Multivalent Interactions of Calcium/Calmodulin-dependent Protein Kinase II with the Postsynaptic Density Proteins NR2B, Densin-180, and α-Actinin-2*

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Dendritic calcium/calmodulin-dependent protein kinase II (CaMKII) is dynamically targeted to the synapse. We show that CaMKIIα is associated with the CaMKII-binding proteins densin-180, the N-methyl-D-aspartate receptor NR2B subunit, and α-actinin in postsynaptic density-enriched rat brain fractions. Residues 819–894 within the C-terminal domain of α-actinin-2 constitute the minimal CaMKII-binding domain. Similar amounts of Thr286-autophosphorylated CaMKIIΔholoenzyme [P-T286]CaMKII bind to α-actinin-2 as bind to NR2B (residues 1260–1339) or to densin-180 (residues 1247–1495) in glutathione-agarose co sedimentation assays, even though the CaMKII-binding domains share no amino acid sequence similarities. Like NR2B, α-actinin-2 binds to representative splice variants of each CaMKII gene (α, β, γ, and δ), whereas densin-180 binds selectively to CaMKIIα. In addition, C-terminal truncated CaMKII monomers can interact with NR2B and α-actinin-2, but not with densin-180. Soluble α-actinin-2 does not compete for [P-T286]CaMKII binding to immobilized densin-180 or NR2B. However, soluble densin-180, but not soluble NR2B, increases CaMKII binding to immobilized α-actinin-2 by ≈10-fold in a PDZ domain-dependent manner. A His6-tagged NR2B fragment associates with GST-densin or GST-actinin but only in the presence of [P-T286]CaMKII. Similarly, His6-tagged densin-180 or α-actinin fragments associate with GST-NR2B in a [P-T286]CaMKII-dependent manner. In addition, GST-NR2B and His6-tagged α-actinin can bind simultaneously to monomeric CaMKII subunits. In combination, these data support a model in which [P-T286]CaMKII can simultaneously interact with multiple dendritic spine proteins, possibly stabilizing the synaptic localization of CaMKII and/or nucleating a multiprotein synaptic signaling complex.

CaMKIIβ plays a ubiquitous and central role in calcium signaling. Alternative splicing of the four CaMKII genes (α, β, γ, and δ) gives rise to ∼30 known mRNA/protein products. CaMKIIα and CaMKIIβ pre-

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2 The abbreviations used are: CaMKII, calcium/calmodulin protein kinase II; [P-T286]CaMKII, Thr286-autophosphorylated CaMKII; NMDA, N-methyl-D-aspartate; NR2B, NMDA receptor 2B subunit; GST, glutathione-S-transferase; PSD, postsynaptic density; AMPA, aminomethylphosphonic acid; ATP, adenosine triphosphate; SDS-PAGE, sodium dodecyl-sulfate polyacrylamide gel electrophoresis; CaMKAP, CaMKII associated protein; PDZ, postsynaptic density-95/disc large/zacona occludens-1.

MATERIALS AND METHODS

Antibodies—Western blotting was performed with mouse anti-CaMKIIα (Affinity Bioreagents), mouse anti-α-actinin (Sigma) antibodies, or mouse anti-NR2B (BD Transduction Laboratories). The goat
anti-CaMKII antisera was described previously (25). Rabbit anti-den-
sin-180 serum was raised against a GST fusion protein including resi-
dues 1247–1290 and 1378–1412 of densin-180. Goat anti-densin-180
serum was raised against a GST fusion protein including residues
451–470 and 652–671 of densin-180. A detailed characterization of the
densin antibodies will be published separately.3

