolar cells. Sections were then washed repeatedly in ICC buffer (3 x 20 min /1 x 60 min). Sections were incubated for 4 h in Cy3-conjugated goat anti-rabbit antibody (Jackson ImmunoResearch, West Grove, PA) diluted 1:200 in ICC buffer and subsequently washed in ICC buffer 3 x 20 min and 1 x 60 min. Sections were overexposed in 50 ml of 5% p-mercaptoethanol in glycerol to retard photobleaching. Sections were analyzed and images collected using a Bio-Rad 600 laser scanning confocal microscope located at the W. M. Keck Center for Advanced Studies of Neural Signaling (University of Washington School of Medicine). Negative controls for immunolabeling studies included each of the following steps: omission of primary antibody; incubation in preimmune sera at matching dilutions from rabbits used for polyclonal production; and adsorbed controls using purified CaBP1 (for UW72) or CaBP5 (UW98/600 nm final concentration) to abolish immunolabeling.

Protein Determination, SDS-PAGE, and Immunoblotting

The protein concentration was determined by the Bradford method (23). SDS-PAGE was performed according to Laemmli (24) using 15% polyacrylamide gels. The electrophoresis of protein onto Immobilon-P (Millipore) was carried out using a Hoefer mini-gel system. For immunoblotting, membranes were blocked with 5% (w/v) gelatin in 20 ml Tris/HCl, pH 8.0, containing 150 mM NaCl and 0.05% Tween 20, and incubated for 1–2 h with primary antibody at dilutions of 1:1,000. A secondary antibody conjugated with alkaline phosphatase (Promega, Madison, WI) was used at 1:5000. Antibody binding was detected using 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium.

Models of CaBP

Homology models of h-CaBP1, h-CaBP2, and h-CaBP5 were created on the basis of the crystal structure of h-CaM in its Ca2+-bound state (Protein Database entry: 1CLL at 1.75 Å resolution (25)), making use of the sequence alignment between these proteins (Fig. 1A). Because the N-terminal end of CaM is significantly shorter than that of the h-CaBPs, for example, residues 1–19 of h-CaBP1 and residues 1–22 of h-CaBP5 were omitted from the model.

The models were generated with the HOMOLOGY module of IN-SIGHTII software (Molecular Simulations, Inc., San Diego, CA) using established homology modeling protocols (26). In short, protein backbone coordinates were taken from CaM for all helices, strands, EF-hands, and loops with identical lengths. The coordinates of conserved side chains were kept. Nonconserved side-chains were built from a rotamer data base.

The 4-residue insertion in the long central helix of CaBPs between the two domains of the proteins was assumed not to distort or cause significant bending of this structural element. Therefore, the residues were built in helical conformation. Experimental support for this approach comes from a mutation study with CaM in which 2 residues were deleted from the central helix. A crystal structure showed only helix shortening leading to a different separation of the two protein domains and a change in relative orientation of the domains (27). Coordinates for the loop with the 1-residue insertion were obtained by transplantation from an appropriate Protein Database entry. Finally, 2000 steps of conjugate gradient energy minimization were executed to alleviate small irregularities in the structure.

RESULTS

Molecular Cloning of Novel Subfamily of Ca2+-binding Proteins from the CaM Superfamily—A search of the EST data base with a query sequence corresponding to the Ca2+-binding loops of GCAP1 resulted in the identification of a partial sequence of a novel Ca2+-binding protein with similarity to CaM, termed CaBP1 (EST AA364517 and AA363865 from a human pineal gland cDNA).

The complete sequence of h-CaBP1 was cloned by PCR from a human retina cDNA library in two overlapping fragments using primers from the EST AA364517 sequence. The full-length cDNA contains an open reading frame encoding a protein of 227 aa (Fig. 1A, Lh-CaBP1), closely related to CaM (Fig. 1B). The first ATG is set within a favorable context for translation initiation, with an A in position −3 and a G in +4 (28). PCR amplification of the full coding sequence of h-CaBP1 yielded two bands of variable intensities, one with the expected size and a shorter product with a 180-base pair deletion at the 5'-end region. The longer product encodes a protein that differs from S-CaBP1 by a 60-amino acid insert located at the N-terminal region (underlined in Fig. 1A).

b-CaBP1 and m-CaBP1 cDNA were amplified from cDNA libraries, using primers from h-CaBP1. Long and short forms of CaBP1 were cloned from bovine and mouse cDNA libraries. The amino acid sequences show a putative site for N-terminal myristoylation at Gly2 and three putative functional EF-hands, EF-hand 1, 3, and 4 motifs. Changes within the EF-hand 2 motif (Asp in position 1 instead of Asp, Gly in position 5 instead of Pro) are found in all three EF-hands. The sequence alignment between these proteins (Fig. 1) reveals high similarity to any other known protein and has an overall aa composition similar to the rest of the protein.

