

# A Myristoylated Calcium-binding Protein that Preferentially Interacts with the Alzheimer's Disease Presenilin 2 Protein

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**Abstract.** It is well established that mutations in the presenilin 1 and 2 genes cause the majority of early onset Alzheimer's disease (AD). However, our understanding of the cellular functions of the proteins they encode remains rudimentary. Knowledge of proteins with which the presenilins interact should lead to a better understanding of presenilin function in normal and disease states. We report here the identification of a calcium-binding protein, calmyrin, that interacts preferentially with presenilin 2 (PS2). Calmyrin is myristoylated, membrane-associated, and colocalizes with PS2

when the two proteins are overexpressed in HeLa cells. Yeast two-hybrid liquid assays, affinity chromatography, and coimmunoprecipitation experiments confirm binding between PS2 and calmyrin. Functionally, calmyrin and PS2 increase cell death when cotransfected into HeLa cells. These results allude to several provocative possibilities for a dynamic role of calmyrin in signaling, cell death, and AD.

**Key words:** presenilins • Alzheimer's disease • calcium-binding protein • myristoylation • cell death

ALZHEIMER'S disease (AD)<sup>1</sup> is a degenerative disorder characterized clinically by progressive dementia and neuropathologically by the presence of senile plaques and neurofibrillary tangles (NFT). Genetic studies indicate the etiology of AD to be heterogeneous. Mutations in (amyloid precursor protein ( $\beta$ APP), presenilin 1 (PS1), and presenilin 2 (PS2) (reviewed by Hardy, 1997; Cruts and Van Broeckhoven, 1998) are linked to autosomal dominant inheritance of early onset familial AD (i.e., FAD before 65 years of age). Many other genes, including some that act as modifiers or risk factors, appear to be associated with late-onset AD (>65 yr; Corder et al., 1993; Payami et al., 1997; Pericak-Vance et al., 1997; Blacker et al., 1998; Montoya et al., 1998). Approximately 50% of all FAD cases are linked to the presenilin genes where missense mutations are generally found in residues that are conserved between the two proteins with the rare exceptions of in-frame splice deletions and premature truncations. The mechanisms by which mutations in PS and

$\beta$ APP genes cause AD are not known, although mutations in these genes appear somehow interconnected as they increase amyloidogenic A $\beta$  fragment accumulation (reviewed by Hardy, 1997).

Human PS1 and PS2 genes are both ubiquitously expressed, but at low protein levels which have led to difficulties and inconsistencies in their detection and localization. Upon overexpression, the presenilins have been localized to the ER and nuclear envelope (see Kovacs et al., 1996; Janicki and Monteiro, 1997 and references therein) with one group reporting evidence also for cell surface localization (Dewji and Singer, 1997). Endogenous PS1 and PS2 proteins, in turn, have been localized to a variety of structures, including the ER, vesicular structures of the somatodendritic compartment, within axons, at centrosomes and centromeres, and at the plasma membrane (Busciglio et al., 1997; Capell et al., 1997; Li et al., 1997; Ye and Fortini, 1998).

Human PS1 and PS2 proteins are 67% identical, sharing highest similarity in their COOH-terminal sequence and in multiple internal regions that are structurally predicted to form transmembrane domains (TMD). Assuming the presenilins are transmembrane proteins, their topography according to most models is of a protein that weaves through membranes eight times with the NH<sub>2</sub>- and COOH-terminal domains and the large "loop" spanning the putative sixth and seventh TMD all facing the cytoplasm (see Fig. 1; Hardy, 1997).

Several lines of evidence from a variety of species have

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1. *Abbreviations used in this paper:* AD, Alzheimer's disease; FAD, familial AD; MTN, multiple tissue Northern; NF-L, neurofilament light; NFT, neurofibrillary tangles; PS, presenilin; TMD, transmembrane domains.

indicated presenilins play important roles during development. First, the *Caenorhabditis elegans* presenilin homologue, *sel-12*, facilitates Notch-based cell signaling of *lin-12*, a gene involved in cell fate determination and vulva development (see Levitan and Greenwald, 1995). Second, PS1 knockout mice die shortly after birth with the embryos displaying central nervous system defects and abnormal patterning of the axial skeleton and spinal ganglia (Shen et al., 1997; Wong et al., 1997). Third, disruption of the *Drosophila* PS gene is lethal, causing Notch processing defects as well as Notch-like phenotypes (Strul and Greenwald, 1999; Ye et al., 1999). Although these results clearly allude to a role for presenilins in development, the mechanisms by which FAD mutations in presenilin genes cause AD are unknown. Interestingly, mice disrupted of PS1 can be rescued by human transgenes containing FAD-linked PS mutations, indicating that the FAD mutations do not affect PS functions related to embryo development in mammals (Davis et al., 1998; Qian et al., 1998). In contrast, the *C. elegans sel-12* mutant is more able to discriminate between wild-type and FAD bearing PS transgenes, being functionally rescued by expression of wild-type human presenilins but only partially by presenilins containing FAD mutations (Levitan et al., 1996; Baumeister et al., 1997). The FAD-linked PS mutations are thought to confer some unknown detrimental gain of function which correlates with altered  $\beta$ APP processing (see Davis et al., 1998; De Strooper et al., 1998; Qian et al., 1998).

Presenilins have also been implicated in the regulation of programmed cell death (apoptosis). Evidence for such a role was first shown when a cDNA fragment encoding the 103 COOH-terminal amino acids of mouse PS2, termed ALG-3, was isolated in a screen for cDNAs that could rescue T cells from receptor-induced apoptosis (Vito et al., 1996a). This rescue appears to be a consequence of the ALG-3 fragment acting in a dominant negative fashion, since expression of full-length PS2 leads to apoptosis (Vito et al., 1996b). Compared with the apoptosis induced by the overexpression of wild-type PS2 in PC12 and HeLa cells, the FAD PS2-N141I mutation causes even higher levels of apoptosis (Deng et al., 1996; Wolozin et al., 1996; Janicki and Monteiro, 1997). Likewise, PS1 overexpression also sensitizes cells to apoptosis (Guo et al., 1996, 1997; Wolozin et al., 1998). The mechanisms by which presenilins induce apoptosis are not fully understood, but perturbations in calcium, oxidative stress (Guo et al., 1996; Keller et al., 1998), destabilization of  $\beta$ -catenin (Zhang et al., 1998), and increased signaling by heterotrimeric GTP-binding proteins have been implicated (Wolozin et al., 1996; Smine et al., 1998).

To gain a better understanding of presenilin function, we used the yeast two-hybrid system to identify proteins with which human PS2 interacts. Using the loop region of PS2 as bait we have isolated and characterized a recently identified calcium-binding protein that binds preferentially to PS2 compared with PS1 loop sequence. This protein, calmyrin, displays several interesting properties including myristoylation, membrane-association, and colocalization with PS2 in cotransfected cells. Like presenilin, calmyrin causes cell death when overexpressed in HeLa cells, and interestingly, coexpression of PS2 and calmyrin promotes an additive increase in cell death. The interaction of this

calcium-binding protein with PS2 may therefore be important in presenilin function.

## Materials and Methods

### Primer List

B3': 5'GCTGAGTACGCTCGAGGTAGGGGAGCTGGAGGGC3'; B5': 5'CGCTTCTGGAATTC<sup>u</sup>CCCCAAAGGGCCTCTGAG3'; C3': 5'GCTATCGCTCGAGCCACACCATGGCAGATG3'; D5': 5'CGCTTC-TGGAATTC<sup>u</sup>CCCCACGGTTGGCATG3'; E3': 5'TATCGCTTAAGT-CGACGATGTAGAGCTGATGGG3'; E5': 5'CGGTACGTGAATTC-AAGAAGGCGCTGCC3'; F3': 5'GCTAGCATCGCTCGAGATAC-TTGGAAATTTTGG3'; F5': 5'CGTCATCAGCAATTC<sup>u</sup>CCCGAAA-GGTCCACTTCG3'; G3': 5'CTCGCTAGCCTCGAGCCACACCA-TGTGTGAGG3'; L3': 5'TCGTGAGGATCCCTCGAGCTACTGGAG-CCGCGACAGGC3'; L5': 5'CTAGACCTGAATTC<sup>u</sup>CCAATGGCGA-CCTCGACCC3'; M3': 5'CGAGTAGCATGTGACACAGGACAAT-CTTAAAGGA3'; M5': 5'GCTACACTAGCCGCGGAATTCGGCA-CGAGGCG3'; N3': 5'CGAGTAGCATGTGACTCACAGGACAA-TCTTAAA3'; N5': 5'GCTACACTAGCCGCGGCCACCATGGAG-CAAAAGCTCATTCTGAAGAGGACTTGAATCGCCGGGGG-CGATGGG3'. Restriction enzymes sites incorporated into the primers to aid in cloning are underlined.

### Yeast Two-Hybrid Library Screening

The yeast two-hybrid screen for PS2 interacting proteins was performed essentially as described by Golemis et al. (1996), with the necessary plasmids and cDNA library obtained from Dr. Roger Brent (Harvard Medical School, Cambridge, MA). The PS2-loop bait (designated PS2-loop B, see Fig. 1 A) was constructed by PCR amplification of the region from human PS2 (Janicki and Monteiro, 1997) using primers B5' and B3'. The resulting 150-bp PCR fragment was double digested with EcoRI and XhoI and ligated into the corresponding sites of the pEG202 LexA fusion plasmid. This bait construct and the LacZ reporter plasmid pSH18-34 were cotransformed into yeast strain EGY48 which was then transformed with  $\sim 5 \mu\text{g}$  of human fetal brain cDNA library in pJG4-5.  $1.5 \times 10^7$  of the resulting transformants were plated on Gal/Raf/CM-ura-his-trp-leu plates to screen for transcriptional activation of the chromosomally integrated LEU2 reporter gene. 100 Leu+ yeast colonies were picked to a Glu/CM-ura-his-trp master plate, then replica-plated to Glu/CM-ura-his-trp-leu, Gal/Raf/CM-ura-his-trp-leu, Glu/Xgal/CM-ura-his-trp and Gal/Raf/Xgal/CM-ura-his-trp plates to test for galactose-dependent *leu2* and *lacZ* expression. Dual expression of the reporters was displayed by 15 colonies, from which the library plasmids were recovered in *Escherichia coli* strain JBe15 and subsequently transformed back into EGY48 containing pSH18-34 and the pEG202LexA/PS2-loop B bait or one of several negative controls to test the specificity of the interaction with PS2-loop B. The library plasmids that produced a strong and specific interaction with PS2-loop B were recovered in *E. coli* strain DH1 and the DNA sequence of their inserts was determined.