**Coimmunoprecipitation**—Adult male Sprague-Dawley rats were sac-
ricfied by decapitation. Forebrains were quickly removed and homoge-
nized on ice in 0.32 M sucrose containing 4 mM HEPES, pH 7.4 (Buffer 1,
8 ml per forebrain) using a Potter-Elvehjem Teflon-glass device. All
remaining steps were performed at 4 °C. Following centrifugation
(800 × g, 10 min), the supernatant was removed and re-centrifuged
(9,200 × g, 15 min). The 9,200 × g pellet was resuspended in Buffer 1 (8
ml per forebrain) and centrifuged again (10,200 × g, 15 min). The new
pellet (crude synaptosomes) was resuspended in Buffer 2 (20 mM
HEPES/NaOH, pH 8.0, 0.1 M NaCl, 5 mM EDTA, 1% Triton X-100, 0.1
mM phenylmethylsulfonfyl fluoride, 1 mM benzamidine, 20 μg/ml soy-
bean trypsin inhibitor, 5 μg/ml leupeptin, 1 μM microcystin; 4 ml per
forebrain), incubated on ice for 30 min, and then centrifuged (100,000 ×
g, 30 min). The supernatant was diluted in Buffer 2 to 2 mg/ml protein
and used for immunoprecipitations using anti-CaMKII, anti-densin, or
preimmune antibodies (5 μl of goat serum per 2 mg of protein) (see
above). Protein G-Sepharose beads (Pierce, 25 μl) were added after 2 h,
and the incubation continued overnight. Beads were collected by cen-
trifugation and washed five times with Buffer 2 (1 ml each). Proteins
were eluted in 2× SDS-PAGE sample buffer and analyzed by Western
blot.

**GST- and His-tagged Fusion Proteins**—The α-actinin-2 cDNA was a
generic gift from Dr. Alan Beggs (Harvard). Desired fragments of
α-actinin-2, NR2B, or densin-180 cDNAs were amplified by PCR using
oligonucleotide primers containing BamHI (5‘) and EcoRI (3’) restric-
tion enzyme sites as described (10, 14). PCR products were ligated into
the pGEX-2T (Amersham Biosciences) or pRSET-A (Qiagen) vectors
and transformed into BL21-DE3 Gold bacteria. After induction of protein expression, glutathione S-transferase (GST) or His,
fusion proteins were purified using glutathione-agarose beads (Sigma, 25 μl of packed resin) in pull-down (PD) buffer (50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 0.5% Triton X-100; 500-μl final volume). Where indicated, His-tagged
fusion proteins (∼250 nm each) were included with the beads. Beads
were sedimented by centrifugation and washed in 500 μl of PD Buffer 6
times for 5 min each. Beads were transferred to new microcentrifuge
tubes, and sedimented proteins were eluted with SDS-PAGE sample
buffer, resolved by SDS-PAGE, and transferred to nitrocellulose mem-
branes. Membranes were stained using Ponceau S and digitally scanned
to quantify protein loading using ImageJ (rsb.info.nih.gov/ij/). Pilot
studies showed that pixel densities of individual protein bands are
linearly related to the amount of protein loaded on the gel in the range of
0.04–2.5 μg per lane for both GST and CaMKIIα (0.7–42 pmol).

**CAIMKII Is Associated with Multiple CaMKII-binding Proteins in
PSD-enriched Synaptosomal Fractions**—PSD-enriched synaptosomal
fractions immobilized GST fusion proteins (1–5 μg) were incubated with [32P-
T286]CaMKII (50 nM subunit), washed and autoradiographed, essen-
tially as described (25). Where indicated, [32P-T286]CaMKII was incu-
bated on ice for 30 min with soluble GST fusion proteins before addition
to the membrane. Films exposed within the linear range were digita-
lly scanned, and band intensities (determined using ImageJ) were normal-
ized to GST protein loading (see above).

**RESULTS**

**CaMKII Is Associated with Multiple CaMKII-binding Proteins in
PSD-enriched Fractions**—PSD-enriched synaptosomal fractions iso-
lated from adult rat forebrain were enriched in CaMKII and three
CaMKII-binding proteins, α-actinin, NR2B, and densin-180. About
50% of the CaMKIIα, but less than 20% of the CaMKIIβs, were solubi-
lized from the PSD-enriched fraction using Triton X-100. CaMKII
immunoprecipitates from this solubilized extract also specifically con-

3 Y. Jiao, A. J. Robison, Y. Nikandrova, and R. J. Colbran, manuscript in preparation.
tained α-actinin, densin-180, and occasionally NR2B (Fig. 1). Densin-180 immunoprecipitates also contained CaMKII and NR2B, but no detectable α-actinin (Fig. 1). These data confirm previous observations that CaMKII, α-actinin, NR2B, and densin-180 are enriched in PSDs. The effects of solubilization conditions and the differential regulation of each interaction preclude definitive conclusions about the nature of specific protein–protein interactions in PSDs. However, the data are consistent with the existence of multiprotein complexes containing CaMKII in vivo.