Ca2+-binding Proteins

The 60-aa insert has no significant bending of this structural element. Therefore, the residues were built in helical conformation. Experimental support for this approach comes from a mutation study with CaM in which 2 residues were deleted from the central helix. A crystal structure showed only helix shortening leading to a different separation of the two protein domains and a change in relative orientation of the domains (27). Coordinates for the loop with the 1-residue insertion were obtained by transplantation from an appropriate Protein Database entry. Finally, 2000 steps of conjugate gradient energy minimization were executed to alleviate small irregularities in the structure.
that are identical among CaBPs are shaded aa residues are absent in the short spliced forms. The letters underlined in intron/exon junctions in CaBP3 and in alternative splice forms of CaBP1 and CaBP2. The open box motif is in an black background. The sequences included, with their GenBank TM/EMBL accession numbers in parentheses, are: h-CaM (A31920); h-CaM-like protein B (P37235); NCS1: rat neuronal Ca2$^{2+}$ sensor 1 (P36610); VILIP1: human visinin-like protein 1 (U14747); VILIP2: rat visinin-like protein 2 (P35332); VILIP3: (P27482); rat caldendrin (Y17048); human recoverin (S62028); GCAP1 (L36859); GCAP2 (see Ref. 30); GCAP3 (AF110002); chicken visinin (P27728); bovine neurocalcin (JH0616); VILIP1: human visinin-like protein 1 (U14747); VILIP2: rat visinin-like protein 2 (P35332); VILIP3: human visinin-like protein 3 (P57235); NCS1: rat neuronal Ca$^{2+}$ sensor 1 (P36610); CaBP4 (unpublished sequence); CaBP1, CaBP2, CaBP3, and CaBP5 are novel sequences reported in this study.

PCR amplification of the full-length coding sequence of m-CaBP2 yielded two products, the expected 221-aa-long product and a shorter product encoding for a protein of 160 aa. The extra 61 aa, present in the long form of m-CaBP2, localize at the same position where the extra 60 aa are present in the long form of CaBP1. A 10-aa-long fragment (LV/LGPACIFLR) of this extra peptide is 90% similar between both CaBPs. The aa sequences show three putative functional EF-hands, EF-hands 1, 2, and 3, with the EF-hand 2 motif partially deleted in CaBP2. The predicted $M_r$ are 24,834 and 18,279 for the long form and short forms of m-CaBP2, respectively. Screening of a human BAC genomic library with CaBP2 cDNA probe resulted in a BAC genomic clone containing this gene. The locations of the introns were identified by comparison with the genomic structure of CaBP1. Each intron was amplified by PCR with primers designed in the exons, and the PCR products were used to sequence the exon/intron junctions. All the exon/intron junctions were at the same position compared with the other CaBP genes (Fig. 1A). Like CaBP1, the alternatively spliced exon 2A of CaBP2 is isolated from exon 1 and exon 2B by 2 introns (not shown). The deletion of part of the EF-hand 2 motif occurred just at the position of an intron but conserved the open reading frame. The CaBP2 gene is much smaller (~5 kb) than the CaBP1, CaBP3, and CaBP5 genes (~16 kb) (sequences deposited in the GenBank TM/EMBL Data Bank).
Using the sequence of CaBP2, we identified a genomic clone CIT-HSP-1337H24 (GenBank™/EMBL accession number AC005849), containing a novel CaBP4 (~75% similarity with other CaBPs, Fig. 1, A and B). The intron/exon junctions of h-CaBP4 (Fig. 1A) were in identical positions as in other CaBPs, as determined by comparing cDNA sequences with the genomic sequence. CaBP4 is highly conserved between species (data not shown). It appears that CaBP4 gene is expressed at low levels in the retina, as well as in other tissues. CaBP4 was not characterized in this study.

