

# Densin-180 Forms a Ternary Complex with the $\alpha$ -Subunit of $\text{Ca}^{2+}$ /Calmodulin-Dependent Protein Kinase II and $\alpha$ -Actinin

Randall S. Walikonis, Asako Oguni, Eugenia M. Khorosheva, Chung-Jiuan Jeng, Franklin J. Asuncion, and Mary B. Kennedy

Division of Biology, California Institute of Technology, Pasadena, California 91125

Densin-180 is a transmembrane protein that is tightly associated with the postsynaptic density in CNS neurons and is postulated to function as a synaptic adhesion molecule. Here we report the identification of the  $\alpha$ -subunit of  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase II (CaMKII) and  $\alpha$ -actinin-4 as potential binding partners for the densin-180 intracellular segment. We demonstrate by yeast two-hybrid and biochemical assays that the intracellular portion of densin-180, the  $\alpha$ -subunit of CaMKII (CaMKII $\alpha$ ), and  $\alpha$ -actinin interact with each other at distinct binding sites and can form a ternary complex stabilized by multiple interactions. Densin-180 binds specifically to the association domain of CaMKII $\alpha$  and does not bind with high affinity to holoenzymes of CaMKII that contain  $\beta$ -subunit. The PDZ (PSD-95, Dlg, Z0-1) domain of densin

contributes to its binding to  $\alpha$ -actinin. A distinct domain of  $\alpha$ -actinin interacts with the kinase domains of both  $\alpha$ - and  $\beta$ -subunits of CaMKII. Autophosphorylation of CaMKII increases its affinity for densin-180 from an  $\text{EC}_{50}$  of  $>1 \mu\text{m}$  to an  $\text{EC}_{50}$  of  $<75\text{--}150 \text{ nM}$ . In contrast, phosphorylation of densin-180 by CaMKII at serine-1397 only slightly decreases its affinity for CaMKII. The specific interaction of densin-180 with holoenzymes of CaMKII containing only  $\alpha$ -subunit and the increased affinity of CaMKII for densin-180 after autophosphorylation suggest that densin-180 may be involved in localization of activated CaMKII synthesized in dendrites.

*Key words:* postsynaptic density; synaptic plasticity; protein phosphorylation; synapse; spine; neuronal cytoskeleton

The postsynaptic density (PSD) in glutamatergic synapses contains a highly ordered array of proteins that initiate and modulate signal transduction in the postsynaptic neuron (Kennedy, 1997, 1998). Although many of the proteins assembled at the PSD have been identified (Husi et al., 2000; Walikonis et al., 2000), the physical and functional interactions among these proteins are only beginning to be deciphered. Densin-180 (hereafter referred to as densin) is the founding member of a newly described family of proteins termed the LAP [leucine-rich repeat (LRR) and PDZ (PSD-95, Dlg, Z0-1)] family, characterized by an LRR near the N terminus and one or more PDZ domains at the C terminus (Bilder et al., 2000). Other members of the LAP family, which include LET-413, scribble, and ERBIN (Bilder and Perrimon, 2000; Borg et al., 2000; Legouis et al., 2000), are associated with cell membranes at specialized domains. They play essential roles in sorting of membrane proteins to their appropriate location and in organizing signaling and structural proteins at cellular junctions.

Unlike the other LAP proteins, which are cytosolic membrane-associated proteins, densin is a transmembrane glycoprotein (Apperson et al., 1996). Its domain structure suggests that the N terminal 137 kDa, containing the LRR, and a sialylated mucin-homology region are extracellular, whereas the C terminal 27.4 kDa, comprising a 17.5 kDa membrane proximal region and a 9.9

kDa PDZ domain, are intracellular. We recently confirmed that densin spans the membrane when it is expressed recombinantly in heterologous cells (C.-J. Jeng and M. B. Kennedy, unpublished observations). In its transmembrane domain structure, densin resembles the platelet adhesion molecule GPIIb $\alpha$ , which contains extracellular LRRs that bind von Willebrand factor, and a short intracellular actin-binding domain (Apperson et al., 1996). The full range of functions that densin performs in the spine and PSD is not yet clear. The results presented here suggest a role in organizing both structural and signaling systems.

To gain insight into the functions of densin, we have used the yeast two-hybrid method to screen for proteins that interact with its putative cytosolic region. Here we show that the cytosolic domain can form a ternary complex with  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase II (CaMKII) and  $\alpha$ -actinin, both of which are enriched in spines and associated with the PSD (Kennedy et al., 1990; Wyszynski et al., 1998). The binding of densin to CaMKII, both *in vitro* and in synaptosomes, is selective for holoenzymes composed only of  $\alpha$ -subunits. These data suggest that densin may be important for localization, and/or translocation to the PSD (Shen and Meyer, 1999) of CaMKII holoenzymes synthesized in dendrites (Ouyang et al., 1999) after activation of synaptic NMDA receptors in hippocampal neurons. Dendritically synthesized holoenzymes are likely composed primarily of  $\alpha$ -subunits (Burgin et al., 1990).

Received Sept. 18, 2000; revised Oct. 20, 2000; accepted Oct. 30, 2000.

This work was supported by National Institutes of Health Grants NS28710 and NS17660 (M.B.K.) and by a fellowship from the FRAXA (Fragile-X) Research Foundation (R.S.W.) and from the John Douglas French Alzheimer's Foundation (C.-J.J.).

Correspondence should be addressed to Mary B. Kennedy, Division of Biology 216-76, California Institute of Technology, Pasadena, CA 91125. E-mail: kennedym@its.caltech.edu.

Copyright © 2001 Society for Neuroscience 0270-6474/01/210423-11\$15.00/0

## MATERIALS AND METHODS

**Yeast two-hybrid screen.** A yeast two-hybrid screen was performed in yeast strain Y190 containing HIS3 and  $\beta$ -galactosidase ( $\beta$ -gal) reporter genes under the control of the GAL1 activating sequence. The cDNA encoding the putative intracellular region of densin (densin(intra); amino acids 1249–1495) (Apperson et al., 1996) was inserted into pAS2-1 for expression as a fusion with the GAL4 DNA-binding domain (Clontech, Palo

Alto, CA). A human brain cDNA library inserted into pACT2 (Clontech), a vector encoding the GAL4 activation domain, was screened for expression of proteins that interact with the intracellular region of densin.

The pAS2-1:densin(intra) construct and the brain cDNA library in pACT were sequentially transformed into yeast by the lithium acetate method (Gietz and Schiestl, 1995). Interaction was assessed by growth on His medium and by expression of  $\beta$ -galactosidase according to the Clontech manual. Specificity of interaction was tested by the mating assay as described in the Clontech manual. Plasmids were isolated from yeast with the EZ Yeast Plasmid Miniprep kit according to the manufacturer's instructions (Geno Technology, St. Louis, MO).

**Delineation of binding domains by yeast two-hybrid assay.** Truncation mutants of cDNA inserts were generated by restriction digest or by PCR and subcloned into pACT2 or pAS2-1. PCR was conducted in buffer supplied with *Taq* polymerase (Life Technologies, Grand Island, NY) or with *Vent* polymerase (New England Biolabs, Beverly, MA), as appropriate, with the addition of 50  $\mu$ M sense and antisense primers, 2 mM deoxynucleoside 5'-triphosphates, 2.5 U of *Taq* or *Vent*, and 4 ng/ $\mu$ l cDNA. The sequences of all cDNA constructs were verified by restriction mapping and sequencing. Regions of the  $\alpha$ -actinin-4 cDNA amplified by PCR include those encoding residues 464–806, 464–834, 464–871, 835–879, and 835–871. A cDNA encoding residues 633–879 was made by digesting clone IJ12 with *Bam*HI and *Xho*I. Another cDNA encoding residues 872–879 was made by synthesizing sense and antisense DNA strands that encode these eight amino acids. Each cDNA was inserted into pACT2.

The cDNAs encoding kinase and association domains of the  $\alpha$ - and  $\beta$ -subunits of CaMKII were inserted into either pAS2-1 or pACT2. cDNAs encoding the kinase domain (residues 1–316) and the association domain (residues 314–478) of CaMKII $\alpha$  were generated by PCR and inserted into pAS2-1. Similarly, cDNAs encoding the kinase domain (residues 1–316) and the association domain (residues 315–542) of CaMKII $\beta$  were amplified and ligated into both pAS2-1 and pACT2. The cDNA encoding the entire CaMKII $\beta$  was inserted into pAS2-1.

For tests of interaction by expression of  $\beta$ -galactosidase, we judged colonies that turned blue in 1 hr to contain strongly interacting proteins and colonies that turned blue within 8 hr to contain weakly interacting proteins. For tests of interaction by expression of HIS3, we judged yeast colonies that appeared on His<sup>-</sup> plates within 2 d to contain strongly interacting proteins and those that appeared within 3–5 d to contain weakly interacting proteins. Constructs encoding a PDZ domain as a fusion with the DNA-binding domain would often weakly autoactivate  $\beta$ -galactosidase expression but not HIS3 expression. Therefore, interactions with proteins that contain a PDZ domain fused to the DNA-binding domain were judged by growth on His<sup>-</sup> plates.

**Construction and expression of fusion proteins.** cDNAs encoding three intracellular regions of densin were inserted into pGEX vectors and expressed in *Escherichia coli* to form fusion proteins with glutathione-S-transferase (GST). A fusion protein containing the entire intracellular domain of densin (residues 1249–1495) was made by inserting the cDNA encoding this region into pGEX-5X-1; a cDNA encoding the membrane proximal region of densin (residues 1266–1423) was inserted into pGEX-2T. A cDNA encoding residues 1374–1495, encoding the PDZ domain, was inserted into pGEX-2T as described previously (Apperson et al., 1996). We term these fusion proteins GST:densin(intra), GST:densin(prox), and GST:densin(PDZ), respectively. A cDNA encoding residues 638–879 of  $\alpha$ -actinin was inserted into pGEX-5X-2. The proper orientation of insertion for each construct was verified by restriction mapping and sequencing. Fusion proteins were expressed in *E. coli* DH5 $\alpha$  and purified as described previously (Omkumar et al., 1996).

A His: $\alpha$ -actinin-4 fusion protein was generated by inserting the cDNA encoding residues 638–879 of  $\alpha$ -actinin-4 into the pET28c vector (Novagen, Madison, WI). Expression of the fusion protein was induced in *E. coli* BL21 (DE3) according to the instructions supplied with the vector. Fusion proteins were harvested and absorbed onto beads substituted with Ni-nitrilotriacetic acid (Qiagen, Valencia, CA) according to the manufacturer's instructions. A portion of the washed beads with bound fusion protein was removed and stored. The His:actinin(COOH) fusion protein was eluted from the remaining beads with elution buffer (in mM: 50 NaH<sub>2</sub>PO<sub>4</sub>, pH 8.0, 300 NaCl, and 250 imidazole).