The CaMKII-binding Region of α-Actinin—The α-actinin isoforms are inverted dimers in the native state, containing N-terminal actin-binding domains, four spectrin repeats, and two putative C-terminal EF hand domains (Fig. 2A) (27). To investigate whether α-actinin-2 binds directly to CaMKII and to delineate the CaMKII-binding domain, GST fusion proteins containing various portions of the C-terminal domain of α-actinin-2 were used in co-sedimentation assays. GST-actinin-(547–894) effectively bound Thr286-autophosphorylated CaMKIIα (\([\text{P-}^{286}\text{CaMKII}]\)) with similar efficacy to the GST-densin-(1247–1542) control (Fig. 2B and also see Fig. 3). Similar amounts of non-phosphorylated CaMKIIα and [P-^{286}]CaMKII associated with GST-actinin-(547–894) under these conditions. Truncation of 80 amino acids from the C terminus (GST-actinin-(547–814)) reduced CaMKII binding to the very low nonspecific levels detected with GST alone. Moreover, C-terminal fragments (GST-actinin-(809–894) and GST-actinin-(819–894)) bound similar amounts of [P-^{286}]CaMKII as did GST-actinin-(547–894). Interestingly, the CaMKII-binding C-terminal domain (75 amino acids) displays 70% amino acid sequence identity (84% similarity) across three α-actinin isoforms that interact with CaMKII (15, 16) (Fig. 2D). Deletion of the more highly conserved extreme N- or C-terminal regions within this domain to yield GST-actinin-(839–894) or GST-actinin-(819–885) abrogates CaMKII binding (Fig. 2, B and C). Thus, determinants for [P-^{286}]CaMKII interactions with α-actinin-2 appear to be distributed within a minimal functional binding domain of residues 819–894.

It is well established that [P-^{286}]CaMKII interacts with residues 1290–1309 of NR2B and with residues 1355–1382 of densin-180 (10, 11, 13, 14, 21). Comparable amounts of [P-^{286}]CaMKII bound to GST-actinin-(547–894), GST-NR2B, or GST-densin-(1247–1542) (Fig. 3, A and C). The ratio of binding of CaMKII to GST-densin-(1247–1542) and to GST-actinin-(547–894) was 0.9 ± 0.1 and 0.8 ± 0.2, respectively, when normalized to NR2B (Fig. 3C). However, in gel overlay assays, [P-^{286}]CaMKII bound to GST-actinin-(547–894) very weakly when

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**FIGURE 1.** CaMKII associates with multiple proteins in PSD-enriched fractions. A Triton X-100-solubilized PSD-enriched fraction was immunoprecipitated using anti-CaMKII serum (CaMKII), anti-densin-180 serum (densin), or preimmune serum (PIS) and immune complexes were immunoblotted (IB) as indicated. The anti-CaMKII immune complex also contained α-actinin and densin-180, whereas the anti-densin-180 complex also contained CaMKII and NR2B. These data are representative of three similar experiments.

**FIGURE 2.** CaMKII directly binds residues 819–894 α-actinin-2. A, schematic domain structure of α-actinin dimers. ABD, actin-binding domain; R1–4, spectrin repeat domains; EF, putative EF hand domains. B, glutathione-agarose cosedimentation assays using [P-^{286}]CaMKII and the indicated GST-actinin fusion proteins or GST-densin-(1247–1542). Sedimented proteins were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and visualized by Ponceau S for total protein (top) or by immunoblotting for CaMKII (bottom). Asterisks on the Ponceau-stained membrane mark full-length (intact) GST fusion proteins, and the arrowhead indicates the CaMKII bands. Data are representative of three to eight experiments. C, summary of glutathione-agarose cosedimentation studies mapping the CaMKII binding domain. D, amino acid sequence alignment of the minimal CaMKII binding domain of α-actinin-2 with corresponding regions of α-actinin-1 and α-actinin-4. Identical residues are indicated by black boxes, with conservative changes in gray boxes.
Multivalent CaMKII Interactions