Unusual Structures of CaBP3 and CaBP5 Genes That Partially Overlap on Complementary Strands of DNA—Cloning of CaBP5 was helped by EST W22993 (deposited by Dr. J. Nathans, John Hopkins School of Medicine, MD) obtained from adult human retina cDNA. This clone was found through searching EST data bases with CaBP1 as a query sequence. EST AA318398 (deposited by A. R. Kerlavage, the Institute for Genomic Research, Rockville, MD) also covers part of h-CaBP5. h-CaBP5 was cloned by PCR from a human retina cDNA library in two overlapping fragments using a primer from EST W22993 and a primer from the arm of the vector. The translation initiation ATG is difficult to assign, as there is no consensus pattern for eukaryotic translation initiation. Moreover, the second in frame Met residue shows a putative myristoylation site. The first ATG has been assigned (Fig. 1A) as the first in frame Met residue because the sequence is conserved between species from this first ATG and, furthermore, the presence of Gly close to the second ATG is not myristoylated as shown below in myristoylation analysis. This open reading frame codes for a protein of 173 aa with three putative functional EF-hands (EF1, EF3 and EF4). b-CaBP5 and m-CaBP5 have been cloned from bovine and mouse retina cDNA libraries, respectively, following the same procedure. h-CaBP5 shares 96% similarity with m-CBP5 and b-CaBP5. h-CaBP5 shares 58% similarity with h-CaM (Fig. 1B).

Using primers from EST W22993, covering part of h-CaBP5, a fifth CaBP cDNA has been cloned by PCR from human retina cDNA library. The open reading frame encodes a protein of 192 aa with only two putative functional EF-hands, EF3 and EF4 (h-CaBP3, Fig. 1A). Attempts to clone CaBP3 from bovine and mouse have not been successful so far. Two-thirds of CaBP3 cDNA has 99.5% similarity to CaBP5 in both translated and untranslated regions at the cDNA 3′-end. At the 5′-end, CaBP3 sequence is complementary and reverse to CaBP5 sequence, suggesting an overlapping organization of both genes.

A BAC genomic clone was obtained by screening of a human BAC genomic library with the full-length CaBP3 cDNA probe. The exon/intron junctions of CaBP3 gene were obtained by sequencing of PCR products or the BAC clone. The CaBP3 gene consists of 7 exons spanning >15 kb of the genomic clone (Fig. 2B). The sequence of CaBP3, complementary and reverse to CaBP5, is encoded by exons 1–3A. To prove that both CaBP genes are on complementary strands, a PCR was carried out with a single primer (K28) from exon 4, located outside the

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**Fig. 2. Organization of h-CaBPs genes.** The coding regions are shown in black boxes, and noncoding regions of exon 1 and exon 6 are shown in white boxes. Introns are shown as thick lines. A, organization of the h-CaBP1 gene. The gray outlined box indicates the position of the closest exon specific to the caldendrin gene. The numbers below CaBP1 indicate the position of the exon/intron junctions in the CaBP1 cDNA. B, organization of h-CaBP3 and h-CaBP5 genes. Gray boxes indicate the position of exons in nonoverlapping complementary strands. The gray outlined box indicates the extra exon (exon 3B) of the CaBP3 gene that is part of exon 3 in the CaBP5 gene. Arrows indicate selected primers (K, forward primers; k, reverse primers) used to amplify the introns. Numbers below CaBP3 and above CaBP5 indicate the position of the exon/intron junctions in the CaBP3 and CaBP5 cDNAs. C, agarose-gel separation of PCR products for CaBP3 and CaBP5 gene analysis. Selected PCR products (indicated by arrows) generated with primers indicated above the gel, were cloned or purified and partially sequenced to determine the organization of the CaBP3 and CaBP5 genes.
The CaBP5 gene consists of 6 exons spanning >14 kb of the genomic clone. In comparison, CaBP3 has an extra exon (exon 3B) of 10 bases which is part of exon 5 in CaBP5. Exons 4, 5, and 6 of CaBP3 have 99.5% similarity with exons 4, 5, and 6 of CaBP5. The lengths of CaBP5 introns are the same as the lengths of CaBP3 introns except for intron 3. Intron 3 of CaBP5 corresponds to introns 3A and 3B of CaBP3. Intron 3 is ~1 kb for CaBP5 (Fig. 2B, k54-k28): Intron 3A + 3B covers ~3 kb for CaBP5 (Fig. 2B, k44-k28). All intron/exon junctions of CaBP5 follow the gt-ag rule. One consequence of the partial overlapping organization of these CaBP genes is that the overlapping intron/exon junctions of CaBP3 do not have consensus gt-ag sequences (U2-type intron) but have complementary ct-ac sequences, which is not the rare consensus at-ac sequence.