DNA and protein sequences were analyzed using MacVector 6.5 (Oxford Molecular). Homology searches of the NCBI databases were performed using the BLAST program. Three of seven putative interactors were independent clonings of the same cDNA which we named calmyrin. For further experiments, clone 7, a library plasmid which contained full-length calmyrin cDNA was digested with EcoRI and XhoI and subcloned into pBluescript KS(-) or pGST/His T1 vector (Pharmacia Biotech, Inc.).

### Yeast Two-Hybrid Liquid Assay

The specificity of the calmyrin interaction was tested against three PS2-loop region constructs, two different PS1-loop constructs of which one was further mutated to the corresponding PS2 sequence, one PS2 COOH-terminal construct, and a lamin control. A conserved 31-amino acid loop region (designated PS2-loop C) was obtained by using primers B5' and C3' to PCR generate a 93-bp fragment. The more divergent region of the loop (designated PS2-loop D) was generated using primers D5' and B3'. A construct encoding the final 40 amino acids of PS2 (designated PS2-Cterm) was created using primers E5' and E3'. The corresponding loop B and loop C regions in PS1 were PCR amplified using primers F5' with F3' or G3', respectively, from a full-length PS1 clone obtained from Dr. S.S. Sisodia (University of Chicago, IL). The PS1-loop C region, which differs

by only three amino acids from the corresponding PS2-loop (containing a threonine instead of a proline at position 281 (see Fig 1 A, numbered according to PS1), a leucine in place of an isoleucine at position 282, and a threonine for an alanine at position 291), was mutated at each of the three divergent residues, singly, and in every possible combination to the corresponding PS2 sequence using appropriate PCR primers and the QuikChange site-directed mutagenesis method (Stratagene). A control bait construct which contained the first 31 amino acids of lamin B was obtained by PCR with primers L5' and L3' from lamin B cloned in pBlue-script KS(-) (Mical and Monteiro, 1998).

All PCR-amplified regions were digested with EcoRI and XhoI, cloned into pEG202, and confirmed by DNA sequencing. These various baits were transformed into EGY48 and found by immunoblotting of yeast extracts to express appropriately sized lexA-PS fusion polypeptides. Three isolates from yeast transformed with the calmyrin in pJG4-5 (clone 7) plus each PS bait or the control lamin bait were assayed for  $\beta$ -galactosidase enzyme activity in liquid cultures using ONPG (*O*-nitrophenyl  $\beta$ -D-galactopyranoside) as a substrate (Reynolds and Lundblad, 1989).

### Northern Blot Analysis

<sup>32</sup>P-labeled DNA probes were prepared via standard random primer labeling of 100 ng of full-length calmyrin cDNA or human  $\beta$ -actin cDNA control. A human multiple tissue Northern (MTN) blot and a human brain multiple tissue Northern blot II (CLONTECH Laboratories, Inc.) were hybridized with the calmyrin probe at 68°C overnight, washed in 0.1× SSC at 50°C, and exposed to film. The blots were then stripped of the calmyrin probe and reprobbed with the  $\beta$ -actin control (CLONTECH Laboratories, Inc.).

### Bacterial GST Fusion Protein Expression

The original pGST construct or the pGST construct containing the complete calmyrin sequence fused COOH-terminally and in-frame with GST was transformed into CAG456 bacteria. Unfused GST and GST/calmyrin fusion protein induction with IPTG, incubation with glutathione agarose, and elution with reduced glutathione were as described in Janicki and Monteiro (1997).

### Cloning of Eukaryotic Expression Constructs

The pGEM-CMV vector, a CMV-driven expression plasmid containing a COOH-terminal myc-tag (described in Janicki and Monteiro, 1997), was used for protein expression in HeLa cells. A calmyrin construct containing an in frame COOH-terminal myc epitope was created by PCR amplifying the calmyrin fragment from pBS-calmyrin with primers M5' and M3' resulting in a ~600-bp PCR product that was digested with SacII and SalI and ligated into pGEM-CMV.

An NH<sub>2</sub>-terminal myc-tagged calmyrin construct was also created by PCR using primer N5' with primer N3' to introduce eleven residues of the myc epitope (Monteiro et al., 1994) followed by four residues encoded by 5' untranslated calmyrin sequence linked to the complete calmyrin coding sequence. The resulting ~600-bp PCR product was digested with SacII and SalI and ligated into pGEM-CMV.

An untagged full-length calmyrin expression construct was created by digesting pBS-calmyrin with SacII and XhoI, gel isolating the ~650-bp fragment, and ligating it to SacII/SalI linearized pGEM-CMV. The cloning and expression of both full-length PS2 and the PS2 construct deleted of loop and COOH-terminal sequence [pPS2(268aa) + Myc] were described previously (Janicki and Monteiro, 1997). Expression of full-length wild-type neurofilament light (NF-L) subunit was achieved using the CMV-NF-L expression construct (Lee et al., 1993).

### Polyclonal Antibody Production

Purified GST/calmyrin protein and GST/PS2(NH<sub>2</sub>-terminal) fusion protein (described in Janicki and Monteiro, 1997) were sent to Covance Research Products for inoculation into rabbits. The specificity of these rabbit antibodies was determined by immunoblotting (Janicki and Monteiro, 1997) and immunofluorescent staining of HeLa cell transfected with calmyrin or PS2. For immunoblotting, the anti-calmyrin and anti-PS2 antibodies were used at a 1/500–1/700 dilution and detected with horseradish peroxidase-conjugated goat anti-rabbit secondary antibodies and Super-Signal Substrate (Pierce Chemical Co.).

### HeLa Cell Culture and DNA Transfection

HeLa cells were grown in DME supplemented with 10% FBS and transiently transfected with appropriate plasmid DNAs as calcium phosphate precipitates (Janicki and Monteiro, 1997). Alternatively, 20  $\mu$ g of DNA and 2 × 10<sup>6</sup> HeLa cells were electroporated at 960  $\mu$ F and 0.3 kV.

### Cell Staining and Immunofluorescence Microscopy

HeLa cells were transfected directly on glass coverslips, fixed, and antibody stained as described in Janicki and Monteiro (1997). Antibodies used were rabbit anti-calmyrin serum (diluted 1:250), goat anti-PS2(NH<sub>2</sub>-terminal) antibody (diluted 1:150; Santa Cruz Biotechnology, Inc.), rabbit anti-lamin serum (diluted 1:200; Mical and Monteiro, 1998), rabbit anti-NFL serum (diluted 1:250; generated in this lab using recombinant-purified, bacterially expressed, mouse neurofilament light chain), M30 CytoDEATH mouse anti-cytokeratin 18 antibody (diluted 1:50; Boehringer Mannheim), fluorescein- and rhodamine-conjugated donkey anti-rabbit, anti-goat, and anti-mouse antibodies (Jackson ImmunoResearch Laboratories, Inc.). Fluorescence staining of cells was visualized on an inverted Leica DM IRB microscope and images were captured using a Photometrics SenSys camera and manipulated with IPLab Spectrum and Multi-probe software (Scanalytics) on a Power Macintosh. Confocal microscopy and image processing was performed using the ×100 objective of a Leica confocal and imaging system (Leica Inc.) with the kind help of Dr. Timothy Mical and Dr. Joseph Gall (Carnegie Institution, Baltimore, MD).

### Mouse Tissue Lysates and Primary Cultures

Spleen, brain, kidney, liver, heart, and skeletal muscle tissues were dissected from an adult mouse, chopped with a razor blade in 1–2 ml lysis buffer (Monteiro and Mical, 1996), homogenized on ice, briefly sonicated on ice, and centrifuged at 2,000 rpm for 5 min. Tissue lysate supernatants were collected, their protein concentration was determined by the BCA Protein Assay (Pierce), and 100  $\mu$ g of each sample was separated by SDS-PAGE, transferred to nitrocellulose filters, and immunoblotted with the rabbit anti-calmyrin antibody.

Kidney and heart tissues from 8–12 2-d-old mice were chopped with a razor blade, resuspended in 2 ml 0.25% collagenase in PBS, vortexed, incubated at 37°C for 15 min, centrifuged, and washed 3× with PBS. Cells were cultured in EGM medium supplemented with BBE (Clonetics) and 10% FBS for 2–7 d. For immunofluorescence, cells were cultured directly onto coverslips and fixed and stained as described above.

### Cell Fractionation

Nondetergent soluble and insoluble fractions of HeLa cells were prepared essentially as described by Gerace and Globel (1980). HeLa cells (~1 × 10<sup>6</sup>) were collected 24 h after transfection by scraping the cells in ice-cold PBS and centrifugation at 10,000 *g*. The cells were resuspended in 0.25 ml 10 mM triethanolamine-HCL (pH 7.4), 10 mM KCl, 1.5 mM MgCl<sub>2</sub>, 5 mM iodoacetamide, and 1 mM Pefabloc (Boehringer Mannheim). After 10 min incubation on ice the cells were disrupted with 10 gentle strokes in a 0.5-ml Potter-Elvehjem homogenizer. Next, 0.25 ml 10 mM triethanolamine-HCL (pH 7.4), 270 mM KCl, 1.5 mM MgCl<sub>2</sub>, 5 mM iodoacetamide, and 1 mM Pefabloc were added and after mixing, the homogenates were centrifuged at 100,000 *g* for 15 min in a Beckman TLX ultracentrifuge. The supernatants were removed and the pellets resuspended in lysis buffer (Monteiro and Mical, 1996) to a volume equal that of their respective supernatants.