**Preparation of synaptosome and PSD fractions.** A synaptosome fraction and a "One-Triton" PSD fraction were prepared from rat forebrain as described previously (Carlin et al., 1980; Cho et al., 1992). Synaptosomes were purified from forebrain homogenates by differential and density

gradient centrifugation and then extracted with 0.5% Triton X-100 for 15 min to form the One-Triton PSD fraction. Protein concentrations were determined by a modified method of Lowry (Peterson, 1983).

**Immunoblots.** Proteins were separated by SDS-PAGE under reducing conditions and electrophoretically transferred to nitrocellulose membranes. The membranes were blocked at least 2 hr in 5% nonfat milk in TBST (10 mM Tris, pH 7.5, 200 mM NaCl, and 0.2% Tween 20) followed by incubation with primary antibodies. Antibodies against CaMKII included mouse monoclonal antibody 6G9, which recognizes CaMKII $\alpha$  (diluted 1:5000), rabbit antiserum Darlene (1:5000), which recognizes nonphosphorylated  $\alpha$ - and  $\beta$ -subunits of CaMKII, and rabbit antiserum Darcy (1:500), which recognizes CaMKII $\beta$ . Antibodies against densin included M3 or CT245 (Apperson et al., 1996), diluted 1:2500 and 1:5000, respectively. PSD-95 was detected with rabbit antiserum Frances against recombinant PSD-95. Anti-GST rabbit antiserum (1:3500) was purchased from Sigma (St. Louis, MO) and anti-T7 mouse monoclonal antibody (1:10,000) was obtained from Novagen. Bound antibodies were detected by the alkaline phosphatase method with reagents purchased from Boehringer Mannheim (Indianapolis, IN), or by chemiluminescence with reagents purchased from Pierce (Rockford, IL), according to the manufacturer's instructions.

**Phosphorylation reactions.** CaMKII (12  $\mu$ g) was autophosphorylated by incubation in 100  $\mu$ l of phosphorylation mix (50 mM Tris, pH 8.0, 0.7 mM CaCl<sub>2</sub>, 0.4 mM EGTA, 10 mM MgCl<sub>2</sub>, 10 mM dithiothreitol, 100  $\mu$ M ATP, 0.2 nM to 4  $\mu$ M calmodulin, and 1 mg/ml BSA). The mix was prewarmed to 30°C for 5 min. CaMKII was added, and the solution was incubated for 5 min. Control reactions in which CaMKII was not phosphorylated contained the same reagents, except that CaCl<sub>2</sub> and calmodulin were omitted. Reactions were stopped by addition of 4  $\mu$ l of 500 mM EDTA and placed on ice.

Phosphorylation of fusion proteins was conducted as described previously (Miller et al., 1988; Omkumar et al., 1996). Phosphorylation reactions contained 0.25–7.4  $\mu$ M GST fusion proteins in the phosphorylation mix described above with 100  $\mu$ M [ $\gamma$ -<sup>32</sup>P]ATP (1000 cpm/pmol) in a final volume of 100  $\mu$ l. The mixtures were preincubated at 30°C for 3 min, after which 40 ng of rat brain CaMKII was added. Phosphorylation was terminated at indicated times by the addition of SDS-PAGE sample buffer, and the sample was boiled for 3 min. Twenty microliters of each sample was fractionated by SDS-PAGE, the gels were stained with Coomassie blue, and radioactive bands were identified by autoradiography. The radioactive bands were cut from the gel, and their content of [ $\gamma$ -<sup>32</sup>P]PO<sub>4</sub> was quantified by detection of Cerenkov radiation in a Beckman LS 7800 scintillation counter (Beckman Coulter, Fullerton, CA).

**Measurement of binding specificity for CaMKII by pull-down assay.** GST: $\alpha$ -actinin, GST:densin(intra), and GST were cross-linked to glutathione-coated agarose beads with dimethylpiperimidate (DMP) (Harlow and Lane, 1988). The beads containing bound fusion proteins were washed twice with 10 volumes of 0.2 M sodium borate, pH 9.0, and suspended in 10 volumes of the same solution. DMP was added to a final concentration of 20 mM, and the suspension was rotated for 30 min at room temperature. The beads were washed with 0.2 M ethanolamine, pH 8.0, and then incubated in the same solution for 2 hr at room temperature. The cross-linked beads were washed in 0.02 M Na phosphate buffer, pH 7.4, 0.15 M NaCl (B-buffer). After examining the content of fusion protein remaining in the supernatant, we estimated that the beads contained ~1  $\mu$ g of fusion protein per microliter of beads.

To study the specificity of binding of nonphosphorylated CaMKII, rat forebrain CaMKII (12  $\mu$ g), purified from rat forebrain as described previously (Miller and Kennedy, 1985), was diluted to 0.4 mg/ml in B-buffer and mixed with 20  $\mu$ l of a 1:1 suspension of beads containing cross-linked GST:actinin, GST:densin, or GST. Parallel experiments were performed to study the specificity of binding of autophosphorylated CaMKII. CaMKII (36  $\mu$ g) was autophosphorylated for 5 min in 120  $\mu$ l of reaction mix as described above. After autophosphorylation, 40  $\mu$ l of the mix, containing 12  $\mu$ g of CaMKII, was mixed with 20  $\mu$ l of a 1:1 suspension of beads containing cross-linked GST:actinin, GST:densin, or GST, and 40  $\mu$ l of 2 $\times$  B-buffer. The suspensions were rotated for 1 hr at room temperature, washed twice in B-buffer containing 0.1% Triton X-100, and washed once in B-buffer. Bound protein was eluted by boiling the beads in SDS-PAGE sample buffer. The eluted proteins were fractionated by SDS-PAGE and transferred to a nitrocellulose membrane. Nonphosphorylated CaMKII was detected by immunoblot with the antiserum Darlene, which recognizes the nonphosphorylated form of  $\alpha$  and  $\beta$  CaMKII. Phosphorylated CaMKII was detected by immunoblot

with a mixture of antibody 6G9 and the antiserum Darcy, which recognize the  $\alpha$ - and  $\beta$ -subunits of CaMKII, respectively.

**Membrane overlay assays.** GST:densin(intra), GST:densin(prox), GST:densin(PDZ), and GST were separated by SDS-PAGE and transferred to a nitrocellulose membrane. Approximately 2.5  $\mu$ g of the major band of each fusion protein was loaded in each lane. The membrane was blocked by incubation in 5% nonfat dry milk in TBST for 2 hr. Purified forebrain CaMKII was autophosphorylated, diluted in TBST to a concentration of 10  $\mu$ g/ml, and incubated with the membrane for 15 hr at room temperature. The membrane was washed three times in TBST and subsequently incubated with antibody 6G9 against CaMKII $\alpha$  for 3 hr. Bound antibodies were detected by the alkaline phosphatase method (Harlow and Lane, 1988).

To test the effect of autophosphorylation of CaMKII and phosphorylation of densin on the affinity of binding between them, GST:densin(intra) (50  $\mu$ g) or GST (50  $\mu$ g) was phosphorylated by CaMKII in the phosphorylation mix described above with or without CaCl<sub>2</sub> and calmodulin. The solution was prewarmed for 5 min at 30°C, 3  $\mu$ g of CaMKII was added, and the solution was incubated for another 15 min. Reaction was stopped by the addition of 4  $\mu$ l of EDTA. Glutathione-conjugated agarose beads were added to the mix, and the slurry was rotated end-over-end for 20 min. The supernatant was removed, and the beads were washed once in TBS (50 mM Tris, pH 8.0, and 150 mM NaCl), twice in TBS plus 0.1% Triton X-100, and twice in TBS. Bound GST fusion proteins were stripped from the beads by addition of SDS-PAGE sample buffer, followed by boiling. The proteins were fractionated by SDS-PAGE and transferred to a nitrocellulose membrane. In parallel experiments, we demonstrated that ~40% of the GST fusion proteins were recovered on beads, resulting in ~10  $\mu$ g of protein in each lane of the gel. The membrane was blocked in 5% milk in TBST for 2 hr. The membrane was placed in a Decaprobe multilane screening apparatus (Hofer, San Francisco, CA). CaMKII was autophosphorylated as described above, diluted to 10  $\mu$ g/ml in TBST, and placed over individual lanes for 12 hr. The membrane was washed in TBST, and CaMKII bound to the fusion protein was detected by immunoblot with antibody 6G9.

The region of densin that binds to  $\alpha$ -actinin was delineated in a membrane overlay assay. His: $\alpha$ -actinin (8.5  $\mu$ g/lane) was separated by SDS-PAGE and transferred to a nitrocellulose membrane. Individual lanes were overlaid with 10  $\mu$ g/ml GST:densin(intra), GST:densin(PDZ), GST:densin(prox), or GST in TBST. The membrane was incubated for 16 hr and washed three times with TBST. Bound GST:densin was detected by immunoblot with antiserum against GST (Sigma).

**Coimmunoprecipitation of densin and CaMKII.** Synaptosomes were solubilized in immunoprecipitation (IP) buffer (20 mM Tris-HCl, pH 7.5, 0.1 M NaCl, 4 mM KCl, 5 mM CaCl<sub>2</sub>, 2.5 mM EDTA, 20 mM NaHCO<sub>3</sub>, and 2% Triton X-100) and incubated on ice for 1 hr. Insoluble material was removed by brief centrifugation. Solubilized synaptosomal fractions were incubated for 16 hr at 4°C with 60  $\mu$ l of 50% (w/v) protein-A-agarose (Pierce) previously loaded with M3 antibodies against densin (Apperson et al., 1996) or mouse IgG. Beads were washed three times in IP buffer and once with IP buffer without Triton X-100. The immune complexes were eluted from the beads by boiling for 5 min in 30  $\mu$ l of SDS-PAGE sample buffer. Eluates were fractionated by electrophoresis on 8% SDS-PAGE minigels. Immunoprecipitated proteins were detected by immunoblot with antibody M3 against densin, antibody Darlene against CaMKII, and antibody Frances against PSD-95.

**Detection of formation of ternary complex.** Glutathione-agarose beads loaded with 10  $\mu$ g of GST:densin(prox), GST:densin(PDZ), or GST were incubated with 12  $\mu$ g of CaMKII, 7.5  $\mu$ g of His:actinin(COOH), or both proteins in combination, in a total volume of 50  $\mu$ l of B-buffer. The solution was rotated for 2 hr at room temperature. The beads were washed three times in B-buffer, suspended in SDS-PAGE sample buffer, and boiled to elute bound proteins. Eluted proteins were fractionated by SDS-PAGE and transferred to a nitrocellulose membrane. CaMKII was detected by immunoblot with antibody 6G9, and His:actinin(COOH) was detected by immunoblot with anti-T7 antibody.