The introns of CaBP5 (Fig. 1A, arrows above the sequences) localize at the same positions as the introns of CaM except intron 4, which is 2 aa upstream from intron 4 of CaM (Fig. 2C, arrows below the sequences). The location of CaBP5 introns is identical to the position of CaBP1 introns (Fig. 1A). CaBP Genes Are Not Clustered on One Chromosome—In contrast to S100 proteins (chromosome 1Q12–25) (29) or GCAP1 and GCAP2 (chromosome 6p.21.1) (30), CaBPs do not cluster on one chromosome in humans. The CaBP1 gene is located on chromosome 12, based on the characterization of the 44N10 genomic clone (GenBank™/EMBL accession number Z97197); the CaBP2 gene was found on band 11q13.1 by fluorescence in situ hybridization (FISH) analysis using a BAC genomic clone as a probe (present studies, data not shown); CaBP3 and CaBP5 genes were localized to band 19q13.33 by FISH analysis using a BAC genomic clone as a probe (present studies, data not shown); and the CaBP4 gene is located on chromosome 11, based on the characterization of the genomic clone CIT-HSP-13.37H24 (GenBank™/EMBL accession number AC005849).

The Expression Patterns among CaBPs Differ Significantly—Tissue distribution of CaBPs mRNA was analyzed through diverse approaches. Northern blot analyses with CaBP cDNAs as probes showed a transcript of 1.7 kb in retina and in brain for CaBP1 (Fig. 3A), weak bands of 1.5 and 1.3 only in the retina for CaBP2 (Fig. 3B), and a product of ~1.9 kb only in retina for CaBP3/5 (Fig. 3C). A Master RNA Blot containing polyclonal antibodies in rabbits. One of these antibodies, UW72, was used to immunolabel CaBP1-expressing neurons in mouse retina. The antibody immunolabeled two distinct subsets of neurons in the inner nuclear layer (INL) (Fig. 4A). One group of CaBP1-positive cells has somata located in the center of the INL and...
the other, at the inner border of the INL. CaBP1-positive cells located at the inner border of the INL have a morphology and distribution characteristic of amacrine cells. The middle of the INL contains somata from a mixed population of neurons (rod and cone bipolar neurons) and glia (retinal Müller cells). In double-labeling studies undertaken to identify this subset of CaBP1-expressing cells, UW72 and anti-PKC (a marker for retinal rod bipolar cells) only rarely co-localized within the same INL neurons, indicating that CaBP1 is not expressed at detectable levels in the vast majority of rod bipolar cells. Similar double-labeling studies with antibodies to glial fibrillary acidic protein and cellular retinaldehyde binding protein (Müller cell markers) demonstrate that CaBP1 is not expressed in this cell type (data not shown). CaBP1-expressing cells in the center of the INL resemble cone bipolars, but definitive identification of these cells awaits future studies.

The polyclonal antibody UW89, which was specific for CaBP5 (see Fig. 4B), immunolabeled rod, and cone bipolar cells in the retinas from a variety of mammalian species, including mouse, bovine, baboon, and human. UW89 immunolabeling is most intense in the perikarya of these retinal neurons, located in the outer half of the INL. Fine processes of these bipolar cells are also immunolabeled, including dendrites in the outer plexiform layer (OPL) and axons ramifying in three distinct strata in the inner plexiform layer (IPL). Bipolar cells comprise approximately 40% of the cells in the INL, and in rod dominant retinas like the mouse, the vast majority of these bipolar cells are rod bipolar cells. Double-labeling studies with UW89 and rod bipolar cell-specific antibodies (PKC) confirm that virtually all PKC-positive rod bipolars are also UW89 immunopositive. These double-labeled neurons have axons that terminate in layer 5 of the inner plexiform layer, a characteristic of rod bipolar cells. A subset of cells are UW89-positive but PKC-negative. These cells have nuclei located in the center of the INL and possess axons that ramify in two layers within the outer portion of the inner plexiform layer; both morphological features are consistent with cone bipolar cells. Double-labeling studies with horizontal cell markers (calbindin) and Müller cell markers (glial fibrillary acidic protein and cellular retinalde-