Triton X-100-treated HeLa cell fractions were prepared by lysing the transfected cells in 0.5 ml ice-cold 1% Triton X-100, 10 mM triethanolamine-HCL (pH 6.9), 140 mM KCl, 1.5 mM MgCl<sub>2</sub>, 5 mM iodoacetamide, and 1 mM Pefabloc. After 10 min incubation the lysates were centrifuged at 140,000 *g* for 15 min. Supernatants were collected and the pellets resuspended in lysis buffer. Equivalent volumes of the supernatant and pellet fractions of the detergent-treated and untreated cells were separated by SDS-PAGE and immunoblotted using the rabbit anti-calmyrin antibody or the rabbit anti-lamin antibody. The same cell fractionation procedure was used on primary cell cultures prepared from mouse kidney.

### Myristoylation Experiments

After transfection, sodium pyruvate to a final concentration of 1 mM and 0.1–0.2 mCi <sup>3</sup>H-myristic acid (Amersham Life Science Inc.) were added to the fresh media in each cell culture dish. At ~24 h after transfection, cells

were scraped off the bottom of the dish and the media was collected and centrifuged 5 min at 3,000 rpm. The cell pellet was washed with PBS, centrifuged, resuspended in 400  $\mu$ l lysis buffer (50 mM Hepes, 100 mM KCl, 0.3% NP40, 1 mM EDTA, 1 mM EGTA, pH 7.5, + protease inhibitor cocktail with aprotinin, leupeptin, and PMSF; Boehringer Mannheim), and homogenized on ice. Insoluble material was pelleted and the supernatant was collected and diluted with an equal volume of dilution buffer (50 mM Hepes, 1 mM EDTA, and 1 mM EGTA, pH 7.5). 150  $\mu$ l of the lysates were incubated with 5  $\mu$ l of antibody (rabbit anti-calmyrin, rabbit anti-PS2, or rabbit control preimmune serum) for 2 h at 4°C. 45  $\mu$ l of a slurry of protein A-Sepharose beads (Pharmacia Biotech, Inc.) was then added to the lysates and incubated with rotamixing for another 1 h. The beads were pelleted by centrifugation, and after removal of the supernatant, the beads were washed four times with lysis/dilution buffer. All of the immunoprecipitate and one-sixth of the supernatant sample were separated by SDS-PAGE. After Coomassie blue staining and destaining, the gel was soaked for three 15 min changes in DMSO, immersed in 22% PPO (2,5-diphenyloxazole) for 90 min, washed in water, dried, and exposed to film by fluorography for 1 wk to 2 mo.

### Affinity Chromatography

After ~24 h, mock or PS2-transfected HeLa cells (~1  $\times$  10<sup>6</sup> cells) were washed in ice-cold PBS, scraped into PBS, and pelleted by centrifugation for 5 min at 3,000 rpm. The cells were resuspended in 400  $\mu$ l of lysis buffer (see myristoylation section), sonicated, and homogenized on ice. Insoluble material was pelleted and the supernatant was collected and diluted with an equal volume of dilution buffer. All of the soluble lysate was incubated overnight at 4°C with CNBr-activated Sepharose beads (Pharmacia Biotech, Inc.) coupled with an equivalent amount (240  $\mu$ g) of either purified GST or GST/calmyrin. The Sepharose beads were then pelleted by centrifugation and supernatant containing unbound protein was removed. The beads were washed with 2.5 M KCl, resuspended in Laemmli buffer, and 1/2 of the sample was separated by SDS-PAGE and immunoblotted for the presence of PS2 using a goat anti-PS2(NH<sub>2</sub>-terminal) antibody.

### Quantification of Cell Death

Duplicate dishes of HeLa cells (plated at ~3  $\times$  10<sup>5</sup>/100 mM dishes) were transfected with various combinations of pGEM-CMV-calmyrin, pGEM-CMV-PS2, and control vector (a CAT basic expression vector; Promega). After ~48 h, floating cells from each dish were harvested by collecting all of the media, centrifuging 5 min at 3,000 rpm, and removing all but ~0.5 ml of the supernatant. After vortexing, the exact volume of each cell suspension was measured. Cell numbers were counted twice for each sample on a hemacytometer. These cell counts were adjusted according to the initial resuspension volume to give the total number of floating cells per dish. The counts for the two independent dishes of each transfection construct combination were averaged and graphed. There was a direct correspondence between floating cells and apoptotic cells, with ~85% of floating cells showing positive CytoDEATH staining.

## Results

### PS2-Loop Interaction Trap Identifies a Calcium-binding Protein, Calmyrin

Using the yeast two-hybrid system, a human fetal brain cDNA library was screened for proteins that bind the loop region of PS2. Full-length PS2 was unsuitable as bait presumably because it could not be transported into the nucleus due to the presence of hydrophobic transmembrane domains. In addition, as it is one of the most divergent regions between the presenilin proteins, we believed our chances of finding PS2-specific interactors would be increased. After finding that our initial PS2-loop construct (residues 270–361) self-activated transcription, we truncated the bait reducing it to the first 50 amino acids in order to eliminate several acidic residues and designated it PS2-loop B (B for bait; Fig. 1 A). The PS2-loop B bait construct, the lacZ reporter plasmid, and human fetal brain

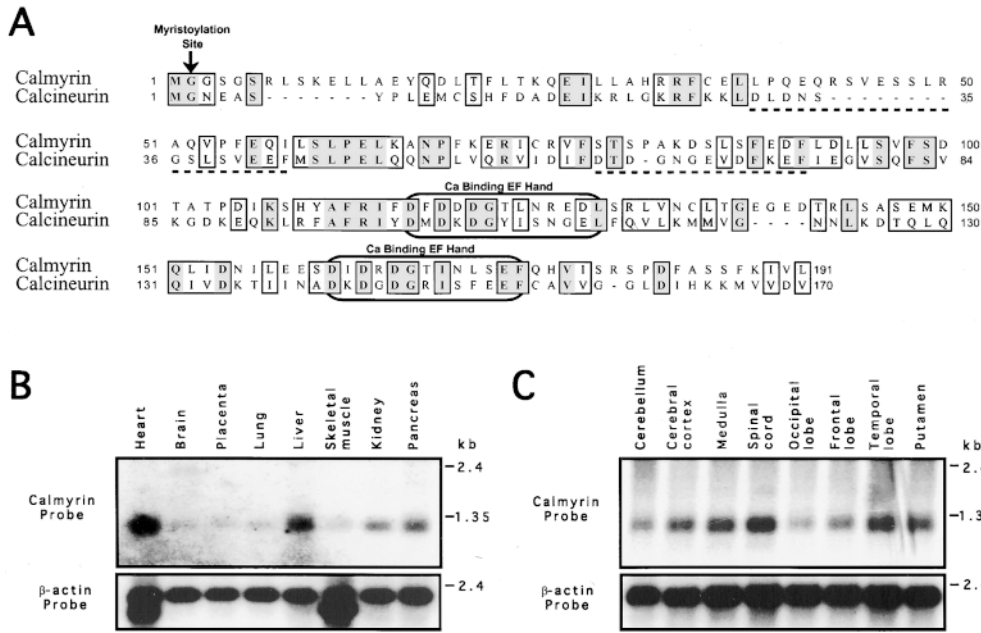
cDNA library plasmids were transformed into yeast, and out of 1.5  $\times$  10<sup>7</sup> primary transformants screened, 15 putative interactors were isolated. Isolated library prey plasmids were tested for their ability to reproduce the specific interaction phenotype when coexpressed with the loop bait but not with unrelated baits (such as human lamin B). Clones that produced the specific interaction phenotype were sequenced and identified via BLAST homology database search. Interestingly, three of the interactors were independent cDNAs all containing the full coding sequence of a recently identified calcium-binding protein, but with varying NH<sub>2</sub>-terminal untranslated extensions. Two other groups have recovered this calcium-binding protein in yeast two-hybrid screens and have named it CIB, for its calcium- and integrin  $\alpha$ IIB-binding ability (Naik et al., 1997), and KIP, due to its interaction with eukaryotic DNA-dependent protein kinase, DNA-PKcs (Wu and Lieber, 1997). Rather than pick between these two names we have chosen to refer to this protein as calmyrin (for calcium-binding myristoylated protein with homology to calcineurin) because it describes its inherent properties without bias towards its multiple binding partners.

To quantify the binding specificity of calmyrin to the PS2-loop and to determine if this protein also interacts with the PS1 loop which is 45% identical in amino acid sequence, we measured the  $\beta$ -galactosidase activity in yeast liquid assays. When cotransformed with calmyrin, the PS2-loop B bait produced an 8.5-fold increase in  $\beta$ -galactosidase activity over the lamin B negative control, while the corresponding region of PS1 (PS1-loop B) produced only a 1.9-fold increase in activity (Fig. 1 B). A PS2-COOH-terminal construct, containing the COOH-terminal 39-amino acid sequence downstream of the eighth TMD also did not appear to interact with calmyrin.

To further map the binding site of calmyrin within the PS2-loop, two new baits were constructed which divided the loop into a conserved portion, loop C (28 out of the 31 amino acids are identical to PS1), and a divergent region, loop D (only 33% identity to PS1; Fig 1 A). Since calmyrin did not interact preferentially with the comparable loop region of PS1 (PS1-loop B) we expected the calmyrin binding site to be within the divergent region of the PS2-loop sequence (PS2-loop D). To our surprise, the PS2-loop D bait interacted very weakly with calmyrin, a 2.2-fold increase over control (Fig. 1 B). However, the highly conserved region of the PS2 loop, PS2-loop C, produced a 74-fold increase in activity (Fig. 1 C). In comparison, the corresponding PS1-loop C construct increased activity only 5.8-fold.