**Determination of phosphorylation site for CaMKII on densin.** To identify the CaMKII phosphorylation sites on densin, fusion proteins GST:densin(PDZ) and GST:densin(intra) were exhaustively phosphorylated. The GST:densin fusion proteins (50  $\mu$ g) were added to a phosphorylation mix containing 100  $\mu$ M [ $\gamma$ -<sup>32</sup>P]ATP (1000 cpm/pmol) and preincubated for 15 min at 30°C. Rat forebrain kinase (1.2  $\mu$ g) was added, and incubation continued for 15 min, followed by the addition of another 1.5  $\mu$ g of kinase and incubation for an additional 15 min. The reaction was terminated by bringing the solution to 20 mM EDTA. Glutathione-conjugated agarose beads (100 ml, 50% w/v) were added, and the solution

was rotated at 4° for 25 min. The beads were washed in 125 mM Tris, pH 8.0, and suspended in 25 mM Tris, pH 8.9. Endoproteinase-Lys-C (0.5  $\mu$ g) (Wako Chemicals, Dallas, TX) was added, and the solution was incubated at 30°C for 15 hr. A second aliquot of 0.5  $\mu$ g of endoproteinase-Lys-C was added, and digestion continued for another 4 hr. The reaction was terminated by addition of 10% trifluoroacetate (TFA) to a final concentration of 1%. Beads were pelleted by centrifugation, and the supernatant was collected. The beads were washed twice with 50% acetonitrile in 25 mM Tris, pH 8.9, and the washes were pooled with the supernatant. The pooled solution was evaporated to dryness in a Speed-Vac concentrator (Savant Instruments, Holbrook, NY).

**HPLC fractionation of phosphopeptides.** HPLC was conducted as described previously (Omikumar et al., 1996). Lyophilized peptides were resuspended in 0.1% TFA and fractionated by HPLC on a C18 reverse-phase column (4.1  $\times$  250 mm). The column was developed at 1 ml/min with a gradient of 0–42% acetonitrile. Absorbance at 214 nm was monitored, and 0.5 ml fractions were collected. Radioactivity in each fraction was measured by detection of Cerenkov radiation.

**Mass spectrometry.** Fractions containing radiolabeled peptides were analyzed by matrix-assisted laser desorption ionization, time-of-flight (MALDI-TOF) mass spectrometry in both linear and reflector modes. Peak fractions containing labeled peptides were concentrated and mixed with  $\alpha$ -cyano-4-hydroxycinnamic acid matrix solution, dried, and subjected to MALDI-TOF mass spectrometric analysis. Amino acid sequencing was conducted at the Caltech Protein/Peptide Microanalytical Laboratory with a model 476A automatic protein sequenator (Applied Biosystems, Foster City, CA).

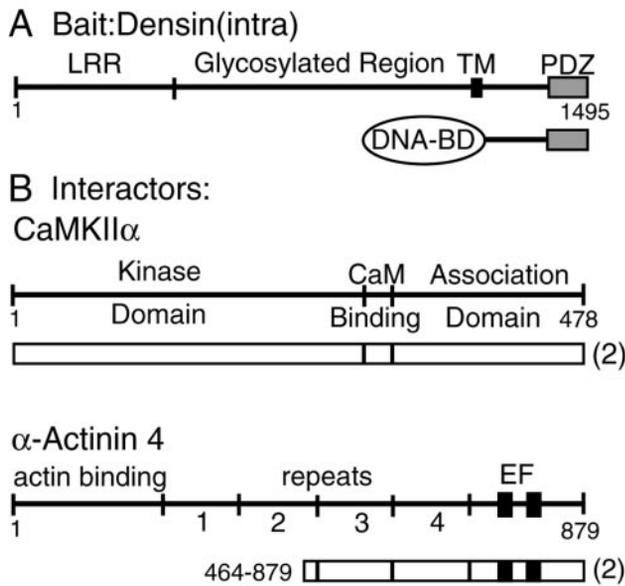
**Phosphorylation of densin in the PSD fraction.** To phosphorylate densin in the PSD fraction, 500  $\mu$ g of PSD fraction was added to the phosphorylation mixture described above with 25  $\mu$ M [ $\gamma$ -<sup>32</sup>P]ATP at 10,000 cpm/pmol. PSD proteins were phosphorylated for 5 min at 30°C. Densin was immunoprecipitated from the reaction mix after diluting it in RIPA buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% deoxycholate, and 0.1% SDS). Forty microliters of CT245 antibodies against densin (Apperson et al., 1996) were added, and the solution was rotated end-over-end overnight. Protein A-agarose beads (100  $\mu$ l of a 1:1 suspension) were added, and the mix was rotated for 2 hr. The beads were collected and washed three times in RIPA buffer. Proteins were eluted by boiling the beads in SDS-PAGE sample buffer and fractionated by SDS-PAGE. Autoradiography of the dried gel revealed a single phosphorylated band of 180 kDa, which was cut from the gel and rehydrated in 25 mM Tris, pH 8.9. Endoproteinase-Lys-C (0.5  $\mu$ g) was added, and the mixture incubated for 4 hr at 30°C. A second aliquot of 0.5  $\mu$ g of endoproteinase-Lys-C was added, and incubation continued for 15 hr at 30°C. The reaction was terminated, and the proteins were concentrated as described above.

**Quantification of binding of CaMKII to GST:densin(intra).** GST:densin(intra) (17  $\mu$ g) was electrophoresed on a preparative slot SDS gel and transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked for 2 hr in 5% nonfat milk in TBST and placed in a Mini-Protean II multiscreen apparatus (Bio-Rad, Hercules, CA). Purified forebrain CaMKII (41  $\mu$ g) was phosphorylated or added to phosphorylation mix without ATP (control nonphosphorylated) for 5 min in 750  $\mu$ l of reaction mixture as described above. Portions of autophosphorylated or control nonphosphorylated kinase (0.01–27.5  $\mu$ g) were diluted to 500  $\mu$ l volume in TBST buffer and incubated overnight at room temperature with individual lanes in the multiscreen apparatus. The membrane was washed three times for 15 min each with TBST, and subsequently incubated for 4 hr with antibody 6G9, which recognizes both phosphorylated and nonphosphorylated CaMKII $\alpha$ , as described above. The membrane was washed three times for 15 min each in TBST, and subsequently incubated for 1 hr with fluorescein-conjugated secondary antibody (Amersham Pharmacia Biotech, Piscataway, NJ) diluted 1:100 in TBST. After washing and drying, the membranes were scanned with a Storm fluorescent imager (Molecular Dynamics, Sunnyvale, CA). The resulting images were digitized and quantified with ImageQuant software (Molecular Dynamics). EC<sub>50</sub> values were calculated with Prism statistical software (GraphPad, San Diego, CA).

## RESULTS

### CaMKII interacts with the intracellular domain of densin

We used the putative intracellular domain of densin as bait in a yeast two-hybrid screen of ~450,000 clones of a human brain cDNA library (Fig. 1A). Interactions between the intracellular



**Figure 1.** Diagram of bait construct and binding partners identified in a yeast two-hybrid screen of a brain cDNA library. *A*, Diagram of the bait construct. The intracellular region of densin was inserted into yeast pAS2-1 vector and used to screen a human brain cDNA library. Interacting clones were identified by  $\beta$ -gal expression and growth of yeast on His<sup>-</sup> plates as described in Materials and Methods. *B*, Binding partners for densin identified in the yeast two-hybrid screen. The numbers in parentheses indicate the number of identical copies of each cDNA isolated in the screen. Both cDNAs encoding CaMKII $\alpha$  contain the entire open reading frame, whereas the cDNAs encoding  $\alpha$ -actinin-4 encode the indicated amino acid residues.

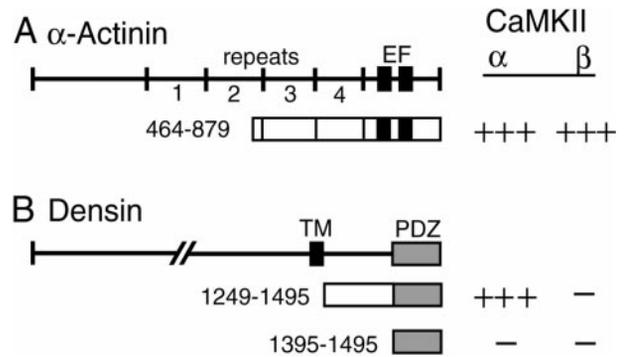
region of densin and proteins expressed from the brain library were detected by expression of  $\beta$ -gal and the HIS3 reporter gene as described in Material and Methods. The screen yielded five cDNA inserts representing three separate genes.

Three of the cDNA inserts encode subunits of human CaMKII. Two identical 3.6 kb cDNAs encode the  $\alpha$ -subunit (Fig. 1*B*) (Nagase et al., 1999). They contain 129 bp of 5' UTR, the 1434 bp coding region, and a 1532 bp 3' UTR. CaMKII $\alpha$  and densin are both highly enriched in the PSD fraction and colocalize in spines at excitatory synapses (Apperson et al., 1996). The third insert encodes CaMKII $\gamma_E$ , which is 84% identical to CaMKII $\alpha$  except for two inserts of 38 and 39 amino acids. The  $\gamma_E$  subunit of CaMKII was originally identified in human islets of Langerhans cells (Breen and Ashcroft, 1997). Its expression in the brain has not been investigated. None of the selected cDNAs contain stop codons between the sequence encoding the GAL4 activation domain and that encoding CaMKII.

After this work was presented in abstract and poster form (Walikonis et al., 1999), and while this manuscript was being prepared, Strack et al. (2000) published a study showing that densin binds CaMKII in the PSD fraction.