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**Fig. 4.** Immunolocalization of CaBP1 and CaBP5 in mouse retina. CaBP1 (UW72) (A) and CaBP5 (UW89) (B) expression is prominent in cell bodies located in the INL. CaBP1 expression is restricted to a small subset of INL neurons, while CaBP5 is expressed by a majority of INL neurons. No immunolabeling is visible in sections of mouse retina incubated in pre-immune sera from UW72 (C) or UW89 (D). Preincubation of UW72 sera with purified CaBP1 (600 nM) abolishes CaBP1 immunolabeling (E). Preincubation of UW89 sera with purified CaBP5 (600 nM) abolishes CaBP5 immunolabeling (F). Sections of mouse retina were double-labeled with antibodies to PKC and either CaBP1 (G) or CaBP5 (H). PKC immunolabels rod bipolar cell bodies and their processes (green). Very few CaBP1-labeled cells (red) are PKC-positive. In contrast, most CaBP5-positive cells (red) are double-labeled by antibodies to PKC (arrowheads, H), confirming their identity as rod bipolar cells. Cone bipolars are labeled by antibodies to CaBP5 but not PKC. Magnification bar: A–F, 50 μm; G–H, 25 μm.
hyde binding protein) confirm that CaBP5 is not expressed at detectable levels in either of these cells types in adult animals. Specificity of UW89 antibody was confirmed by preadsorbing this antibody with an excess of purified CaBP5 prior to immunolabeling, which abolished the signal.

Two Splice Forms of CaBP Show Different Cellular Localization—To identify the subcellular localization of the long and short forms of CaBP1, two constructs carrying fused GFP to the coding regions of CaBP1 were made. pSh-CaBP1-GFP (short) and pLh-CaBP1-GFP (long) were transiently transfected into CHO cells and analyzed using confocal microscopy. S-CaBP1 localized at or near the plasma membrane (Fig. 5 C), whereas L-CaBP1 was associated most likely with the cytoskeletal structures (Fig. 5 D). These data suggest that the splice forms may have distinct localization patterns, producing further diversification among CaBPs.

CaBPs Are Translated and Can Be Isolated from the Retina—To identify CaBPs in bovine retina, soluble proteins were extracted with low ionic strength buffer, separated from membranous material by ultracentrifugation and passed through a UW75-Sepharose column. UW75 was raised against the common domain of CaBP, UW92 (raised against CaBP5), and UW89 (raised against CaBP5). C, retinal CaBPs were purified as described under “Materials and Methods” and blotted on polyvinylidene difluoride. The protein bands were digested with trypsin, and peptides were separated on a reverse phase column. The aa sequence of peptides was obtained using a combination of Edman degradation of matrix-assisted laser desorption/ionization (MALDI). In brackets are the predicted residues from the cDNA sequence and the N-terminal side of trypptic digestion: x represent an undetermined aa, dotted points show that the sequence did not reach the end of the peptide. cont, contaminants from UW72. D, Western blot of retinal CaBPs probed with UW72 in the presence or absence of 100 μM Ca^2+.

CaBP1 and CaBP2 Are Myristoylated—CaBP1 (MGNCVK-
CaBP1 and CaBP2 were used in the assay of CaM kinase II. The activity of CaM kinase II from rat brain was measured as a 

Activation of CaM kinase II by CaBPs—To identify potential targets for CaBPs, we have tested known effector proteins that are regulated by CaM. This approach was justified by close similarity of CaBPs with CaM. Furthermore, it was reported that calbrain inhibited the CaM protein kinase II activity (12). In our assay conditions, all CaBPs were able to stimulate CaM kinase II (Table I, Fig. 8A). These differences most likely resulted from the fact that calbrain represents only a partial clone of CaBP1. From all CaBPs, CaBP2 was the most effective in the CaM kinase II stimulation (Fig. 8A). CaBP2 also bound most avidly to a peptide derived from the CaM-binding domain of CaM kinase II (Fig. 8B), whereas CaBP1 and CaBP5 did not bind significantly.

Inhibition of GRK2 and GRK5 Activities by CaBPs—We also tested whether CaBPs can inhibit GRK activity, known to be potently modulated by CaM (reviewed in Ref. 31). At the highest concentrations tested (CaBP1 ~ 0.8 μM, CaBP2 ~ 6 μM, CaBP5 ~ 20 μM) none of the CaBPs significantly inhibited the activity of GRK2 in either the absence or presence of Gβγ (data not shown). However, CaBPs inhibited GRK5-mediated phosphorylation of rhodopsin (Fig. 9). CaBP1 appears to have a much higher affinity for GRK5 (IC50 ~0.12 μM) compared with CaBP2 (IC50 ~5.5 μM) and CaBP5 (IC50 ~2.5 μM) (Fig. 9). CaBPs also inhibited GRK5 phosphorylation of a soluble sub-