Although the two PS-loop C baits are highly conserved in sequence, they differ by three amino acids, with PS1 containing threonine residues at positions 281 and 291 instead of proline and alanine, respectively (see Fig. 1 A, numbered according to PS1), and a leucine instead of an isoleucine at position 282. We investigated how these three divergent residues influenced calmyrin interaction with the PS-loop C region in yeast two-hybrid assays by introducing the PS2 amino acids into the PS1 bait, so that each of the three divergent residues were mutated singly, and in every possible combination, to the corresponding PS2 sequence. These data indicated that all three residues contributed in different and complex ways towards the in-





**Figure 2.** Calmyrin amino acid sequence and transcript expression pattern. (A) The complete amino acid sequence of calmyrin is shown in comparison to human calcineurin B. The two conserved calcium-binding EF hands are circled and the two EF hands that are disrupted in calmyrin are noted by a dashed line. (B) The human multiple tissue Northern blot and (C) the human brain multiple tissue Northern blot were first probed with  $^{32}\text{P}$ -labeled calmyrin cDNA, stripped, and subsequently probed with  $^{32}\text{P}$ -labeled  $\beta$ -actin cDNA as a control. The  $\sim 1.2$ -kb hybridization band corresponds to the expected size for calmyrin transcript.

idence that it is widely expressed (Naik et al., 1997; Wu and Lieber, 1997) and implying that it plays a common function in most if not all cells. Although mRNA expression was relatively low in brain, a Northern blot of specific brain regions showed that the expression of calmyrin transcripts was easily detectable and fairly uniform (Fig. 2 C).

#### **Tissue Distribution and Subcellular Localization of Endogenous Calmyrin Protein**

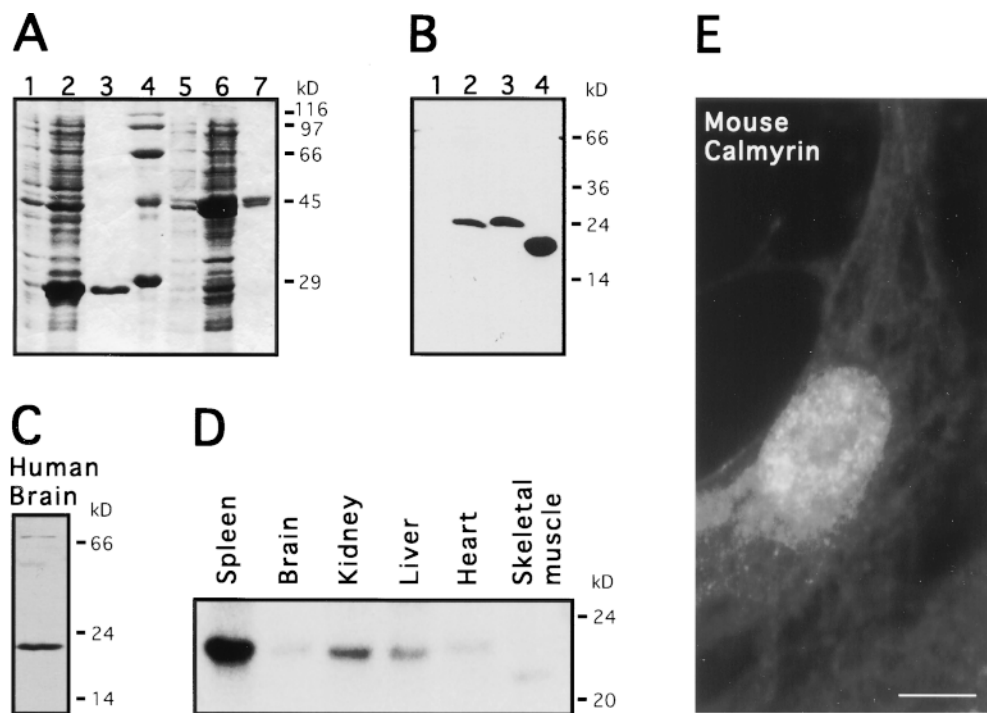
To study further the calmyrin protein, rabbit polyclonal anti-calmyrin antibodies were generated to affinity-purified GST/calmyrin fusion protein (Fig. 3 A, lane 7). By immunoblotting, these antibodies appear to be highly specific for calmyrin as they reacted only with the appropriately sized polypeptides ( $\sim 22$ - $25$  kD) in HeLa cells overexpressing calmyrin cDNAs (Fig. 3 B). Lane 1 of Fig. 3 B shows that at the depicted exposure time the antibodies failed to detect any endogenous calmyrin in untransfected lysate. Only after prolonged exposure did a faint calmyrin band appear (data not shown), indicating that endogenous levels of this calcium-binding protein are relatively low in HeLa cells. However, consistent with our Northern blot analysis, an endogenous immunoreactive band at  $\sim 22$  kD was detected in human adult brain lysate (Fig. 3 C). The anti-calmyrin antibodies also successfully detected the mouse form of this protein in several mouse tissue lysates (Fig. 3 D) due to the high conservation between the human and mouse calmyrin proteins (only five dissimilar residues; Saito et al., 1999). The significance of the faster migrating immunoreactive band in mouse skeletal muscle (Fig. 3 D, lane 6) has not been determined.

Since the subcellular localization of calmyrin was unknown, we used the anti-calmyrin antibody to determine its distribution in mammalian cells by indirect immunofluorescence microscopy. In primary cultures from mouse heart tissue endogenous calmyrin localized to the nucleus and in a reticular-like pattern throughout the cytoplasm

(Fig. 3 E). This staining was clearly distinguishable from the nonspecific background produced when probing with rabbit preinoculation serum (data not shown), and moreover, this staining pattern was reproduced by overexpression of calmyrin upon transfection (see below).

#### **Calmyrin Is Myristoylated and Membrane Associated**

As PS2 is a transmembrane protein and our yeast two-hybrid findings indicated that calmyrin interacts with PS2, the membrane targeting potential of the consensus myristoylation site in calmyrin especially intrigued us. To determine whether calmyrin is myristoylated *in vivo*, we added  $^3\text{H}$ -myristic acid to the media of HeLa cells transfected with untagged calmyrin. For comparison, HeLa cells were also transfected with calmyrin constructs that had myc tags fused at either the  $\text{NH}_2$ - or  $\text{COOH}$ -terminal ends of the protein. The prediction was that the myc tag (MEQKLI-SEEDLN) fused at the  $\text{NH}_2$ -terminal end would disrupt myristoylation since it moved the glycine residue that is essential for myristoylation more downstream (Olshevskaya et al., 1997). After 24 h, the cells were lysed and calmyrin was immunoprecipitated with the anti-calmyrin antibody. Myristoylated proteins were visualized by fluorography after SDS-PAGE (Fig. 4 A). The fluorograph of labeled HeLa cell lysates indicated immunoprecipitated C-myc-tagged calmyrin and untagged wild-type calmyrin were myristoylated as evident by incorporation of the radioactive  $^3\text{H}$ -myristic acid label (band in lanes 4 and 6 indicated by an arrows) while, as expected, the N-myc tagging of the protein prevented myristoylation (absence of band in lane 2). The lower panel of this figure contains an immunoblot of these same HeLa cell lysates to show that both  $\text{NH}_2$ - and  $\text{COOH}$ -terminally tagged calmyrin proteins were expressed efficiently and to equivalent levels, whereas untagged calmyrin accumulated at lower protein levels, explaining the fainter myristoylated calmyrin band seen in lane 6 as compared with lane 4. In fact, when the ratio of



**Figure 3.** Expression and purification of GST/calmyrin protein, characterization of anti-calmyrin antibodies, and detection of endogenous calmyrin protein. (A) Coomassie blue-stained gel documenting the induction and purification of GST (lanes 1–3) and GST/calmyrin (lanes 5–7). Uninduced bacterial lysate (lanes 1 and 5), induced lysate (lanes 2 and 6), glutathione agarose affinity-purified proteins (lanes 3 and 7), and molecular mass markers (4) are displayed. The purified ~45-kD GST/calmyrin fusion protein shown in lane 7 was inoculated into rabbits for the production of polyclonal antibodies. (B) Immunoblot of HeLa cell lysates probed with rabbit anti-calmyrin serum. HeLa cells were transfected with C-myc calmyrin (lane 2), N-myc calmyrin

(lane 3), and wild-type calmyrin (lane 4), or were mock transfected (lane 1). The anti-calmyrin antibodies react with the appropriate size polypeptides only in calmyrin transfected cells. Note the absence of nonspecific background staining and the inability to detect any endogenous calmyrin in HeLa cells at this exposure. (C) Immunoblot of human brain lysate loaded at 100  $\mu$ g total protein and probed with rabbit anti-calmyrin serum. Please note endogenous calmyrin in brain migrates as a 22-kD protein. (D) Immunoblot of various mouse tissues loaded at 100  $\mu$ g total protein and probed with anti-calmyrin to detect endogenous levels of calmyrin. (E) Immunofluorescent localization of endogenous calmyrin in mouse heart primary cell culture indicates that this protein resides in the nucleus and throughout the cytoplasm. Bar, 5  $\mu$ m.

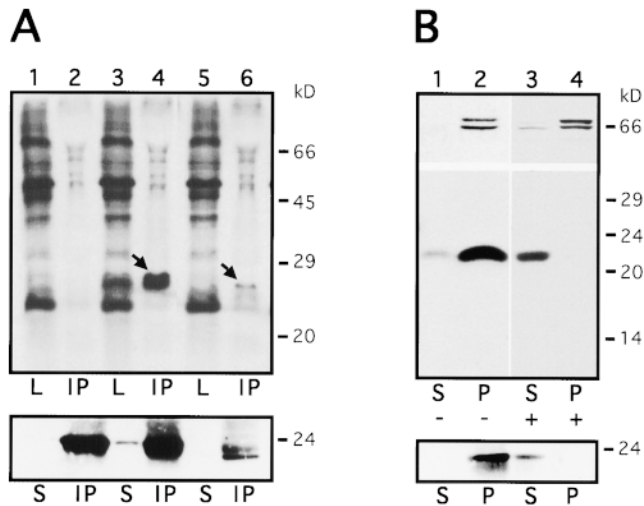
calmyrin protein to radioactive  $^3\text{H}$ -myristic acid labeling is compared for C-myc-tagged and wild-type calmyrin proteins they are similar, which is expected since myristoylation is thought to occur cotranslationally (Wilcox et al., 1987). The reason for higher expression of C- and N-myc-tagged calmyrin proteins is not known but perhaps fusion of the myc epitope affects protein stability or toxicity, allowing the proteins to accumulate to higher levels. Similar attempts to demonstrate myristoylation of endogenous calmyrin in mouse and human cells were unsuccessful, presumably because of low protein expression or the slow turnover of the protein.