#### $\alpha$ -Actinin interacts with the intracellular domain of densin

The two remaining cDNA inserts are identical and encode the COOH-terminal half of human  $\alpha$ -actinin-4 (Fig. 1*B*). The  $\alpha$ -actinins are a family of closely related proteins that contain an NH-terminal actin-binding domain, four spectrin-like repeats, and two EF-hands near the COOH terminus. The sequences of our inserts are contained in a splice variant of human  $\alpha$ -actinin-4 (Honda et al., 1998), starting at bp 1478 and extending for ~2.3



**Figure 2.** Interactions of  $\alpha$ - and  $\beta$ -subunits of CaMKII with  $\alpha$ -actinin and densin in a yeast two-hybrid assay. The numbers to the left of each construct indicate the encoded amino acid residues. *A*, Interactions of  $\alpha$ -actinin with the  $\alpha$ - and  $\beta$ -subunits of CaMKII. The cDNA encoding the COOH-terminal region of  $\alpha$ -actinin was inserted into pACT2 and tested for interactions with the  $\alpha$ - and  $\beta$ -subunits of CaMKII inserted into pAS2-1. *B*, Interactions of densin with the  $\alpha$ - and  $\beta$ -subunits of CaMKII. The cDNA encoding the intracellular portion of densin was inserted into pAS2-1, and the cDNA encoding the PDZ domain of densin was inserted into pACT. The encoded proteins were tested for interaction with the CaMKII subunits inserted into pAS2-1 or pACT. Strong interaction or no interaction is indicated by +++ or -, respectively.

kb. The encoded protein begins within the second spectrin-like repeat and continues to the COOH terminus.

#### $\alpha$ -Actinin interacts with both the $\alpha$ - and $\beta$ -subunits of CaMKII in the yeast two-hybrid assay

$\alpha$ -Actinin contains multiple protein-binding domains and interacts with other proteins located in the PSD (Krupp et al., 1999). Therefore, we tested whether the portion of  $\alpha$ -actinin-4 encoded by our cDNA interacts directly with the subunits of CaMKII in a yeast two-hybrid assay, as described in Materials and Methods. We found a strong interaction between both  $\alpha$ - and  $\beta$ -subunits of CaMKII and the C-terminal half of  $\alpha$ -actinin-4 (Fig. 2*A*).

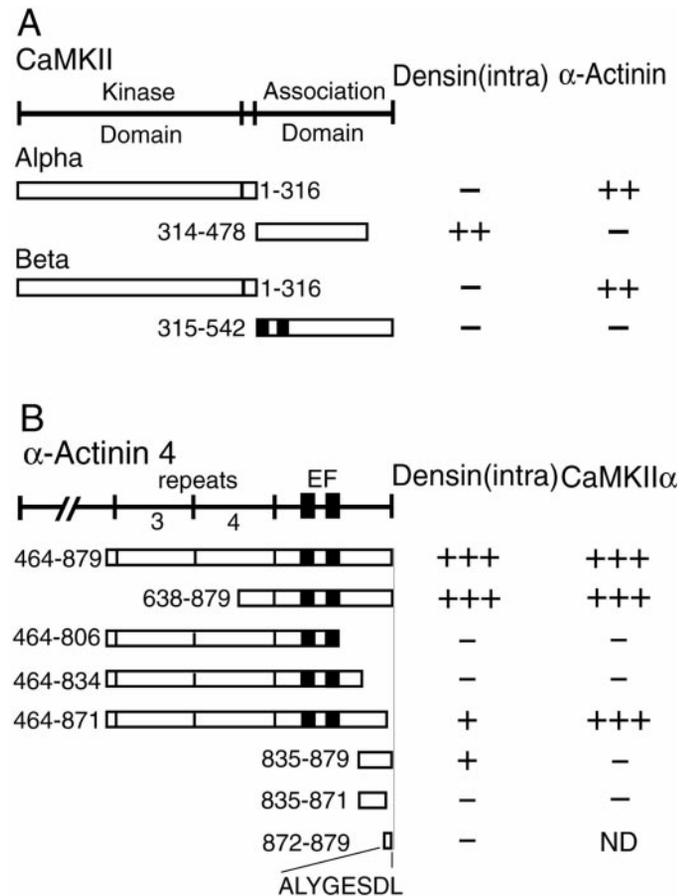
#### Densin interacts only with the $\alpha$ -subunit of CaMKII in the yeast two-hybrid assay

We did not isolate any cDNAs encoding the  $\beta$ -subunit of CaMKII in our screen of a human library for binding partners of densin. Therefore, we tested whether CaMKII $\beta$  interacts with the intracellular domain of densin in a yeast two-hybrid assay, as described in Materials and Methods, and we found that it does not (Fig. 2*B*). Thus, in contrast to  $\alpha$ -actinin-4, densin interacts with CaMKII $\alpha$  but not with CaMKII $\beta$  in this assay.

#### Identification of domains of interaction among densin, CaMKII, and $\alpha$ -actinin by yeast two-hybrid assay

The region of densin that binds to CaMKII was identified by yeast two-hybrid assay. A cDNA encoding only the PDZ domain of densin was inserted into the bait vector and tested for interaction with CaMKII $\alpha$ . The PDZ domain did not bind to CaMKII $\alpha$  (Fig. 2*B*), suggesting that the binding site on densin for CaMKII $\alpha$  is in the region between the transmembrane domain and the PDZ domain. The binding region was further delineated by biochemical experiments (see below).

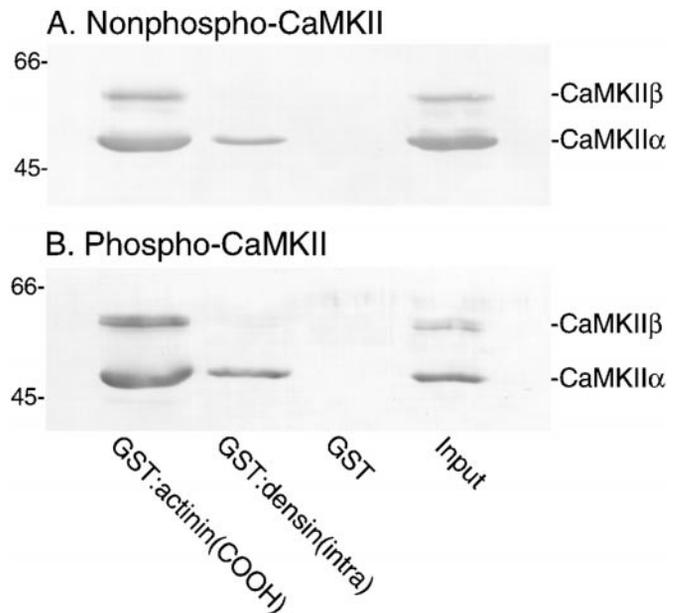
The regions of the CaMKII subunits that bind to densin and  $\alpha$ -actinin were identified by dividing the cDNAs encoding the CaMKII subunits into portions encoding the kinase domain and the association domain. Each of these domains were then tested in a two-hybrid assay for binding to the intracellular region of densin and to the C-terminal half of  $\alpha$ -actinin (Fig. 3*A*). The kinase domains of both  $\alpha$ - and  $\beta$ -subunits of CaMKII bound to



**Figure 3.** Identification of regions of interaction among densin, CaMKII, and  $\alpha$ -actinin by yeast two-hybrid assay. **A**, Interaction of regions of CaMKII with densin and  $\alpha$ -actinin. The cDNAs encoding the kinase or association domains of  $\alpha$ - and  $\beta$ -subunits of CaMKII were inserted into pAS2-1 or pACT2, and the encoded proteins were tested for interactions with the intracellular portion of densin (inserted into pAS2-1) and the COOH-terminal region of  $\alpha$ -actinin (inserted into pACT2). Interaction or no interaction is denoted by ++ or -, respectively. **B**, Interaction of regions of  $\alpha$ -actinin with densin and CaMKII. The cDNAs encoding the intracellular region of densin and the  $\alpha$ -subunit of CaMKII were each inserted into pAS2-1 and tested for interaction with regions of  $\alpha$ -actinin inserted into pACT2. Strong interactions are indicated by +++, weak interactions by +, and no interaction by -. ND, not done. The numbers refer to the residues encoded by each cDNA. The sequence of the COOH-terminal 8 residues of  $\alpha$ -actinin is indicated by single-letter abbreviation.

$\alpha$ -actinin-4. In contrast, the association domain of the  $\alpha$ -subunit bound to densin, whereas neither the kinase nor the association domains of the  $\beta$ -subunit bound, consistent with the results of assays with full-length CaMKII $\beta$ . Thus,  $\alpha$ -actinin and densin bind to distinct regions within CaMKII.

The regions of  $\alpha$ -actinin that bind to densin and CaMKII were identified by two-hybrid assay (Fig. 3B). Amino acids 464–879 of  $\alpha$ -actinin bind to the intracellular domain of densin. Deletion of the COOH-terminal 45 residues (835–879) abolished binding, whereas deletion of the COOH-terminal 8 residues reduced, but did not abolish, binding. The COOH-terminal 45 residues alone interact with densin, although more weakly than constructs containing upstream sequence. Elimination of the last eight residues from this shorter sequence abolished binding, but these residues alone were not sufficient for binding. We conclude that  $\alpha$ -actinin binds to densin at a site that lies primarily between residues 835



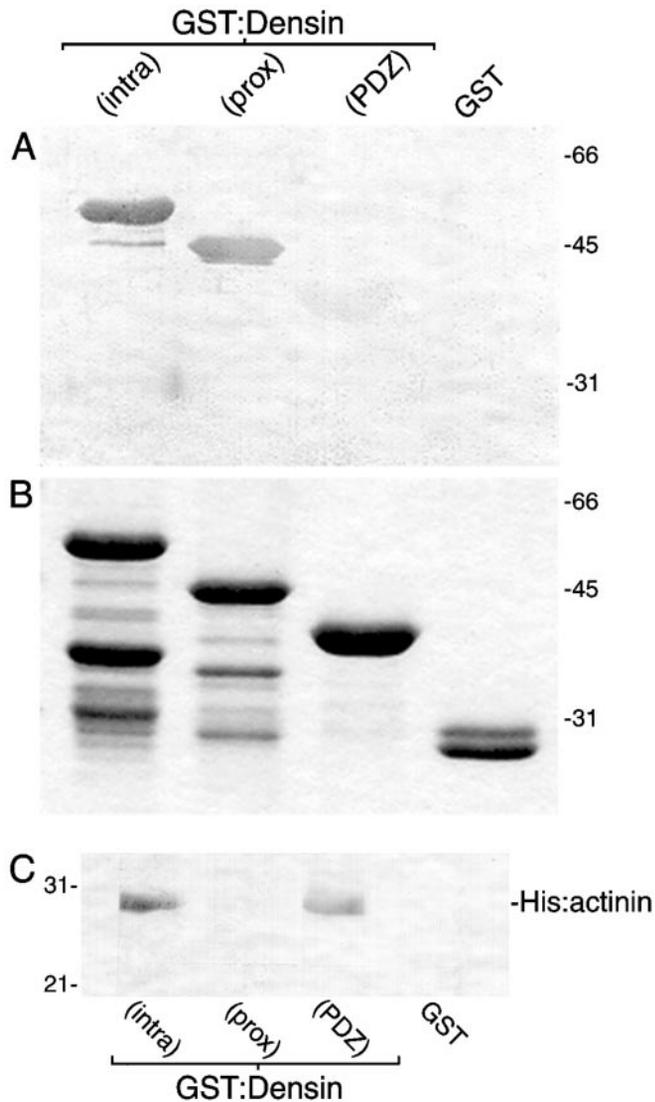
**Figure 4.** Subunit specificity of interaction of CaMKII with  $\alpha$ -actinin and densin. The indicated fusion proteins were cross-linked to agarose beads and subsequently incubated with 12  $\mu$ g of either nonphosphorylated (7  $\mu$ M) (**A**) or phosphorylated (3  $\mu$ M) (**B**) rat forebrain CaMKII. CaMKII was eluted and detected by immunoblot with the antiserum Darlene (**A**), which recognizes the nonphosphorylated form of both  $\alpha$ - and  $\beta$ -subunits of CaMKII, or with a mixture of antibody 6G9 and antiserum Darcy (**B**), which recognize the  $\alpha$ - and  $\beta$ -subunits of CaMKII, respectively. The lane labeled input contains 50% (**A**) or 25% (**B**) of the CaMKII that was originally incubated with the beads. The relative positions of the  $\alpha$ - and  $\beta$ -subunits are indicated on the right; molecular size markers (in kilodaltons) are indicated on the left.

and 871, although residues upstream of 835 and downstream of 871 may participate in binding or be necessary for proper folding of the binding site.