Binding of each isoform or truncation mutant was normalized to wild type CaMKII. CaMKII genes are highly similar within their N-terminal catalytic and regulatory domains, as assessed by immunoblotting using phospho-Thr286-specific antibodies and by the generation of autonomous kinase activity (data not shown). We previously showed that CaMKII (1–420) exhibited reduced binding to GST-NR2B (20), but under the conditions used here, both GST-NR2B and GST-actinin-(819–894) bound with comparable efficacy to the monomeric kinases and the wild type holoenzyme. However, there was no statistically significant binding of either truncation mutant to GST-densin-(1247–1495) (Fig. 3A and E). These data show that the NR2B and α-actinin-2 binding sites lie within the first 380 amino acids of CaMKII and that formation of a CaMKII holoenzyme is not essential for these interactions. However, CaMKIIα binding to densin-180 appears to require an intact association domain.

Non-competitive Binding of Densin-180, NR2B, and α-Actinin-2 to CaMKII—Residues 819–894 of α-actinin-2 share no obvious amino acid sequence similarity to the CaMKII-binding domains of NR2B or densin-180. To compare mechanisms for interactions of CaMKII with α-actinin-2, NR2B, and densin-180, competition overlay assays were performed. Nitrocellulose membranes containing immobilized GST fusion proteins were incubated with [32P-T286]CaMKII (50 nM) in the absence or presence of each of the soluble GST fusion proteins (up to 1.25 μM). Binding of kinase to immobilized proteins was determined by autoradiography of the washed membranes.

With immobilized GST-densin-(1247–1495), soluble GST-densin-(1247–1495) decreased [32P-T286]CaMKII binding in a concentration-dependent manner (≈55% of control at 1.25 μM, but similar concentrations of soluble GST-actinin-(819–894) or soluble GST-NR2B had no significant effect (Fig. 4). Similarly, with immobilized GST-NR2B, soluble GST-NR2B competed for binding of [P-T286]CaMKII (≈15% binding at 1.25 μM), but soluble GST-densin-(1247–1495) or soluble GST-actinin-(819–894) had no significant effect. Control experiments showed that soluble GST had no effect on CaMKII binding to any immobilized protein (data not shown). It was not possible to test for potential effects of higher competitor concentrations because of the limited solubility of these GST fusion proteins.

As shown above, [32P-T286]CaMKII binds very weakly to immobilized α-actinin-2 in overlay assays. However, soluble GST-densin-(1247–1495) increased ≈8-fold the apparent interaction of CaMKII with immobilized GST-actinin-(819–894), whereas soluble GST-NR2B and soluble GST-actinin-(819–894) had no effect (Fig. 4). In combination, these data demonstrate that interactions of NR2B, densin-180, and α-actinin-2 with [P-T286]CaMKII are non-competitive and suggest that densin-180 may facilitate binding of CaMKII to α-actinin-2.

FIGURE 3. CaMKII isofrom binding to α-actinin-2, NR2B, and densin-180: role of the C-terminal association domain. A, Thr286 autophosphorylated CaMKII (wild type WT or C-terminal truncated), CaMKIIγ, or CaMKIIδ, were incubated with GST, GST-densin, GST-NR2B, or GST-actinin (819–894). The top panel shows a Ponceau stained gel of the GST fusion protein preparations used in these experiments. The lower panels show CaMKII immunoblots of proteins isolated on glutathione-agarose (P-40% of total pellet) and the soluble proteins remaining in the supernatant (S-2% of total). B, CaMKIIγ overlay assay. GST fusion proteins were immobilized on nitrocellulose membranes and incubated with wild type [32P-T286]CaMKII. Membranes were washed and autoradiographed. C, quantification of wild type (WT) CaMKII binding to the three GST fusion proteins in pull-down (top) and overlay (bottom) assays. Binding to GST-actinin and GST-densin was normalized to binding to GST-NR2B in each experiment and averaged across three to eight experiments; data are shown as mean ± S.E. D and E, quantitative comparison of CaMKII isofrom (D) and CaMKIIγ truncation mutant (E) binding to densin, NR2B, and α-actinin-2 in glutathione-agarose cosedimentation assays. Binding of each isofrom or truncation mutant was normalized to wild type CaMKIIα for each GST fusion protein, and then averaged across three to eight experiments. Data are expressed as mean ± S.E. *p < 0.001; †p < 0.01 versus binding to wild type CaMKIIα.

compared with the strong binding to GST-NR2B and GST-densin-(1247–1542) (Fig. 3). In fact, binding of [32P-T286]CaMKII to immobilized GST-actinin-(547–894) was not consistently above the very weak non-specific binding with GST alone (Fig. 3B and C). These data suggest that, unlike NR2B and densin-180, the CaMKII-binding domain of α-actinin-2 is unable to refold into a binding-competent conformation on the membrane.