Once we established that calmyrin was indeed myristoylated, next we determined whether this protein was associated with the membranes of fractionated cells. Transfected HeLa cells were fractionated in the absence of any detergents into a soluble (cytosolic) supernatant and an insoluble (membrane and cytoskeletal) pellet. Equivalent amounts of supernatant and pellet cell fractions were separated by SDS-PAGE and immunoblotted for the presence of lamins and calmyrin (Fig. 4 B, lanes 1 and 2). Lamins A and C, cytoskeletal components used as a control of the fractionation process, were detected as 68- and 66-kD polypeptides in the insoluble pellet as expected (Gerace and Blobel, 1980; Mical and Monteiro, 1998). The majority (>85%) of the calmyrin was found in the insoluble fraction. Since this manner of cell fractionation does

not distinguish membrane components from other insoluble structures, the cells were also fractionated in the presence of 1% Triton X-100 which solubilizes membranes. After this procedure, the calmyrin protein shifted to the soluble (membrane) fraction whereas the lamins, as expected, remained insoluble (Fig. 4 B, lanes 3 and 4). The same fractionation was performed on primary cultures of mouse kidney and showed an analogous pattern of membrane localization for endogenous calmyrin (Fig. 4 B, lower panel). Interpreted together, these cell fractionation results provide strong biochemical evidence that calmyrin is associated with cell membranes.

#### ***Calmyrin Accumulates in the Nucleus and Cytoplasm, but When Coexpressed with PS2 these Two Proteins Colocalize at the ER***

On account of the faint staining of endogenous calmyrin in primary and established cell cultures, calmyrin was forcibly expressed in HeLa cells by transient transfection of untagged and myc-tagged calmyrin constructs for further immunofluorescent localization studies. As seen in Fig. 5 A, cells expressing untagged calmyrin had strong staining in the nucleus and cytoplasm, a pattern very similar to the subcellular localization of endogenous calmyrin detected in mouse cells. At higher magnification, many of these transfected cells showed clear calmyrin staining of thin



**Figure 4.** Immunoprecipitation of myristoylated calmyrin and localization of calmyrin to the membrane fraction. (A) Fluorography of myristoylated proteins immunoprecipitated with rabbit anti-calmyrin from HeLa cells after transfection with N-myc calmyrin (lane 1 and 2), C-myc calmyrin (lane 3 and 4), or wild-type calmyrin (lane 5 and 6) and 24 h incubation with  $^3\text{H}$ -myristic acid. The cell lysates (L) and immunoprecipitated (IP) samples are shown. The myristoylated calmyrin band is present in lanes 4 and 6 (indicated by the arrows), but absent from lane 2. The lower panel shows an immunoblot of the supernatants (S) and immunoprecipitants (IP) of these same transfected cell lysates to confirm the expression level and successful immunoprecipitation of calmyrin. (B) Immunoblotting of calmyrin-expressing HeLa cell lysates for lamins A and C (68 and 66 kD) and calmyrin (22 kD) after fractionation in the absence (-) of detergent (lanes 1 and 2) or in the presence (+) of 1% Triton X-100 (lane 3 and 4) into a soluble supernatant (S) and an insoluble pellet (P). The lower panel shows the identical fractionation of endogenous calmyrin in mouse kidney primary cell culture.

projections from the cell surface as well as a reticular staining in the cytoplasm consistent with membrane targeting to the plasma membrane and ER (Fig. 5 C). Cells expressing C-myc calmyrin had greater variation in staining with many showing prominent localization to the ER and plasma membrane and often less staining in the nucleus (Fig. 5 D). Double immunofluorescence staining for calreticulin, an ER marker protein, and calmyrin showed that within the cytoplasm a notable portion of calmyrin colocalized with calreticulin (data not shown), corroborating the impression that in these transfected cells calmyrin localization includes, but is not limited to, ER membranes. In contrast, cells expressing N-myc calmyrin showed predominant nuclear staining, more diffuse cytoplasmic staining, and less staining at the plasma membrane which was especially evident in low expressing cells (Fig. 5 E). This observed reduction in membrane association was not surprising considering our previous finding that this  $\text{NH}_2$ -terminally tagged construct failed to be myristoylated. To address whether the bright nuclear staining was due to calmyrin localization within the nuclear envelope or throughout the nucleoplasm, we double stained wild-type calmyrin transfected HeLa cells for calmyrin and lamins A/C. According to confocal microscopy, lamins A and C

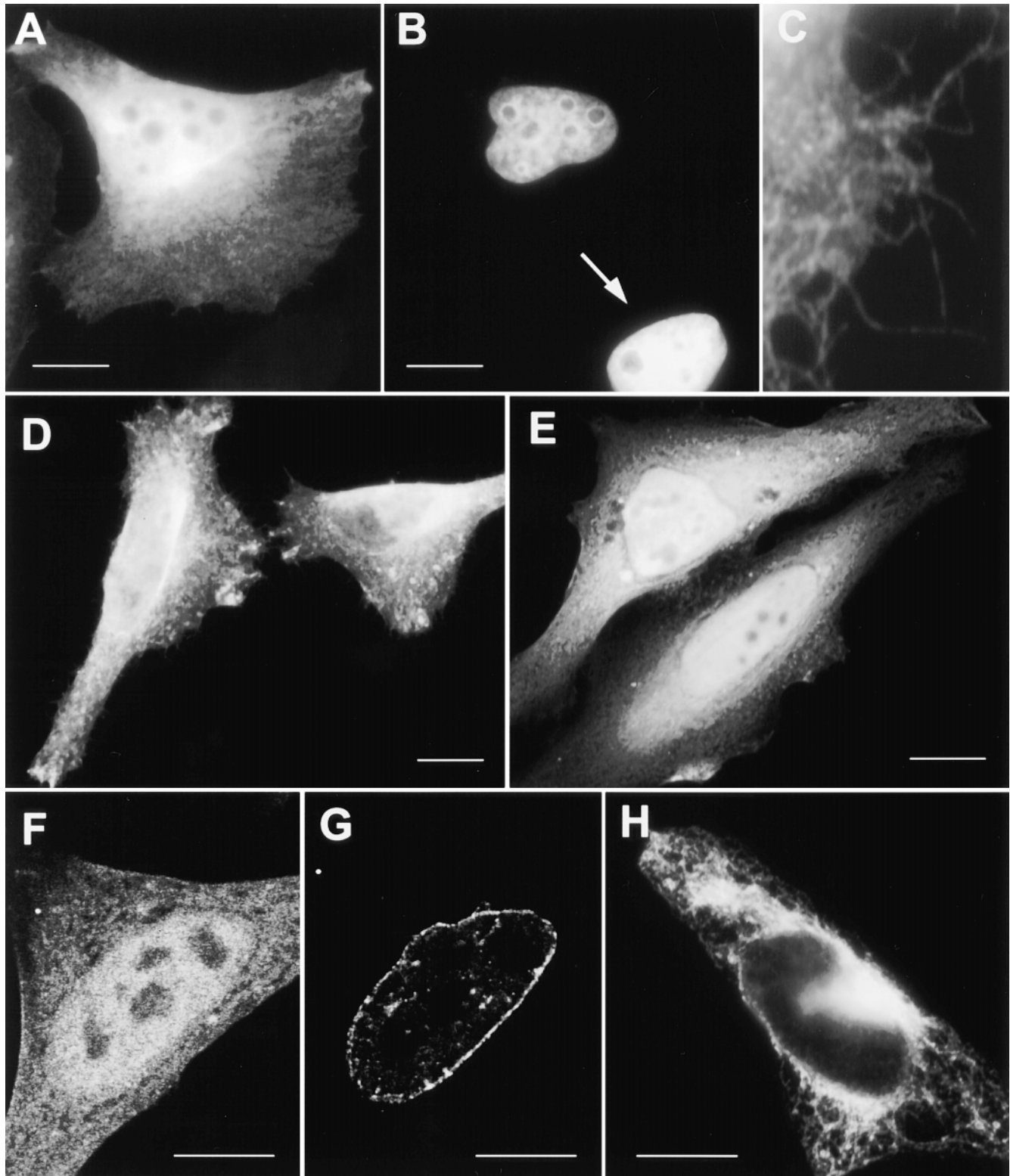
had rim fluorescence (Fig. 5 G) consistent with their known localization as a caged meshwork of filaments tethered to the inner nuclear envelope (see Mical and Monteiro, 1998). In the same confocal Z-section ( $1.0\text{-}\mu\text{m}$  section) where lamins had rim fluorescence, calmyrin immunoreactivity was present throughout the cell and clearly within the nucleoplasm (Fig. 5 F). Overall, these results indicated calmyrin localizes to many different cellular compartments, consistent with the protein having dynamic targeting properties. Of particular interest to this study was the comparison of calmyrin and PS2 staining patterns when overexpressed individually in HeLa cells. Although the two staining patterns overlapped in part, especially the ER reticular staining of untagged and C-myc calmyrin, PS2 staining was readily distinguishable by its exclusive ER and nuclear envelope staining pattern (Fig. 5 H).

When calmyrin was coexpressed with PS2, its staining pattern was dramatically altered such that it colocalized almost completely with PS2 (Fig. 6). As exemplified by the two cells shown in panels A and B, the calmyrin protein was less apparent in the nucleus in coexpressing cells than in cells transfected solely with calmyrin (Fig. 5 A). Another indication that these two proteins bind each other was seen in a small subset of cells where calmyrin and PS2 colocalized distinctively in unusual intranuclear spots (Fig. 6 C). The intranuclear spots did not colocalize with anti-centromere staining by double immunofluorescent microscopy (data not shown) suggesting that they are distinct from the PS-immunoreactive structures observed by Li et al. (1997). The shift in calmyrin localization and the nearly identical staining patterns between PS2 and calmyrin (see merged images) in these coexpressing cells provide persuasive evidence that these two proteins interact *in vivo*. Furthermore, when calmyrin was cotransfected with a PS2 construct deleted of the loop and all sequence COOH-terminal of it, the staining patterns displayed significantly less overlap; as seen by patchy aggregates of PS2 which excluded calmyrin (Fig. 6 D, indicated by arrows). The failure of this PS2 deletion construct to completely colocalize with calmyrin in aggregates, which contrasts with the colocalization of the wild-type PS2 protein and calmyrin in nuclear inclusions, enhances our view that the PS2-loop region facilitates binding of calmyrin.