The region of  $\alpha$ -actinin that binds to CaMKII is also near the C-terminus. The terminal 8 residues are not involved in binding, because deletion of these residues did not alter the strength of binding. Deletion of the COOH terminal 45 residues (835–879) abolished binding; nevertheless, the COOH terminal 45 amino acids alone were not sufficient for binding to CaMKII. Therefore, the binding site for densin on  $\alpha$ -actinin may reside around residue 835 and be split in these two constructs. We conclude that  $\alpha$ -actinin binds to CaMKII $\alpha$  at a site just downstream of the EF hands, between residues 806 and 871.

### Biochemical studies of interactions among densin, CaMKII, and $\alpha$ -actinin

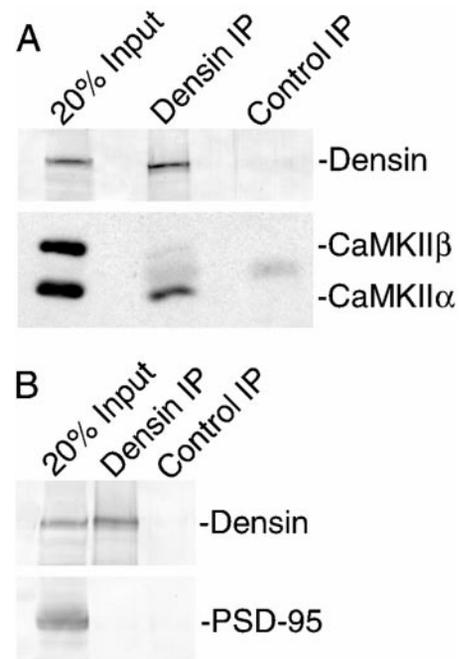
To test whether densin and  $\alpha$ -actinin will bind CaMKII *in vitro*, we covalently linked a fusion protein containing the entire intracellular domain of densin [GST:densin(intra)], the C-terminal half of  $\alpha$ -actinin-4 [GST:actinin(COOH)], and GST to glutathione-agarose beads as described in Materials and Methods. The beads with attached fusion proteins were incubated with purified rat forebrain CaMKII and washed. Bound CaMKII was eluted and detected by immunoblot with an antibody that recognizes both  $\alpha$ - and  $\beta$ -subunits. GST:actinin(COOH) and GST:densin(intra) bound CaMKII, but GST alone did not (Fig. 4). CaMKII purified from rat forebrain is a mixture of dodecameric holoenzymes containing varying numbers of  $\alpha$ - and  $\beta$ -subunits. The average composition is nine  $\alpha$ -subunits and three  $\beta$ -subunits



**Figure 5.** Identification of regions of interaction among densin, CaMKII, and  $\alpha$ -actinin by blot overlay assays. *A, B*, Interaction of densin with CaMKII. Fusion proteins containing the intracellular portion (*intra*), the membrane proximal (*prox*), or the PDZ domain (*PDZ*) of densin, or GST alone were transferred to a nitrocellulose membrane after fractionation by SDS-PAGE. Each lane contained  $\sim 2.5$   $\mu$ g of the most prominent band of the fusion protein. The membrane was overlaid with 10  $\mu$ g/ml autophosphorylated CaMKII. *A*, Immunoblot of membrane after overlay with CaMKII. Bound CaMKII was detected with antibody 6G9 against CaMKII $\alpha$ . *B*, Coomassie-stained gel indicating the relative positions of fusion proteins after SDS-PAGE. Positions of molecular size markers (in kilodaltons) are shown on the right. *C*, Interaction of  $\alpha$ -actinin with densin. His:actinin(COOH) was transferred to a nitrocellulose membrane after fractionation by SDS-PAGE. Individual lanes were overlaid with GST:densin(*intra*), GST:densin(*prox*), GST:densin(*PDZ*), or GST, as indicated. Bound densin fusion proteins were detected with an antibody against GST. The position of His:actinin(COOH) is shown on the right, and those of molecular size markers (in kilodaltons) on the left.

(Bennett et al., 1983).  $\alpha$ -Actinin bound holoenzymes containing both  $\alpha$ - and  $\beta$ -subunits (Fig. 4). In contrast, densin bound selectively to holoenzymes containing only  $\alpha$ -subunits. This experiment demonstrates that densin does not bind with high affinity to holoenzymes that contain  $\beta$ -subunits.

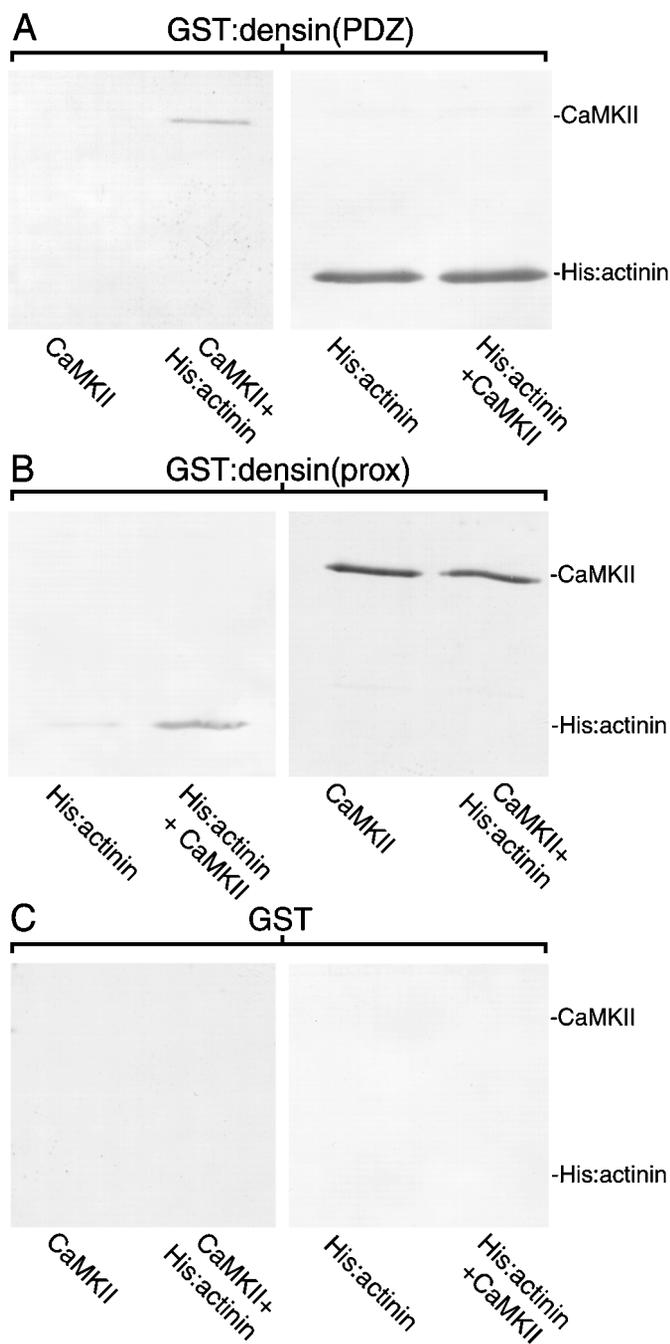
The region of densin that binds CaMKII was further delineated by preparing overlays of blots of GST fusion proteins



**Figure 6.** Coimmunoprecipitation of densin and CaMKII from rat brain. A rat brain synaptosomal fraction was immunoprecipitated with antibodies against densin (*Densin IP*) or nonimmune mouse IgG (*Control IP*) as described in Materials and Methods. Immune complexes were purified on Protein A-agarose beads and fractionated by SDS-PAGE. Proteins were transferred to nitrocellulose membranes and immunolabeled with antibodies against the proteins, the positions of which are indicated on the right. Lanes labeled *input* contain 20% of the amount of synaptosomal fraction used for the immunoprecipitation. *A*, Immunoblot of immunoprecipitated proteins detected with antibody M3 against densin and antiserum Darlene against nonphosphorylated  $\alpha$ - and  $\beta$ -subunits of CaMKII. *B*, Immunoblot of precipitated proteins from an immunoprecipitation identical to that in *A*. Proteins were detected with antibody M3 against densin and antiserum Frances against PSD-95.

containing portions of the intracellular domain of densin. GST:densin(*intra*) and fusion proteins containing the membrane proximal domain [GST:densin(*prox*)] and the PDZ domain [GST:densin(*PDZ*)] were subjected to SDS-PAGE and transferred to a nitrocellulose membrane. CaMKII from rat forebrain was autophosphorylated, diluted to 10  $\mu$ g/ml, and incubated with the membrane as described in Materials and Methods. The membrane was washed, and bound CaMKII was detected by incubation with an antibody against the  $\alpha$ -subunit (Fig. 5*A*). CaMKII bound to the GST fusion proteins containing the intracellular domain and the membrane proximal domain, but did not bind to the fusion protein containing only the PDZ domain. This result is consistent with the results of two-hybrid assays (Fig. 2*B*) and demonstrates that densin binds CaMKII in the membrane proximal half of its putative intracellular region.