CaMKII Isoform Selectivity—Proteins encoded by the four CaMKII genes are highly similar within their N-terminal catalytic and regulatory domains (89–93% amino acid sequence identity) but are more diverse in the C-terminal association domains (53–89% amino acid sequence identity) (28). Representative splice variants of each gene were autophosphorylated at Thr286 (CaMKIIα) or at Thr286 (CaMKIIβ/γ/δ) and then used in glutathione-agarose cosedimentation assays. The four CaMKII isoforms bound with similar efficacy to GST-NR2B and GST-actinin-(819–894). In contrast, GST-densin-(1247–1495) selectively interacted with CaMKIIα (Fig. 3); binding of CaMKIIβ and CaMKIIγ was reduced ~13-fold relative to binding of CaMKIIα. Although GST-densin-(1247–1495) appeared similarly ineffective in binding CaMKIIδ, by immunoblotting (Fig. 3A), this could not be quantitated from the Ponceau-stained membrane, because CaMKIIδ and GST-densin-(1247–1495) co-migrate on the gel.

Role of the C-terminal Association Domain of CaMKIIα—The role of the C-terminal domain in binding to each of the CaMKAPs was investigated using CaMKIIα truncations at either residue 420 or residue 380. Both proteins are monomeric but can be Thr286-autophosphorylated to a similar extent as the wild type kinase using modified reaction conditions, as assessed by immunoblotting using phospho-Thr286-specific antibodies and by the generation of autonomous kinase activity (data not shown). We previously showed that CaMKII(1–420) exhibited reduced binding to GST-NR2B (20), but under the conditions used here, both GST-NR2B and GST-actinin-(819–894) bound with comparable efficacy to the monomeric kinases and the wild type holoenzyme. However, there was no statistically significant binding of either truncation mutant to GST-densin-(1247–1495) (Fig. 3A and E). These data show that the NR2B and α-actinin-2 binding sites lie within the first 380 amino acids of CaMKII and that formation of a CaMKII holoenzyme is not essential for these interactions. However, CaMKIIα binding to densin-180 appears to require an intact association domain.

Non-competitive Binding of Densin-180, NR2B, and α-Actinin-2 to CaMKII—Residues 819–894 of α-actinin-2 share no obvious amino acid sequence similarity to the CaMKII-binding domains of NR2B or densin-180. To compare mechanisms for interactions of CaMKII with α-actinin-2, NR2B, and densin-180, competition overlay assays were performed. Nitrocellulose membranes containing immobilized GST fusion proteins were incubated with [32P-T286]CaMKII (50 mM) in the absence or presence of each of the soluble GST fusion proteins (up to 1.25 μM). Binding of kinase to immobilized proteins was determined by autoradiography of the washed membranes.

With immobilized GST-densin-(1247–1495), soluble GST-densin-(1247–1495) decreased [32P-T286]CaMKII binding in a concentration-dependent manner (≈55% of control at 1.25 μM, but similar concentrations of soluble GST-actinin-(819–894) or soluble GST-NR2B had no significant effect (Fig. 4). Similarly, with immobilized GST-NR2B, soluble GST-NR2B competed for binding of [P-T286]CaMKII (≈15% binding at 1.25 μM), but soluble GST-densin-(1247–1495) or soluble GST-actinin-(819–894) had no significant effect. Control experiments showed that soluble GST had no effect on CaMKII binding to any immobilized protein (data not shown). It was not possible to test for potential effects of higher competitor concentrations because of the limited solubility of these GST fusion proteins.