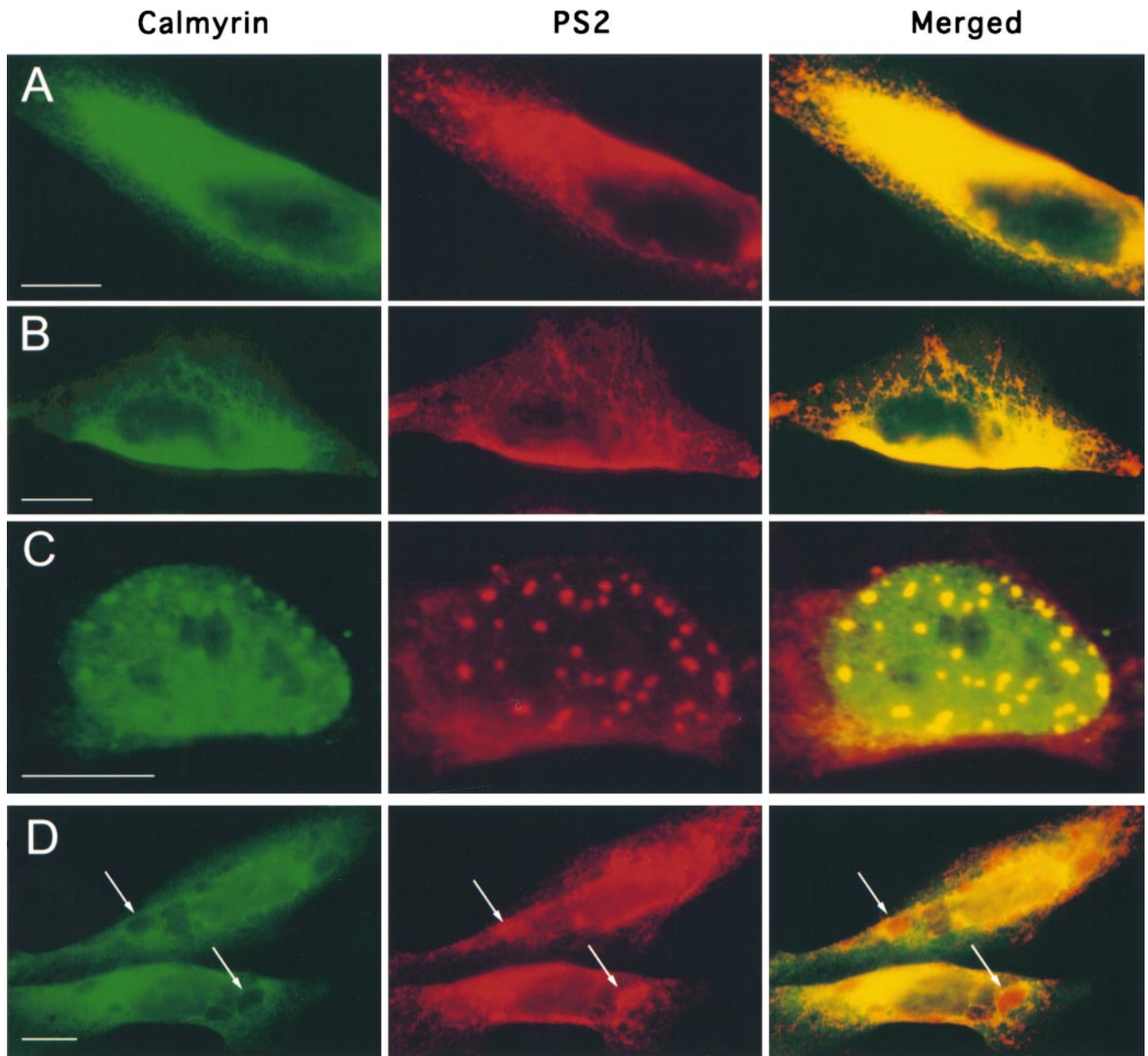
#### **Affinity Chromatography and Immunoprecipitation Confirm Binding between Calmyrin and PS2**

Despite results from yeast two-hybrid assays, cell fractionation experiments, and histological colocalization, which all consistently argue for an interaction between calmyrin and PS2, our initial attempts at demonstrate binding of the two proteins *in vitro* proved difficult. After trying various combinations of affinity chromatography and immunoprecipitation with GST fusion proteins, *in vitro* translated proteins, and HeLa cell-expressed proteins under several different buffer conditions, two of these experiments provided further evidence for the binding of calmyrin and PS2. First, HeLa cell lysates of overexpressed PS2 were incubated with purified GST-calmyrin, or GST alone, (shown in Fig. 3 A) that had been covalently coupled to Sepharose. The two Sepharose columns were then washed,





**Figure 5.** Immunofluorescent localization of calmyrin protein in transfected HeLa cells. (A and B) Field of two cells transfected with wild-type calmyrin and stained with anti-calmyrin (A) and DAPI (B) reveals that the calmyrin antibody is specific since the lower-right untransfected cell (see arrow) has a bright DAPI stained nucleus but has no fluorescein calmyrin staining. In the cell overexpressing calmyrin, the protein localizes to the nucleus and cytoplasm. Note that within the nucleus, calmyrin accumulation closely resembles the DAPI chromatin staining. (C) Close-up from a calmyrin-expressing cell to highlight the localization of calmyrin to the plasma membrane projections. (D) The fluorescein staining pattern of cells overexpressing C-myc calmyrin appears slightly different from (A) with less of the protein in the nucleus. (E) Contrastingly, N-myc calmyrin protein accumulates predominantly in the nucleus and produces a more diffuse cytoplasmic staining. (F and G) Confocal images of a cell overexpressing wild-type calmyrin and stained with anti-calmyrin (F) and anti-lamin (G) indicate that calmyrin localization is not restricted to the nuclear envelop. (H) An example of the ER staining pattern of overexpressed PS2 is also shown. Bar, 5  $\mu$ m.



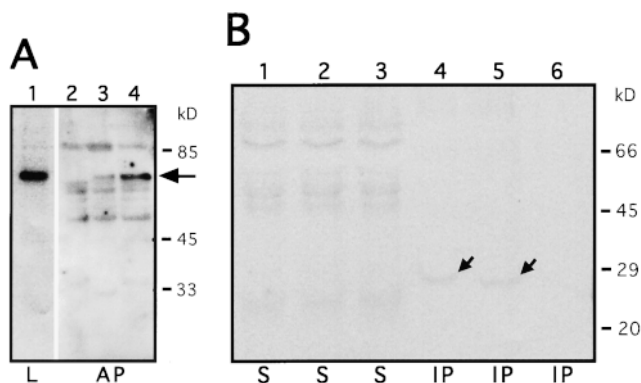
**Figure 6.** Immunofluorescent colocalization of calmyrin with PS2 in transfected HeLa cells. (A–C) HeLa cells cotransfected with wild-type calmyrin and full-length PS2 were stained 24 h after transfection with rabbit anti-calmyrin and goat anti-PS2 antibodies. Green (left-hand panels) is the fluorescein labeled calmyrin, red (center panels) is the rhodamine labeled PS2, and yellow (right-hand panels) is a merged image indicating when the fluorescein and rhodamine signals colocalize. As exemplified by the three selected cells, when co-expressed calmyrin shows a pattern of localization nearly identical to PS2. While most cotransfected cells show a staining pattern consistent with ER localization (A and B), a small subset of cells appear to colocalize calmyrin and PS2 in punctuate intranuclear aggregates (C). (D) Similar staining of HeLa cells cotransfected with wild-type calmyrin and loop/COOH-deleted PS2 show patchy aggregates (see arrows) of PS2 (red) which exclude calmyrin (green). Bar, 5  $\mu$ m.

and retention of PS2 was determined by immunoblotting with anti-PS2 antibody. Fig. 7 A shows that GST-calmyrin Sepharose bound PS2 with approximately threefold greater affinity (lane 4, see arrow) than control GST-coupled Sepharose (lane 3). The second verification of binding was the coimmunoprecipitation of myristoylated calmyrin protein from cotransfected HeLa cell lysates with anti-PS2 antibodies (Fig. 7 B, lane 5). The myristoylated calmyrin protein did not immunoprecipitate when the pre-

immune anti-PS2 serum was used (Fig. 7 B, lane 6) but, as expected, could be immunoprecipitated with the anti-calmyrin antibody (Fig. 7 B, lane 4).

#### ***Overexpression of Calmyrin Causes Apoptosis***

Since we had previously shown that overexpression of PS2 in HeLa cells causes apoptosis (Janicki and Monteiro, 1997), we wished to determine what effect overexpression



**Figure 7.** Affinity chromatography and coimmunoprecipitation provide further evidence for binding between calmyrin and PS2. (A) Immunoblot of PS2-expressing HeLa cell lysates (L) which were affinity purified (AP) over GST-sepharose or GST/calmyrin sepharose probed with goat anti-PS2. The PS2 transfected lysate (1), untransfected lysate affinity purified over GST-sepharose (2), PS2 transfected lysate affinity purified over GST-sepharose (3), and PS2 transfected lysate affinity purified over GST/calmyrin-sepharose (4) are shown. The arrow indicates the PS2 band which appears three-fold more intense in lane 4 than in lane 3. (B) Fluorography of myristoylated proteins immunoprecipitated with rabbit anti-calmyrin (lanes 1 and 4), rabbit anti-PS2 (lanes 2 and 5), or rabbit preimmune serum (lanes 3 and 6) from C-myc calmyrin and PS2 cotransfected HeLa cells incubated for 24 h with  $^3\text{H}$ -myristic acid. Supernatants (S) and immunoprecipitates (IP) are shown. Arrows indicate a more prominent myristoylated calmyrin band in lanes 4 and 5 compared with the control lane 6.

of calmyrin would have on cell viability. To detect apoptosis we used the M30 CytoDEATH antibody. This mouse monoclonal binds an epitope of cytokeratin 18 which is exposed only after caspase cleavage, an early event in apoptosis (Caulin et al., 1997; Boehringer Mannheim). Consistent with our previous findings, Fig. 8 A shows that a subset (two out of three) of cells overexpressing PS2 appeared apoptotic (notably only those that had rounded up) according to both CytoDEATH positive staining and condensed nuclei. Similarly, when calmyrin was overexpressed, analogous apoptosis was observed (Fig. 8 B).