We identified the region in densin that binds  $\alpha$ -actinin with a blot overlay assay. A histidine-tagged fusion protein containing the C-terminal half of  $\alpha$ -actinin [His:actinin(COOH)] was subjected to SDS-PAGE and transferred to a nitrocellulose membrane. Individual lanes of the membrane were overlaid with GST:densin(*intra*), GST:densin(*prox*), GST:densin(*PDZ*), or GST. The blot was washed, and bound fusion proteins were detected with an antibody against GST. The fusion protein containing the entire intracellular domain of densin and that containing only the PDZ domain bound to the His:actinin(COOH) (Fig. 5*C*), whereas the fusion protein containing only the mem-



**Figure 7.** Formation of ternary complexes of densin, CaMKII, and  $\alpha$ -actinin *in vitro*. Agarose beads coated with 10  $\mu$ g of GST:densin(PDZ), GST:densin(prox), or GST were incubated with 12  $\mu$ g of purified forebrain CaMKII, 7.5  $\mu$ g of His:actinin(COOH), or a combination of the two proteins as described in Materials and Methods. Proteins (listed under each lane) were added to the beads and incubated for 2 hr. Bound proteins were eluted and detected by immunoblot with antibody 6G9 against CaMKII $\alpha$  and anti-T7 antibodies that recognize His:actinin(COOH). Immunoblots of CaMKII and His:actinin(COOH) are shown after the elution of proteins from GST:densin(PDZ), (A), GST:densin(prox) (B), and GST (C).

brane proximal domain did not bind to  $\alpha$ -actinin. These data indicate that, in contrast to CaMKII,  $\alpha$ -actinin binds to the PDZ domain of densin. The yeast two-hybrid data reported in Figure 3B suggest, however, that the interaction is not a classical PDZ

domain interaction because the last eight amino acids of  $\alpha$ -actinin are not sufficient for binding.

### Coimmunoprecipitation

To test whether densin interacts with CaMKII *in vivo*, we used antibodies against densin to precipitate it with associated proteins from homogenates of synaptosomes solubilized in 2% Triton X-100, as described in Materials and Methods. CaMKII $\alpha$  was present in the densin immunoprecipitates and not in control immunoprecipitates containing nonimmune IgG (Fig. 6A). PSD-95, another PSD protein, did not coimmunoprecipitate with densin (Fig. 6B), indicating that the interaction with CaMKII is specific, and supporting the hypothesis that densin binds directly to CaMKII *in vivo*. These experiments do not, however, rule out the possible presence of an unknown third protein mediating interaction between densin and CaMKII. Interestingly, little CaMKII $\beta$  was detected in the immunoprecipitates (Fig. 6A), supporting the conclusion that densin preferentially associates with holoenzymes containing only CaMKII $\alpha$  *in vivo*.

In similar experiments, we were unable to detect  $\alpha$ -actinin in samples containing immunoprecipitated densin. Interactions between densin and  $\alpha$ -actinin may be transient or may be disrupted by detergents required to solubilize proteins in the PSD.

### Formation of a ternary complex containing densin, CaMKII, and $\alpha$ -actinin

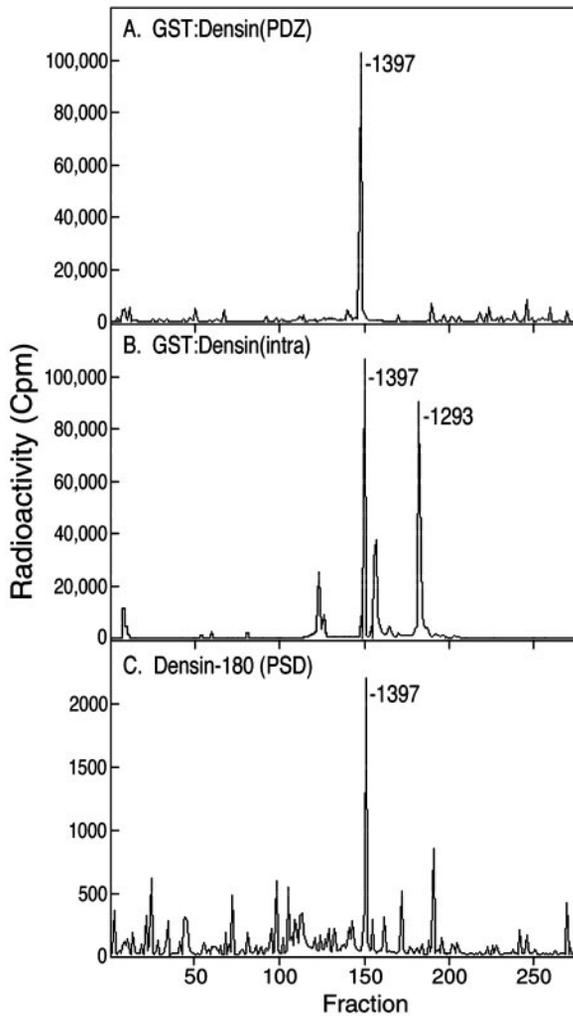
We used pull-down assays to test whether densin can form ternary complexes with CaMKII and  $\alpha$ -actinin. Glutathione-agarose beads loaded with GST:densin(PDZ) were incubated with solutions containing purified rat forebrain CaMKII alone, CaMKII plus His:actinin labeled with a T7 epitope tag, or His:actinin alone. After washing, bound proteins were eluted from the beads and subjected to immunoblot with antibodies against CaMKII $\alpha$  or against T7 (Fig. 7A). As predicted from previous experiments (Fig. 5A), CaMKII alone did not bind to GST:densin(PDZ); however, it did bind to the column when His:actinin was present in the solution. The presence of CaMKII in the solution did not decrease binding of  $\alpha$ -actinin to densin, indicating that there is no competition between  $\alpha$ -actinin and CaMKII for binding. No CaMKII or  $\alpha$ -actinin bound to beads loaded with GST (Fig. 7C). These results show that CaMKII can bind to  $\alpha$ -actinin at the same time that  $\alpha$ -actinin is bound to the PDZ domain of densin.

In a similar experiment, beads loaded with GST:densin(prox) were incubated with solutions containing His:actinin(COOH) alone, His:actinin(COOH) plus CaMKII, or CaMKII alone. As in Figure 7A, bound proteins were eluted and subjected to immunoblot with antibodies against T7 or against CaMKII $\alpha$ . Very little His:actinin(COOH) alone bound to the column, whereas much more bound when CaMKII was present in the solution (Fig. 7B). The presence of  $\alpha$ -actinin did not decrease binding of CaMKII to densin, again indicating that there is no competition between  $\alpha$ -actinin and CaMKII for binding. These results indicate that  $\alpha$ -actinin can bind to CaMKII at the same time that CaMKII is bound to the membrane proximal region of densin.

Taken together, the experiments indicate that densin, CaMKII, and  $\alpha$ -actinin can form a ternary complex.

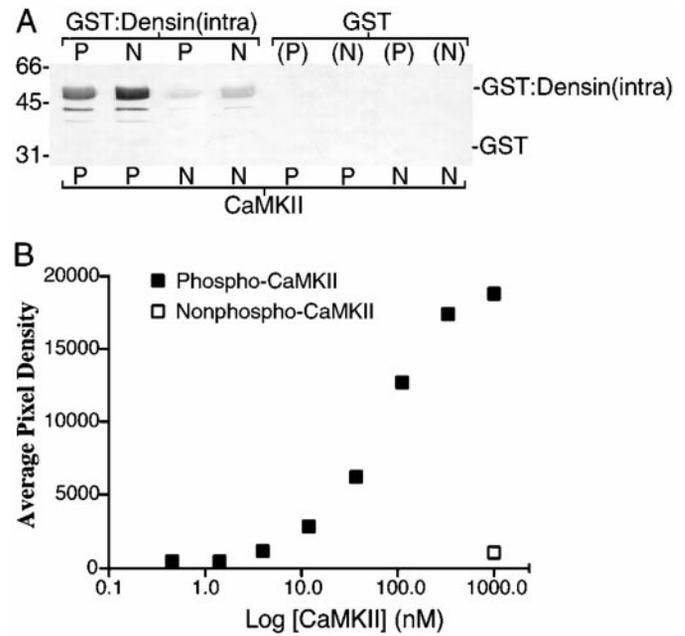
### Identification of the CaMKII phosphorylation site on densin

Densin is phosphorylated to a stoichiometry of  $\sim$ 1 pmol of phosphate per picomole of densin by endogenous CaMKII in the PSD fraction (Apperson et al., 1996). We found that GST:densin(intra) (amino acids 1249–1495) or GST:densin (PDZ) (amino



**Figure 8.** Phosphopeptides of densin generated after phosphorylation by CaMKII and digestion by endoproteinase-Lys-C. GST:densin(PDZ) and GST:densin(intra) were phosphorylated by purified forebrain CaMKII as described in Materials and Methods. The fusion proteins were digested with endoproteinase-Lys-C, and resulting peptides were fractionated by HPLC on a C18 reverse-phase column. Proteins in the PSD fraction were phosphorylated by endogenous CaMKII. Densin was immunoprecipitated from the PSD fraction and fractionated by SDS-PAGE. The band containing densin was cut from the gel and digested in the gel with endoproteinase-Lys-C. Peptides eluted from the gel were fractionated by HPLC. Radioactivity of each fraction was measured as described in Materials and Methods. *A*, Radiolabeled peptides derived from GST:densin(PDZ). *B*, Radiolabeled peptides derived from GST:densin(intra). *C*, Radiolabeled peptides generated from densin-180 in the PSD fraction. The numbers above the peaks at fraction 150 and fraction 181 indicate the phosphorylated amino acid residue present in that peak.

acids 1374–1495) are also phosphorylated by CaMKII in a  $\text{Ca}^{2+}$ -dependent manner. To identify the site of phosphorylation, we exhaustively phosphorylated the fusion proteins in the presence of purified rat brain CaMKII and  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ , as described in Materials and Methods. Phosphorylated fusion proteins were digested with endoproteinase Lys-C, and the peptides were fractionated by HPLC. We identified  $^{32}\text{P}$ -labeled peptide peaks by measuring radioactivity in each fraction. Proteolyzed GST:densin(intra) and GST:densin(PDZ) produced single large radioactive peptide peaks with identical mobilities (Fig. 8*A, B*, fraction 150). GST:densin(intra) produced an additional smaller peak of slower mobility (Fig. 8*B*, fraction 181). MALDI-TOF mass spec-