### Table I

| Ca2+-binding protein | Maximal activity | Activation EC50 |
|----------------------|-----------------|-----------------|
|                      | nmol/min/mg     | ppm             |
| CaM                  | 187.5 ± 19      | 138 ± 12        |
| CaBP1                | 14.5 ± 3        | 360 ± 120       |
| CaBP2                | 62.5 ± 12       | 241 ± 16        |
| CaBP5                | 6.25 ± 1.1      | ND              |

The activity of CaM kinase II from rat brain was measured as a Ca2+-dependent phosphorylation of syntide-2 (BioMol Inc.) for 30 min at 30 °C (12). The reaction was carried out in 50 mM Hepes, pH 7.5, containing 10 mM MgCl2, 100 μM [γ-32P]ATP (800,000 cpm/nmol), 2 mM CaCl2, 80 μM syntide-2, 0.2 mM Ca2+-binding proteins, and 0.4 ng/μl CaM kinase II (purchased from BioMol Inc.). ND, not determined.

Collectively, these data indicate that CaBPs are sensitive to Ca2+ and function as a Ca2+-sensitive sensor within the cells.

![Fig. 8](image-url)

**Fig. 8.** Activation of CaM kinase II by CaBPs and interaction of CaBP2 with the peptide derived from the CaM-binding domain of CaM kinase II. A, the activity of purified CaM kinase II was measured in the presence of 2 μM Ca2+-binding proteins using syntide-2 as a substrate (Table I). The short forms CaBP1 and CaBP2 were used in the assays. B, binding of CaBP2 to the peptide derived from the CaM-binding domain of CaM kinase II. The peptide 290LKKFNA-RKKLKGAILTMLA309 was coupled to CNBr-activated Sepharose, and CaM and CaBP2 were added in the presence of 50 mM Ca2+. The column was washed with 10 mM BTP, pH 7.5, containing 100 mM NaCl. Bound proteins were eluted with 1 mM EGTA. CaBP1 and CaBP5 did not bind to this column.
respond to changes in [Ca\(^{2+}\)] at the molecular level, it is essential to identify all molecules that interact with their physiological targets. A large number of Ca\(^{2+}\)-binding proteins (CaBPs) have provided an evolutionary mechanism for diversification of Ca\(^{2+}\)-signaling on the cellular level (4, 5). GRK5, the most closely related to the prototype CaM and other neuron-specific Ca\(^{2+}\)-binding proteins (Fig. 1B), but they differ from them in three major ways. First, some of the CaBPs are myristoylated, or have a Cys residue that could be palmitoylated. CaBP1 and CaBP2 are also produced in two splice forms (or three for CaBP1, considering caldendrin), respectively. As shown in this study, each of the two CaBP1 variants displays unique localization to specific regions of the cell (e.g. cytoskeleton versus the plasma membrane) (Fig. 5). In general, the N-terminal part of CaBPs is the most divergent from CaM. Second, the central \(\alpha\)-helix is extended by one turn (4 aa) as compared with the corresponding 28-amino acid-long central helix in CaM (Fig. 10). Because this \(\alpha\)-helix in CaM undergoes major conformational changes upon Ca\(^{2+}\) coordination and is involved in the interaction with the target molecules, further structural studies will be important to elucidate what structural changes this additional turn may cause to the overall conformation of these proteins. Third, the EF2-hand motif is likely to be nonfunctional because of the following changes in this loop: the presence of a Gly-Gly motif, only one negative charge within this Ca\(^{2+}\)-binding loop, and substitution of Glu in position 4 of CaM by Asp (CaBP1); deletion of 3 aa followed by a flexible -Gly-Gly- allowing a shorter Ca\(^{2+}\)-binding loop (CaBP2); Arg/Lys substitution of a critical Asp in position 1 (CaBP4 and CaBP5). Inactivation of specific EF-hand motifs of CaM may have provided an evolutionary mechanism for diversification of Ca\(^{2+}\)-signaling (Figs. 10 and 11). Similar examples of inactivation may be found in the EF1-hand motif (GCAPs), the EF1- and EF4-hand motifs (recovery, neurocalcin, and others).

\[\text{CaBP1} \quad 0 \quad 0.08 \quad 0.25 \quad 0.8 \mu M\]
\[\text{CaBP2} \quad 0 \quad 0.6 \quad 2 \quad 6 \mu M\]
\[\text{CaBP5} \quad 0 \quad 2 \quad 6 \quad 20 \mu M\]

**FIG. 9. Inhibition of GRK5 activity by CaBP1.** Purified GRK5 (0.8 pmol) were used to phosphorylate rod outer segment membranes (2 \(\mu M\) rhodopsin) in the presence of indicated concentrations of CaBPs. Proteins were separated on a 10% SDS-polyacrylamide gel and visualized by autoradiography. \(^{32}\)P incorporation into proteins was determined by excising and counting the radioactive bands. The activity of GRK5 in the absence of CaBPs. The short forms CaBP1 and CaBP2 were used in the assays.