Cotransfection of PS2 and calmyrin induced even higher apoptosis. To convey more clearly the high levels of apoptosis that resulted from overexpressing these two proteins, example images captured at low magnification are provided. Fig. 8 C shows that at 16 h after cotransfection almost 13% of PS2-expressing cells (which presumably also expressed calmyrin since PS2 and calmyrin staining showed a near 1:1 correspondence [data not shown]) on coverslips were positive for CytoDEATH staining. By 40 h the proportion of apoptotic cells had increased to ~50% of the PS2-stained cells. The high level of apoptosis seen on coverslips was striking, especially since this method only captured a brief "window" of the cells progression into apoptosis as during programmed cell death HeLa cells lose their adherence on coverslips and float away into the media. This phenomenon explains the reduction in total cells, and most notably PS-expressing cells (only 10 cells), remaining on the coverslip at 40 h after cotransfec-

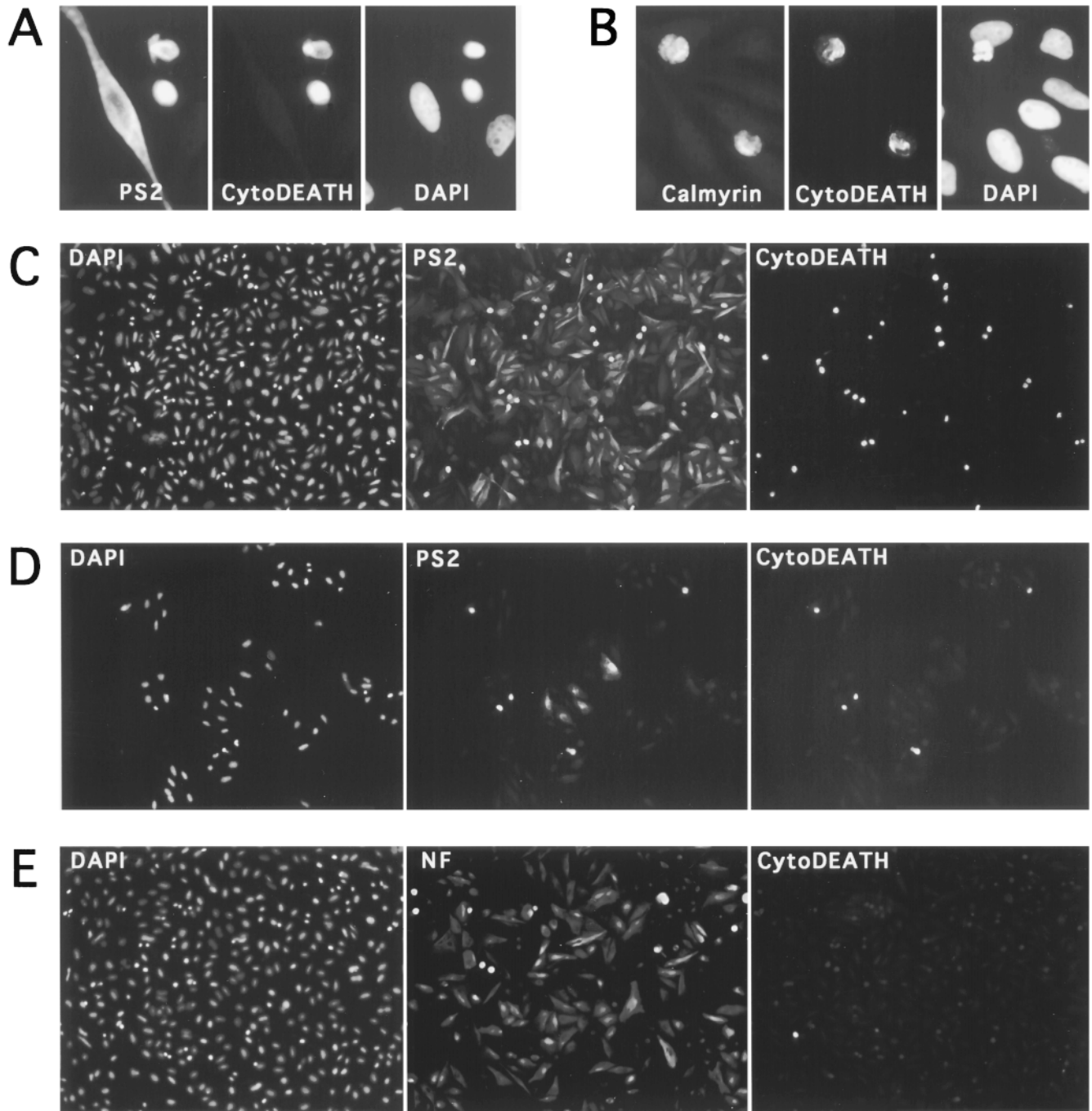
tion (Fig. 8 D). In the examples shown in Fig. 8 only a subset of PS overexpressing cells were apoptotic, indicating a time-dependent process, whereas the corollary that all apoptotic cells were also overexpressors always held true. In contrast when a control protein, the neurofilament light (NF-L) subunit, was overexpressed in HeLa cells (Fig. 8 E), minimal apoptosis (< 1%) was detected and total cell counts and expression levels remained high even at 40 h. It is remarkable that the single apoptotic cell in this field did not stain for NF-L and that, conversely, there are several rounded-up and highly expressing cells (presumably those in mitosis), none of which appeared apoptotic. Fields of cells overexpressing calmyrin or PS2 individually showed levels of apoptosis above the neurofilament background, but less than coexpressors. Unfortunately, due to variation between (and even within) coverslips, this method did not prove suitable for statistically significant quantification.

Because CytoDEATH labeling on coverslips only captured a narrow "window" of cells undergoing apoptosis, we decided to quantify the total amount of cell death accumulated over time by counting the total number of floating cells in the media after transfection with various amounts of plasmid DNAs encoding calmyrin and PS2. This simple method was more reliable in quantifying cell death. As graphed in Fig. 9, transient overexpression of PS2 increased cell death in a dose-dependent manner, whereas cell death induced by calmyrin overexpression reached a plateau at 10  $\mu\text{g}$  of transfected DNA. More interestingly, when both proteins were coexpressed in the linear cell death range of their respective DNAs, cell death increased 5.9-fold over the control, compared with 2.7- and 2.4-fold for the same respective transfection amounts of calmyrin and PS2 individually, suggesting that these two proteins have additive effects in promoting cell death. When these floating cells were collected and stained with the CytoDEATH antibody, ~85% of the cells stained positive for this marker of apoptosis, bolstering our belief that counting floating cells is a reliable measure of cell death.

## Discussion

In this study, we demonstrate by several criteria that human PS2 protein interacts with a recently discovered calcium-binding protein which we refer to as calmyrin. First, calmyrin interacts with PS2-loop sequence in yeast two-hybrid assays. Second, the two proteins bind to each other by affinity chromatography and can be coimmunoprecipitated. Third, the two full-length proteins colocalized when coexpressed in vivo. The interaction of calmyrin with PS2 is also noteworthy since it is the first protein, to our knowledge, that interacts preferentially with PS2 (at least by yeast two-hybrid analysis) suggesting distinct functions for the highly homologous presenilin proteins.

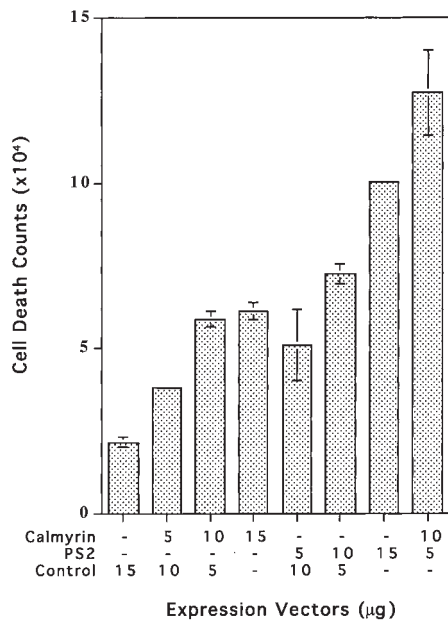
Two lines of evidence favor the PS2-loop region as the critical site of calmyrin interaction: reduced in vivo colocalization when calmyrin was coexpressed with a loop-deficient PS2 construct and increased yeast liquid culture binding of calmyrin to the PS2-loop rather than the PS2-COOH-terminal domain. Deletion analysis indicated that calmyrin binding was mediated primarily by the  $\text{NH}_2$ -terminal 31 amino acids of the PS2-loop. Remarkably, de-



**Figure 8.** Immunofluorescent staining with CytoDEATH indicates that overexpression of PS2 and calmyrin increases HeLa cell apoptosis. (A) An example field of four cells shows that two of the three cells overexpressing PS2 are apoptotic according to CytoDEATH positive staining and condensed nuclei. (B) Similarly, an example field of eight cells shows that both cells which overexpress calmyrin also appear apoptotic. (C)  $10\times$  field of calmyrin and PS2 cotransfected cells after 16 h. Total cells in the field are indicated by DAPI staining, expressing cells are labeled with anti-PS2 antibody (staining for the presence of PS2 and calmyrin in cotransfected cells showed a near 1:1 correspondence between the overexpression of these two proteins), and apoptotic cells are detected with the CytoDEATH anti-cytokeratin 18 antibody. Note the presence of  $>30$  apoptotic cells which all also stain positive for PS2 expression. (D)  $10\times$  field of calmyrin and PS2 cotransfected cells after 40 h. Note the reduction in total cells and the lower percentage of PS2-expressing cells at this later time point. (E)  $10\times$  field of control neurofilament-transfected cells after 40 h. Please note that even at this later time point, a high percentage of anti-neurofilament positive cells remain, while in contrast only one CytoDEATH apoptotic cell can be detected.