As shown above, [32P-T286]CaMKII binds very weakly to immobilized α-actinin-2 in overlay assays. However, soluble GST-densin-(1247–1495) increased ≈8-fold the apparent interaction of CaMKII with immobilized GST-actinin-(819–894), whereas soluble GST-NR2B and soluble GST-actinin-(819–894) had no effect (Fig. 4). In combination, these data demonstrate that interactions of NR2B, densin-180, and α-actinin-2 with [P-T286]CaMKII are non-competitive and suggest that densin-180 may facilitate binding of CaMKII to α-actinin-2.
Mechanism for Potentiation of CaMKII Binding to α-Actinin-2 by Densin-180—Facilitation of CaMKII binding to immobilized α-actinin-2 by soluble densin-180 in the overlay assay (above) may be influenced by interactions of CaMKII with both densin-180 and α-actinin-2, as well as by direct interaction of densin-180 with α-actinin-2 (15). To determine whether the densin-180 PDZ domain interacts with the C terminus of α-actinin under our conditions, biotinylated GST-densin-(819–1382) was incubated with immobilized α-actinin-2 constructs in overlay assays. Densin interacted with GST-actinin-(819–894) and GST-actinin-(839–894), but not with GST-actinin-(819–885), which lacks the C-terminal 9 amino acids (Fig. 5A). Thus, densin-180 interacts with the consensus PDZ-binding motif in α-actinin-2 under our overlay conditions.

We next assessed the role of direct CaMKII interaction with α-actinin-2 in densin-enhanced binding to immobilized α-actinin-2. Soluble GST-densin-enhanced by 16 ± 3-fold the binding of [32P-T286]CaMKII to GST-actinin-(839–894) (Fig. 5B), which cannot bind CaMKII directly in cosedimentation assays (Fig. 2), but retains an intact C terminus. These data suggest that CaMKII interactions with actinin are not involved in the densin-enhanced immobilization of [32P-T286]CaMKII by denatured GST-actinin-(819–894).

We next investigated contributions of the densin-180 PDZ domain and the C terminus of α-actinin-2 to the densin-enhanced binding of [32P-T286]CaMKII to α-actinin-2. The potentiation was essentially abrogated if the PDZ domain was deleted from soluble densin (GST-densin-(1247–1405)), or if nine amino acids were deleted from the C terminus of the immobilized α-actinin-2 (GST-actinin-(819–885)) (Fig. 5B). Moreover, preincubation of immobilized GST-actinin-(819–894) with soluble GST-densin-(1247–1542) potentiated (∼5-fold) the apparent interaction of [32P-T286]CaMKII (added only after washing to remove excess soluble densin) (data not shown). These data are consistent with a model in which densin-180 simultaneously interacts with the C terminus of α-actinin via its PDZ domain (residues 1405–1492), and with CaMKII via the independent CaMKII-binding domain (residues 1355–1382).
**Multivalent CaMKII Interactions**

His$_6$-densin associated with GST-densin-(1247–1542) only when CaMKII was present (Fig. 6A). Moreover, similar stoichiometric amounts of CaMKII associated with GST-densin-(1247–1542) in the presence or absence of mixed His$_6$-tagged CaMKAPs.

To determine the role of the interaction between the PDZ domain of densin-180 and the C terminus of α-actinin in CaMKII-mediated assembly of this multiprotein complex, we performed similar assays using GST-densin-(1247–1405), which lacks the PDZ domain. GST-densin-(1247–1405) bound CaMKII equally well in the presence or absence of the His$_6$-tagged CaMKAPs, but bound the three His$_6$-tagged CaMKAPs in the presence, but not the absence, of CaMKII (Fig. 6B). Therefore, a PDZ-mediated interaction of densin and α-actinin is not necessary for their simultaneous binding to CaMKII.

The role of CaMKII holoenzyme structure in assembly of this multiprotein complex was examined using the monomeric truncation mutant CaMKIIα-(1–420). Although CaMKIIα-(1–420) cannot bind densin-180, it is capable of interacting with both NR2B and α-actinin (Fig. 3). CaMKIIα-(1–420) facilitated the association of His$_6$-actinin with GST-NR2B on glutathione-agarose (Fig. 7). The amount of His$_6$-actinin isolated was somewhat less than that recovered with the wild type holoenzyme in side-by-side experiments (not shown), but this might be expected, because there is only one available binding site for α-actinin when a CaMKII monomer binds to a GST-NR2B molecule, whereas binding of a CaMKII holoenzyme to GST-NR2B immobilizes 12 binding sites for α-actinin. Thus, single CaMKII subunits are capable of binding both NR2B and α-actinin simultaneously.