**Figure 9.** Effect of phosphorylation of densin and CaMKII on their binding affinity. *A*, GST:densin(intra) was phosphorylated (*P*) or not phosphorylated (*N*) by CaMKII. GST was similarly treated. The proteins were fractionated by SDS-PAGE (10  $\mu\text{g}$ /lane) and transferred to a nitrocellulose membrane. Autophosphorylated (*P*) or nonphosphorylated (*N*) CaMKII (10  $\mu\text{g}$ /ml) to densin in a blot overlay. Bound CaMKII $\alpha$  was detected by immunoblot with antibody 6G9 as described in Materials and Methods. Positions of GST-densin(intra) and GST are indicated on the right. *B*, Quantitative analysis of binding of phosphorylated and nonphosphorylated CaMKII to densin in a blot overlay. Nonphosphorylated GST:densin(intra) was fractionated by SDS-PAGE and transferred to a PVDF membrane. CaMKII (3  $\mu\text{M}$ ) was either autophosphorylated or incubated in a control reaction (nonphosphorylated) and subsequently serially diluted and overlaid on individual lanes. CaMKII was detected by immunoblot with antibody 6G9 and fluorescein-conjugated secondary antibodies. Fluorescence was measured with a Storm Imaging System and quantified with ImageQuant software. The graph is a representative example of three separate experiments.

trometric analysis of the peaks in fraction 150 revealed a single peptide with a mass of 2280.85. Gas phase Edman degradation of the peptide revealed a sequence of 18 residues corresponding to residues 1394–1411 of densin. This sequence contains a single serine at position 4, which corresponds to residue 1397 in densin (Apperson et al., 1996). The calculated mass for this peptide, assuming the presence of a phosphoryl group on the serine and a carboxamidocysteine modification of the cysteine, is 2281.35. We confirmed that this peptide is phosphorylated by performing MALDI-TOF mass spectrometry in reflector mode. In addition to the peak of mass 2281 in the linear mode, a second peak of mass 2185 appeared in reflector mode. These peaks differ by  $\sim 96$  mass units, which matches the mass to change ratio of a phosphoryl group. This pattern of fragmentation in reflector mode is diagnostic for a phosphopeptide. Interestingly, the sequence around serine 1397 does not strictly fit the consensus sequence for a phosphorylation site of CaMKII. After this work was presented in abstract and poster form (Walikonis et al., 1999), and before it was submitted for publication, Strack et al. (2000) published an identification of serine 1397 as a phosphorylation site on densin for CaMKII based on site-directed mutagenesis.

Fraction 181 from GST:densin(intra) contained a single peptide of mass 6932. The sequence of the N-terminal 10 residues was

obtained by gas-phase sequencing and corresponds to a 59 residue Lys-C peptide, containing residues 1271–1329 of densin. It contains a serine at residue 1293 that is within a consensus sequence for phosphorylation by CaMKII. MALDI-TOF mass spectrometry of this peptide in the reflector mode revealed a fragmentation peak of mass 6835, again indicative of loss of a phosphoryl group. Thus, serine 1293 is phosphorylated in the GST:densin(intra) fusion protein.

To examine phosphorylation sites in intact densin, we prepared phospho-densin from the PSD fraction after phosphorylation by endogenous CaMKII as described in Materials and Methods. Densin was immunoprecipitated from the PSD fraction and digested in the same manner as the fusion proteins. The resulting digest contains a phosphopeptide that corresponds to the peptide eluting in fraction 150, containing serine 1397 (Fig. 8C). A peptide corresponding to the peptide in Figure 8B, containing serine 1293, did not appear in the digest of intact densin; thus, although serine 1293 can be phosphorylated by CaMKII in a fusion protein, it may not be phosphorylated in densin *in vivo*.

The  $K_m$  for phosphorylation of serine 1397 in GST:densin(PDZ), averaged from Lineweaver–Burke double-reciprocal plots from four separate experiments, was  $0.68 \pm 0.44 \mu\text{M}$  (data not shown), reflecting the high-affinity binding of densin to CaMKII.

### Effects of phosphorylation on the interaction between densin and CaMKII

We tested whether autophosphorylation of CaMKII or phosphorylation of densin by CaMKII alters the interaction between them. We prepared GST:densin(intra) phosphorylated by CaMKII, as described in Materials and Methods. Nonphosphorylated and phosphorylated densin were subjected to SDS-PAGE and transferred to a nitrocellulose membrane. Individual lanes were incubated with either autophosphorylated or nonphosphorylated CaMKII. After washing, bound CaMKII was detected with an antibody against CaMKII $\alpha$ . Autophosphorylation of CaMKII markedly increased binding to GST:densin(intra), whereas phosphorylation of GST:densin(intra) only slightly decreased binding by CaMKII (Fig. 9A).

To better measure the change in affinity of autophosphorylated CaMKII for densin, we devised a quantitative overlay assay using fluorescein-conjugated secondary antibodies. Autophosphorylated and nonphosphorylated samples of CaMKII were serially diluted and incubated with nitrocellulose membranes containing bands of GST:densin(intra) transferred from SDS gels. Binding of CaMKII was quantified after measurement of fluorescence with a Storm Imaging System and ImageQuant software. Autophosphorylated CaMKII bound to the GST:densin(intra) band with a very low  $EC_{50}$  of  $\sim 75$ – $150$  nM, indicating high affinity for densin. Nonphosphorylated CaMKII bound in this assay with at least 100-fold lower affinity (Fig. 9B). Its affinity was too low to measure with precision in this assay. In contrast to these results, autophosphorylation of CaMKII did not alter its binding to GST:actinin(COOH) (data not shown). These experiments indicate that autophosphorylation of CaMKII increases the affinity of CaMKII for the intracellular region of densin by as much as 100-fold. In contrast, phosphorylation of densin by CaMKII does not dramatically alter binding affinity between the two proteins.

Because  $\alpha$ -actinin binds to the kinase domain of CaMKII (Fig. 3A), we tested whether it is phosphorylated by CaMKII by incubating GST:actinin(COOH) with CaMKII and  $\gamma$ - $^{32}\text{P}$ -labeled ATP under phosphorylating conditions. No phosphate was detected in the fusion protein after a 3 min incubation (data not

shown). Thus, the C-terminal tail of  $\alpha$ -actinin is not phosphorylated by CaMKII.

### DISCUSSION

In this study, we used the putative cytosolic region of the PSD protein densin to screen a human brain cDNA library for interacting proteins by the yeast two-hybrid method. We found that two proteins known to be constituents of the PSD, CaMKII $\alpha$  and  $\alpha$ -actinin (Kennedy et al., 1983; Wyszynski et al., 1997), bind to the COOH-terminal tail of densin. We showed that CaMKII $\alpha$  and  $\alpha$ -actinin also interact and the three proteins can form a ternary complex *in vitro*, suggesting that they may do the same *in vivo*. The PDZ domain of densin binds a COOH-terminal domain in  $\alpha$ -actinin. Sequences just upstream of the densin PDZ domain bind the association domain of CaMKII $\alpha$ .  $\alpha$ -Actinin, in turn, binds the kinase domains of the  $\alpha$ - or  $\beta$ -subunits of CaMKII. CaMKII phosphorylates densin at serine-1397, but phosphorylation of densin only slightly decreases its affinity for CaMKII. In contrast, autophosphorylation of CaMKII increases its affinity for densin at least 100-fold. These results suggest that densin may play a role in the movement of activated CaMKII to the PSD after activation of glutamatergic synapses.

The specificity of densin for binding to CaMKII is particularly interesting. Densin interacts with the  $\alpha$ -subunit in a yeast two-hybrid assay but does not interact with the  $\beta$ -subunit (Fig. 2). When exposed to a mixture of holoenzymes purified from rat forebrain, densin preferentially binds holoenzymes containing only  $\alpha$ -subunits (Fig. 4). Finally, immunoprecipitation of densin from the synaptosome fraction reveals that, *in situ*, densin binds primarily holoenzymes containing only the  $\alpha$ -subunit (Fig. 6A). The CaMKII holoenzyme is a dodecamer assembled apparently randomly from  $\alpha$ - and  $\beta$ -subunits as they are synthesized (Bennett et al., 1983; Shen et al., 1998; Kolodziej et al., 2000). In the forebrain, holoenzymes contain an average of nine  $\alpha$ -subunits and three  $\beta$ -subunits (Bennett et al., 1983). However, this average composition reflects the presence of an array of holoenzymes containing various combinations of  $\alpha$ - and  $\beta$ -subunits. Dendrites of pyramidal cells in the hippocampus contain a high concentration of mRNA encoding the  $\alpha$ -subunit; but mRNA encoding the  $\beta$ -subunit is largely confined to the soma (Burgin et al., 1990). Thus, there is reason to believe that holoenzymes of CaMKII assembled after synthesis in dendrites would contain primarily  $\alpha$ -subunits. CaMKII is synthesized in dendrites in response to synaptic activity that activates NMDA receptors (Ouyang et al., 1999). Activation of NMDA receptors also induces translocation of CaMKII from dendritic shafts into the PSD, apparently triggered by autophosphorylation of CaMKII (Shen et al., 1998). The observation that densin specifically binds CaMKII holoenzymes containing only  $\alpha$ -subunits suggests that densin may participate in translocation or localization of dendritically synthesized CaMKII to the PSD. This hypothesis is supported by our data showing that the affinity of CaMKII $\alpha$  for densin increases when the kinase is autophosphorylated. Nonphosphorylated CaMKII $\alpha$  has a reduced but significant affinity for densin (Figs. 4, 9A). Thus, densin may help to maintain CaMKII anchored at the PSD even after dephosphorylation of the kinase. The formation of a ternary complex among densin, CaMKII, and  $\alpha$ -actinin would provide additional stability.

The magnitude of the change in affinity for densin caused by autophosphorylation of CaMKII is different depending on the method of assay (Strack et al., 2000). In the experiment shown in Figure 9B, affinities were tested at concentrations of CaMKII in

solution between 10 nm and 1  $\mu$ M. We could not detect binding of nonphosphorylated CaMKII to densin at 1  $\mu$ M. However, in the experiment shown in Figure 4, nonphosphorylated CaMKII bound to densin on an affinity column when presented at a concentration of 7  $\mu$ M. CaMKII is an abundant protein in the brain (Kennedy et al., 1990); if one assumes a uniform, well mixed distribution, its concentration is estimated to be 10–30  $\mu$ M. However, CaMKII is bound to, and appears to move between, several cellular structures; therefore, it is difficult to directly predict whether the change in affinity for densin produced by autophosphorylation would be significant *in vivo*. A likely cause of the observed increase in affinity is a decrease in the dissociation rate. The stochastic movements and associations of the kinase *in vivo* might well be influenced by a change in  $k_{off}$ .