A Novel Family of CaBPs—Changes in [Ca\(^{2+}\)] are a key factor in cellular regulation. These changes are sensed, either directly by proteins, including enzymes, or through specific Ca\(^{2+}\)-binding proteins. To understand Ca\(^{2+}\)-signaling on the molecular level, it is essential to identify all molecules that respond to changes in [Ca\(^{2+}\)], including sensing proteins and their physiological targets. A large number of Ca\(^{2+}\)-binding proteins have been identified, and by far, the largest subfamily are CaM-like proteins. Members of this subfamily are small (\(\sim 200\) aa), acidic (pI 4–5) proteins that contain four EF-hand motifs, from which only selected motifs are functional. Frequently, they are specifically expressed in subsets of neurons (NCBP:GCAPs, recoverin, neurocalcin, and others). In this study, we identified five related novel genes encoding Ca\(^{2+}\)-binding proteins and their several alternatively spliced forms, yielding an even larger group of related proteins. In addition, a peptide sequence related to CaBP was obtained from sequencing retinal proteins purified on an anti-CaBP-specific antibody column. So far, this sequence has not been assigned to any specific gene reported in this study, suggesting that the subfamily of CaBPs, is perhaps even larger.

**DISCUSSION**

**A Novel Family of CaBPs—**Changes in [Ca\(^{2+}\)] are a key factor in cellular regulation. These changes are sensed, either directly by proteins, including enzymes, or through specific Ca\(^{2+}\)-binding proteins. To understand Ca\(^{2+}\)-signaling on the molecular level, it is essential to identify all molecules that respond to changes in [Ca\(^{2+}\)], including sensing proteins and their physiological targets. A large number of Ca\(^{2+}\)-binding proteins have been identified, and by far, the largest subfamily are CaM-like proteins. Members of this subfamily are small (\(\sim 200\) aa), acidic (pI 4–5) proteins that contain four EF-hand motifs, from which only selected motifs are functional. Frequently, they are specifically expressed in subsets of neurons (NCBP:GCAPs, recoverin, neurocalcin, and others).

In this study, we identified five related novel genes encoding Ca\(^{2+}\)-binding proteins and their several alternatively spliced forms, yielding an even larger group of related proteins. In addition, a peptide sequence related to CaBP was obtained from sequencing retinal proteins purified on an anti-CaBP-specific antibody column. So far, this sequence has not been assigned to any specific gene reported in this study, suggesting that the subfamily of CaBPs, is perhaps even larger.

\[^{5}\] F. Haeseleer, I. Sokal, C. L. M. J. Verlinde, H. Erdjument-Bromage, P. Tempst, A. N. Pronin, J. L. Benovic, R. N. Fariss, and K. Palczewski, unpublished observation.
Several reports describe examples of bidirectionally transcribed genes that partially overlap at their 5′- or 3′-ends, mostly in noncoding regions or promoter regions (reviewed in Ref. 34). The BCMA gene has an organization most closely resembling CaBP3 and CaBP5 genes, with the exon/intron splicing sites shared by the sense and antisense transcripts in the coding sequence (35). In prokaryotes, natural antisense RNAs are part of a general mechanism of control of gene expression (reviewed in Ref. 36). This might also be a potential role in eukaryotes (reviewed in Refs. 34 and 37). Although many antisense RNAs have been described in eukaryotes, only a few are spliced and present an open reading frame (ORF). The h-CaBP3 gene is spliced, polyadenylated, and has an ORF of 192 aa. Moreover, the CaBP3 C-terminal half is identical to the C-terminal half of CaBP5 because of the duplication/inversion of the gene. Because no CaBP3 transcripts were detected in bovine and mouse retinas, we analyzed the complementary strand of the bovine and mouse CaBP5 for the presence of a putative ORF. No ORF similar to that in the h-CaBP3 is possible in mouse and bovine retinas because of the presence of a stop codon on the complementary strand.