spite only a three-amino acid difference, the comparable loop region of PS1 interacted with less than one-tenth the strength in similar yeast two-hybrid assays. Site-directed mutagenesis in which the three divergent PS2 residues

were introduced singly and in double combinations into PS1 indicated that, in fact, all three amino acids produce variable effects on the specificity of calmyrin for PS2. Particularly interesting was the pronounced increased in bind-



**Figure 9.** Quantification of cell death due to overexpression of PS2 and calmyrin. Floating cells from duplicate plates of HeLa cells transfected with a total of 15  $\mu\text{g}$  of DNA containing control vector, increasing amounts of calmyrin, increasing amounts of PS2, or both calmyrin and PS2, were collected after 48 h. The number of floating cells per dish exceeding the mock transfection background counts are graphed with standard errors shown.

ing conferred by the conversion of a threonine at positions 281 and 291 in PS1 to proline and alanine, respectively. In contrast, the leucine at position 282 in PS1 when converted to isoleucine as in PS2 caused pleiotropic effects, increasing, decreasing, and inducing no change in interaction depending on its context with the other two residues. These data suggest that minor alterations in the sequence of the PS loop induce conformational changes in this region with dramatic consequences to protein-protein interactions. Furthermore, the loop region is a site associated with several PS-processing phenomena, including proteolytic cleavage, caspase cleavage, as well as abnormal splicing (Perez-Tur et al., 1995; Thinakaran et al. 1996; Kim et al., 1997; Loetscher et al., 1997; reviewed by Hass, 1997). Apart from calmyrin, several other proteins have been found to interact with the PS-loop, namely,  $\gamma$ -catenin, filamin, calselinin, mu-calpain, and armadillo protein p007 (Zhou et al., 1997; Buxbaum 1998; Murayama et al., 1998; Shinozaki et al., 1998; Zhang et al., 1998; Stahl et al., 1999). Our data showing that minor (single amino acid) alterations in the loop sequence can produce dramatic changes in protein binding not only has implications in terms of calmyrin function, but may also have important consequences for the other processing events and binding partners associated with this region. Therefore, it is not surprising that many FAD mutations map to the PS1 loop.

In addition to the importance of protein-protein interactions for localization, our immunofluorescence microscopy and biochemical fractionation studies indicate that the myristoylation of calmyrin is important for the dy-

namic targeting of this protein to several subcellular compartments including: the cytoplasm, long projections of the plasma membrane, and the nucleoplasm. Elegant studies of recoverin, a myristoylated calcium-binding protein involved in signaling in the retina, have established that this protein alternates between conformations in which the myristoyl group is exposed or sequestered, conformations that are dependent on calcium binding (Kennedy et al., 1996; Ames et al., 1997). These calcium-myristoyl switches are a known mechanism for protein targeting and signal transduction. Radiolabeling and biochemical studies show that calmyrin is myristoylated and associated with membranes. At present, we cannot clearly tease apart the roles that myristoylation and protein-protein interactions play in the *in vivo* targeting of calmyrin to membranes. In fact, our evidence suggests that both are important. Yeast two-hybrid assays with loop constructs containing site-directed mutations clearly show the importance of protein-protein interactions in mediating the association between calmyrin and the integral membrane protein, PS2. Additionally, fusion of the Gal4-acidic blob sequence at the  $\text{NH}_2$ -terminal end of calmyrin in the yeast two-hybrid clones would be expected to prevent this fatty acid modification suggesting that myristoylation is not essential for the interaction. Paradoxically, however, it is the myristoylated form of calmyrin that we were able to show coimmunoprecipitated with PS2. Perhaps insertion of the myristoyl group into the lipid bilayer initiates a conformational change that enhances the affinity of calmyrin for PS2. Analogously, the Gal4-acidic blob may have maintained calmyrin in the conformation that was more prone to binding PS2.

Cells overexpressing calmyrin proteins capable of being myristoylated showed greater variation in staining patterns often with increased targeting of calmyrin to the cytoplasm and plasma membrane suggesting that myristoylation may be involved in this dynamic behavior. The calmyrin that localized to the cytoplasm had a reticular-like staining pattern which colocalized with PS2 staining when the two proteins were coexpressed. We believe that the reticular staining represents targeting of calmyrin protein to the ER since we and others have shown that overexpressed PS2 was localized to the nuclear envelope and ER (see Kovacs et al., 1996; Janicki and Monteiro, 1997). Interestingly, in PS2-cotransfected cells relatively little calmyrin was present in the nucleus, and instead, the entire population almost completely colocalized with PS2 at the ER. This redistribution to the ER is consistent with the stoichiometric change of binding sites available for calmyrin once PS2 was overexpressed. However, the possibility that PS2 expression may alter processing or intracellular targeting of calmyrin can not be ruled out.

As myristoylation is known to be important for membrane targeting, it is curious that a significant pool of calmyrin is present within the nucleoplasm despite the lack of a classical nuclear localization signal. Calmyrin is small enough to passively diffuse through the nuclear pores (proteins  $\sim 65$  kD and larger must be actively transported) and may be sequestered within the nucleoplasm by binding to nuclear resident proteins such as DNA-PKcs which has also been shown to bind calmyrin in yeast two-hybrid assays (Wu and Lieber, 1997). The localization of calmyrin to the long projections of the plasma membrane

may similarly represent binding to calmyrin's other known interactor,  $\alpha$ IIB-subunit of integrin. These results indicate that calmyrin may traffic between several proteins and factors suggesting a role for calmyrin in complex signaling processes. How these processes relate to AD, and/or apoptosis is not known but it is interesting that Volado, a novel integrin which dynamically mediate cell adhesion and signal transduction, was recently identified as a new memory mutant in *Drosophila* (Grotewiel et al., 1998). Also DNA-PKcs is the only known eukaryotic protein kinase activated by DNA double-strand breaks which is a lesion induced during apoptosis (reviewed McConnell and Dynan, 1996; Sheih et al., 1997). The possibility that calmyrin, integrins, DNA-PKcs, and PS2 are in any way connected or involved in human diseases is intriguing, yet speculative.

In hypothesizing a physiologic role for the interaction between PS2 and calmyrin, we are especially interested in exploring the involvement of calcium and apoptosis. It is noteworthy that calsenilin, another  $\text{Ca}^{2+}$ -binding protein with sequence similarity to recoverin, binds to the COOH-terminal region of presenilin proteins and like calmyrin redistributes with presenilin proteins in cotransfected cells (Buxbaum, et al., 1998). Calmyrin does not share a high degree of amino acid similarity to calsenilin, instead the protein sequence of calmyrin is most homologous to human calcineurin B, the regulatory subunit of the  $\text{Ca}^{2+}$ -calmodulin-dependent protein phosphatase 2B, which plays important roles in stress, apoptosis, cell calcium signaling, and signal transduction (see Guerini, 1997; Crabtree, 1999; Wang et al., 1999). The greatest homology is found in the regions surrounding calcineurin B's four calcium-binding EF hand motifs and its  $\text{NH}_2$ -terminal myristoylation site. Although these regions are relatively well conserved, sharing 44% overall similarity, calmyrin appears to have only two functional EF hands as the two  $\text{NH}_2$ -terminal motifs contain several insertions that are predicted to disrupt calcium binding. Naik et al. (1997) have demonstrated that calmyrin can indeed bind calcium, but it is unknown whether this property regulates phosphatase activity. If calmyrin behaves similarly to calcineurin B in phosphatase regulation, it may have some relevance to AD where there is speculation that PHF formation and tau hyperphosphorylation occurs due to misregulation of protein phosphatases or kinases (Matsuo et al., 1994; Gong et al., 1996; Kayyali et al., 1997).

Our cell death findings imply that the binding of calmyrin to PS2 may be related to PS2 function in apoptosis. In a previous study we found that overexpression of PS2 in HeLa cells induced apoptosis. The current finding that coexpression of calmyrin with presenilins in HeLa cells increased apoptosis suggests that the two act in concert in a pathway or pathways regulating cell death. Although we have not determined the pathway through which the two proteins function during programmed cell death, the fact that calmyrin is a calcium-binding protein (Naik et al., 1997) raises some obvious possibilities. First, calmyrin may "sense"  $\text{Ca}^{2+}$  changes and subsequently regulate PS2 function. Alternatively, PS2 proteins (including FAD mutants) may alter calcium homeostasis resulting in a change in calcium binding by calmyrin which could then trigger a signal transduction cascade. This latter possibility is attractive

since overexpression of presenilins has been shown to cause perturbations in calcium homeostasis (Guo et al., 1996; Keller et al., 1998). Interestingly, the apoptosis rescue screen in which the PS2 ALG3 fragment was isolated (see introduction) yielded another cDNA named ALG2, which caused antisense inhibition of a calcium-binding protein (Vito et al., 1996). However, when ALG2 was expressed in the sense orientation, this calcium-binding protein induced apoptosis (Lacana et al., 1997). It could be argued that coexpression of any calcium-binding protein with presenilins would cause increased cell death. This is clearly not the case as overexpression of another calcium-binding protein, calbindin D28k, suppressed the proapoptotic functions of PS (Guo et al., 1998). An imbalance in calcium regulation could be catastrophic to the cell due to the central role calcium plays in cellular processes including its participation in the induction phase of apoptosis (reviewed by McConkey and Orrenius, 1997).

Although there is some disagreement as to whether AD involves a perturbation of calcium regulation (see Etcheberrigaray et al., 1998), the consensus of research (opinion) is indicative of such a defect. The uncertainty is in part complicated by lack of reliable and easy methods to measure intracellular calcium, let alone compare them in different individuals. Nevertheless, numerous studies have shown that calcium levels are altered in cells cultured from AD patients, especially those harboring (or transfected with) presenilin genes containing FAD-linked mutations (Ito et al., 1994; Gibson et al., 1997; Mattson et al., 1998).

In summary, our results suggest that calmyrin, a calcium-binding myristoylated protein, may play dynamic and diverse roles in intracellular signaling, and we propose that it is important in the modulation of presenilin function. Understanding the complex interplay between calcium regulation, apoptotic signaling, and protein-protein interactions will no doubt aid in deciphering the mechanisms through which the presenilins function which in turn could provide insight into the pathogenesis of Alzheimer's disease.

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