The  $\alpha$ -actinins are a family of closely related proteins that cross-link actin filaments and tether proteins to the cytoskeleton. We isolated a splice variant of human  $\alpha$ -actinin-4 as a binding partner for densin. The expression of this isoform in the brain has not been studied. However,  $\alpha$ -actinin-1 and -2 are both reportedly present in spines (Wyszynski et al., 1998) and in the PSD fraction (Walikonis et al., 2000), which suggests that they may both be located at excitatory synapses. We have not tested the interaction of densin with these isoforms. However, the  $\alpha$ -actinins are highly similar in sequence. For example, in the region of interaction with densin (from the EF hands to the COOH terminus),  $\alpha$ -actinins-1 and -4 are 88% identical and 97% similar. Thus, it is likely that densin can bind isoforms other than  $\alpha$ -actinin-4. The various  $\alpha$ -actinins are also likely similar in function, although small sequence variations and differential expression or subcellular distribution may impart subtle differences. A role for actin and  $\alpha$ -actinin in organizing proteins at the PSD is suggested by experiments in which cultured hippocampal neurons were treated with latrunculin A, an agent that depolymerizes actin. The treatment resulted in the loss of actin and  $\alpha$ -actinin from dendritic spines. Interestingly, the treatment also caused a loss of CaMKII $\alpha$  from spines, although other PSD proteins, such as PSD-95, remained concentrated at the PSD (Allison et al., 2000). These experiments demonstrate that actin filaments are necessary for continued anchoring of CaMKII to postsynaptic sites, either by binding directly to CaMKII or indirectly through  $\alpha$ -actinin or other actin-associated proteins.  $\alpha$ -Actinin also binds to subunits of the NMDA receptor (Wyszynski et al., 1997), and may, along with the C-terminal tails of the NR2 subunits (Om-kumar et al., 1996; Strack and Colbran, 1998; Leonard et al., 1999), help to tether CaMKII to this receptor.

The affinity of the intracellular tail of densin for CaMKII and  $\alpha$ -actinin provides some clues about the potential functions of densin as an organizing protein. Identification of its extracellular binding partner(s) and isolation of densin mutants will help to clarify its functions further.

## REFERENCES

- Allison DW, Chervin AS, Gelfand VI, Craig AM (2000) Postsynaptic scaffolds of excitatory and inhibitory synapses in hippocampal neurons: maintenance of core components independent of actin filaments and microtubules. *J Neurosci* 20:4545–4554.
- Apperson ML, Moon I-S, Kennedy MB (1996) Characterization of densin-180, a new brain-specific synaptic protein of the *O*-sialoglycoprotein family. *J Neurosci* 16:6839–6852.
- Bennett MK, Erondy NE, Kennedy MB (1983) Purification and characterization of a calmodulin-dependent protein kinase that is highly concentrated in brain. *J Biol Chem* 258:12735–12744.
- Bilder D, Perrimon N (2000) Localization of apical epithelial determinants by the basolateral PDZ protein Scribble. *Nature* 403:676–680.
- Bilder D, Birnbaum D, Borg JP, Bryant P, Huigbretse J, Jansen E, Kennedy MB, Labouesse M, Legouis R, Mechler B, Perrimon N, Petit M, Sinha P (2000) Collective nomenclature for LAP proteins. *Nat Cell Biol* 2:E114.
- Borg J-P, Marchetto S, Le Bivic A, Ollendorff V, Jaulin-Bastard F, Saito H, Fournier E, Adelaide J, Margolis J, Birnbaum D (2000) ERBIN: a basolateral PDZ protein that interacts with the mammalian ERBB2/HER2 receptor. *Nat Cell Biol* 2:407–414.
- Breen MA, Ashcroft SJ (1997) Human islets of Langerhans express multiple isoforms of calcium/calmodulin-dependent protein kinase II. *Biochem Biophys Res Commun* 236:473–478.
- Burgin KE, Waxham MN, Rickling S, Westgate SA, Mobley WC, Kelly PT (1990) *In situ* hybridization histochemistry of Ca<sup>2+</sup> calmodulin-dependent protein kinase in developing rat brain. *J Neurosci* 10:1788–1798.
- Carlin RK, Grab DJ, Cohen RS, Siekevitz P (1980) Isolation and characterization of postsynaptic densities from various brain regions: enrichment of different types of postsynaptic densities. *J Cell Biol* 86:831–843.
- Cho K-O, Hunt CA, Kennedy MB (1992) The rat brain postsynaptic density fraction contains a homolog of the *Drosophila* discs-large tumor suppressor protein. *Neuron* 9:929–942.
- Gietz R, Schiestl R (1995) Transforming yeast with DNA. *Methods Mol Cell Biol* 5:255–269.
- Harlow E, Lane D (1988) *Antibodies: a laboratory manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Honda K, Yamada T, Endo R, Ino Y, Gotoh M, Tsuda H, Yamada Y, Chiba H, Hirohashi S (1998) Actinin-4, a novel actin-bundling protein associated with cell motility and cancer invasion. *J Cell Biol* 140:1383–1393.
- Husi H, Ward MA, Choudhary JS, Blackstock WP, Grant SGN (2000) Proteomic analysis of NMDA receptor-adhesion protein signaling complexes. *Nat Neurosci* 3:661–669.
- Kennedy MB (1997) The postsynaptic density at glutamatergic synapses. *Trends Neurosci* 20:264–268.
- Kennedy MB (1998) Signal transduction molecules at the glutamatergic postsynaptic membrane. *Brain Res Rev* 26:243–257.
- Kennedy MB, Bennett MK, Erondy NE (1983) Biochemical and immunological evidence that the “major postsynaptic density protein” is a subunit of a calmodulin-dependent protein kinase. *Proc Natl Acad Sci USA* 80:7357–7361.
- Kennedy MB, Bennett MK, Bulleit RF, Erondy NE, Jennings VR, Miller SM, Molloy SS, Patton BL, Schenker LJ (1990) Structure and regulation of type II calcium/calmodulin-dependent protein kinase in central nervous system neurons. *Cold Spring Harb Symp Quant Biol* 55:101–110.
- Kolodziej SJ, Hudmon A, Waxham MN, Stoops JK (2000) Three-dimensional reconstructions of calcium/calmodulin-dependent (CaM) kinase II alpha and truncated CaM kinase II alpha reveal a unique organization for its structural core and functional domains. *J Biol Chem* 275:14354–14359.
- Krupp JJ, Vissel B, Thomas CG, Heinemann SF, Westbrook GL (1999) Interactions of calmodulin and  $\alpha$ -actinin with the NR1 subunit modulate Ca<sup>2+</sup>-dependent inactivation of NMDA receptors. *J Neurosci* 19:1165–1178.
- Legouis R, Gansmuller A, Sookhareea S, Boshier JM, Baillie DL, Labouesse M (2000) LET-413 is a basolateral protein required for the assembly of adherens junctions in *Caenorhabditis elegans*. *Nat Cell Biol* 2:415–422.
- Leonard AS, Lim IA, Hemsworth DE, Horne MC, Hell JW (1999) Calcium/calmodulin-dependent protein kinase II is associated with the *N*-methyl-D-aspartate receptor. *Proc Natl Acad Sci USA* 96:3239–3244.
- Miller SG, Kennedy MB (1985) Distinct forebrain and cerebellar isoforms of type II Ca<sup>2+</sup> calmodulin-dependent protein kinase associate differently with the postsynaptic density fraction. *J Biol Chem* 260:9039–9046.
- Miller SG, Patton BL, Kennedy MB (1988) Sequences of autophosphorylation sites in neuronal type II CaM kinase that control Ca<sup>2+</sup>-independent activity. *Neuron* 1:1593–1604.
- Nagase T, Ishikawa K, Suyama M, Kikuno R, Hirose M, Miyajima N, Tanaka A, Kotani H, Nomura N, Ohara O (1999) Prediction of the coding sequences of unidentified human genes. XIII. The complete sequences of 100 new cDNA clones from brain which code for large proteins *in vitro*. *DNA Res* 6:63–70.
- Om-kumar RV, Kiely MJ, Rosenstein AJ, Min K-T, Kennedy MB (1996) Identification of a phosphorylation site for calcium/calmodulin-dependent protein kinase II in the NR2B subunit of the *N*-methyl-D-aspartate receptor. *J Biol Chem* 271:31670–31678.
- Ouyang Y, Rosenstein A, Kreiman G, Schuman EM, Kennedy MB (1999) Tetanic stimulation leads to increased accumulation of Ca<sup>2+</sup>/calmodulin-dependent protein kinase II via dendritic protein synthesis in hippocampal neurons. *J Neurosci* 19:7823–7833.
- Peterson GL (1983) Determination of total protein. *Methods Enzymol* 91:95–119.

- Shen K, Meyer T (1999) Dynamic control of CaMKII translocation and localization in hippocampal neurons by NMDA receptor stimulation. *Science* 284:162–166.
- Shen K, Teruel MN, Subramanian K, Meyer T (1998) CaMKII $\beta$  functions as an F-actin targeting module that localizes CaMKII $\alpha/\beta$  heterooligomers to dendritic spines. *Neuron* 21:593–606.
- Strack S, Colbran RJ (1998) Autophosphorylation-dependent targeting of calcium/calmodulin-dependent protein kinase II by the NR2B subunit of the *N*-methyl-D-aspartate receptor. *J Biol Chem* 273:20689–20692.
- Strack S, Robison AJ, Bass MA, Colbran RJ (2000) Association of calcium/calmodulin-dependent kinase II with developmentally regulated splice variants of the postsynaptic density protein densin-180. *J Biol Chem* 275:25061–25064.
- Walikonis RS, Oguni A, Asuncion F, Kennedy MB (1999) Postsynaptic density proteins densin-180, CaMKII $\alpha$  and  $\alpha$ -actinin interact to form a ternary complex. *Soc Neurosci Abstr* 25:2005.
- Walikonis RS, Jensen ON, Mann M, Provance DWJ, Mercer JA, Kennedy MB (2000) Identification of proteins in the postsynaptic density fraction by mass spectrometry. *J Neurosci* 20:4069–4080.
- Wyszynski M, Lin J, Rao A, Nigh E, Beggs AH, Craig AM, Sheng M (1997) Competitive binding of  $\alpha$ -actinin and calmodulin to the NMDA receptor. *Nature* 385:439–442.
- Wyszynski M, Kharazia V, Shangvi R, Rao A, Beggs AH, Craig AM, Weinberg R, Sheng M (1998) Differential regional expression and ultrastructural localization of  $\alpha$ -actinin-2, a putative NMDA receptor-anchoring protein, in rat brain. *J Neurosci* 18:1383–1392.