Because the overlapping exon/intron junctions of CaBP3 are at the same position as those of the CaBP5 gene, the 5′-donor and 3′-acceptor sites are CT and AC, complementary to GT and AG. It is puzzling how these introns are spliced, because these splice sites cannot be recognized by the U2- or more rare U12-type spliceosome (38, 39). This gene might use a different splicing machinery, as suggested by other authors (35, 40). CaBP3 has both types of splice junctions in its gene, CT and AC in the CaBP5 overlapping part, but GT and AG in the nonoverlapping part. Because of this particular gene organization, the transcription of CaBP5 would interfere with the transcription of complementary CaBP3 through the contact of both transcription complexes. We do not know yet whether CaBP3 is translated in the retina. CaBP3 might: 1) control the level of expression of CaBP5; 2) not be expressed simultaneously with CaBP5; or 3) originate from different alleles. Monoclonal antibodies against CaBP3 will be necessary to demonstrate the expression of CaBP3 in the retina.

Expression and Localization—CaBPs have a unique expression pattern. CaBP1 is highly expressed in the brain and retina, as shown in Fig. 3A; a more detailed expression pattern is shown in Fig. 3D. mRNAs of caldendrin and calbrain, which are identical, in large part or entirely, with CaBP1, have also been found to localize to the cortex, cerebellum, and hippocampus (11, 12). In contrast, CaBP3/5 is retina-specific. CaBP2 also appears to be a minor retinal component. CaBP1 and CaBP5 were also isolated and microsequenced from the retina, proving that both genes are translated.

Immunocytochemical localization of CaBPs imposes a major challenge because of high sequence similarity among these proteins. The localization data from the retina indicate that CaBP1 and CaBP5 are expressed in the inner retina. CaBP1 is present in amacrine and some bipolar cells, whereas CaBP5 is present in rod and cone bipolar cells. The development of antibodies specific for alternative splice forms will aid in the future characterization of these proteins. Biochemical experiments suggest that CaBP5 and the CaBP1 short form are at least partially soluble and can be extracted under low ionic strength buffer. Expression of the long form of CaBP1-GFP in CHO cells suggests that this protein may be associated with the cytoskeleton. The short form of CaBP1 clearly localizes to the plasma membrane. Seidenbecher et al. (11) found caldendrin to be present in cytoskeletal fractions with a further enrichment in the synapse-associated cytomatrix, suggesting an association with the somatodendritic cytoskeleton. We also ob-
Ca²⁺-binding Proteins

served a similar localization pattern in the retinal bipolar cells. Altogether, these data suggest that CaBPs occur within the cell as soluble proteins; significant pools of these proteins (or splice variants), however, are associated with the plasma membrane or cytoskeletal. The presence of two CaBP1 pools could be an important mechanism for targeting the effector proteins to different cellular compartments, for example, proteins that change their localization from the plasma membrane to intracellular stores (e.g., receptors) during cell stimulation.

Functional Aspects—Significant functional and structural information have accumulated on CaM, and this data may have substantial relevance for understanding CaBPs. Ca²⁺ binding to CaM appears to be stepwise, first to the EF3-hand and EF4-hand motifs and then to the EF1-hand and EF2-hand motifs (41). When site II was disabled by a specific mutation, there was a decrease only in the apparent affinity for the partner site I and little or no effect on sites III and IV (42). It is believed that both N-terminal binding sites can bind Ca²⁺ almost independently to produce different components of the Ca²⁺-induced conformational change (43). Once Ca²⁺ is coordinated in all sites, there is likely to be an interaction of the C- and N-terminal domains. Interestingly, a disabling mutation in the EF2-hand motif not only had an effect on the EF1-binding site but also accelerated the release of Ca²⁺ from EF3- and EF4-hand sites (44). This mutant also has decreased affinities for smooth and skeletal muscle myosin light chain kinases, adenyl cyclase, and plasma membrane Ca²⁺-ATPase (45). Thus, some differences between CaM and CaBPs could be attributed to lack of functional EF2-hand and to specific sequence changes among these proteins. Finally, it may be important that CaBPs have an extended 32-aa flexible tether serving the contact sites with the effector molecule. This excitatory properties of CaBPs and CaM suggest the necessity for pre-

CaBPs in vivo. The highly conserved primary and structural properties of CaBPs and CaM suggest the necessity for preserving the contact sites with the effector molecule. This exciting hypothesis requires further biochemical and structural testing to assess the importance of this subfamily in neuronal functioning.

Note Added in Proof—Recently, Menger et al. (Menger, N., Seidenbecher, C. I., Gundelfinger, E. D., and Kreutz, M. R. (1999) Cell Tissue Res. 298, 21–32) reported the immunolocalization of caldendrin in the retina.

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