**DISCUSSION**

CaMKII partially colocalizes with NMDA receptor NR2B subunits, densin-180, and α-actinin in dendritic spines of cultured neurons (10, 29, 30). Moreover, several studies have shown that CaMKII and NR2B, as well as CaMKII and densin-180, can be coimmunoprecipitated from different types of brain extract (10, 11, 15, 31). Although CaMKIIα was detected in α-actinin-1 immunoprecipitates from whole rat brain lysates, α-actinin was not detected in CaMKII α-actinin immunoprecipitates (16). Here we extend these findings by showing that α-actinin is present in CaMKII α-actinin immunoprecipitates from partially solubilized PSD-enriched extracts (Fig. 1), suggesting that CaMKII interacts with PSD-associated α-actinin, presumably α-actinin-2 (32, 33). Densin-180 was also detected in these samples. Moreover, densin-180 immunoprecipitates from these solubilized PSD extracts contained NR2B and CaMKII (Fig. 1). It is unclear how the solubilization conditions affect specific protein-protein interactions. Moreover, Ca$^{2+}$/calmodulin binding and auto-phosphorylation at Thr$^{286}$ or Thr$^{305/306}$ differentially regulate CaMKII interactions with NR2B and α-actinin (10, 11, 13, 20, 21). Nevertheless, these data support the idea that CaMKII can interact with several CaMKAPs found in dendritic spines, including NR2B, densin-180, and α-actinin. The experiments described here dissect the interactions of CaMKII with these three CaMKAPs.

CaMKII was previously reported to bind α-actinin-1 and α-actinin-4 via a poorly defined C-terminal domain (15). Here, we show that CaMKII directly binds to α-actinin-2 in vitro and provide an initial characterization of the molecular determinants for interaction. Together, our data show that the interactions of CaMKII with NR2B, densin-180, and α-actinin are mechanistically distinct in multiple ways. First, the minimal CaMKII-binding domain identified in α-actinin-2 (residues 819–894, Fig. 2) is substantially larger than, and shares no amino acid sequence similarity with, the minimal CaMKII-binding domains of NR2B and densin-180 (14, 20). However, deletion of

![Image](link)
sequences toward the extreme N and C termini of the CaMKII-binding domain that are highly conserved between these α-actinin isoforms abrogates CaMKII binding (Fig. 2). Second, in overlay assays, CaMKII binding to α-actinin-2 did not exceed background levels (presumably because α-actinin-2 failed to refold correctly on the membrane), whereas CaMKII bound effectively to NR2B and densin-180 (Fig. 3). This observation likely explains why initial overlay analyses of brain subcellular fractions failed to detect significant CaMKII-binding proteins with similar electrophoretic mobility to native α-actinin (25). Third, NR2B and α-actinin-2 interacted effectively with all four CaMKII isoforms, whereas densin-180 was selective for CaMKIIα holoenzymes (Fig. 3). Fourth, activation of CaMKII by calcium/calmodulin-binding or autophosphorylation at Thr286 is essential for the interaction with NR2B and potentiates interaction with densin-180 (10, 11, 13–15, 20, 21), whereas binding of CaMKII to α-actinin-2 is independent of Thr286 autophosphorylation. Last, these CaMKAPs do not compete for binding to CaMKII (Figs. 4 and 6). In combination, the data demonstrate that in contrast to AKAPs, which use a conserved molecular determinant (an amphipathic α-helix) to bind cAMP-dependent protein kinase regulatory subunits (34), CaMKAPs use divergent mechanisms to interact with CaMKII, with the potential for unique functional effects beyond simply dictating the subcellular localization of CaMKII.

We also explored the role of CaMKII holoenzyme structure in the interactions with α-actinin-2, densin-180, and NR2B. NR2B and α-actinin-2 bind monomeric truncation mutants of CaMKIIα. Thus, binding determinants for NR2B and α-actinin lie within the first 380 amino acids (Fig. 3), which contain the catalytic and regulatory domains. Consistent with these findings, Bayer et al. (13) have shown that catalytic domain mutations affect binding of CaMKII to NR2B, and yeast two-hybrid studies have shown that α-actinin interacts with residues 1–316 of CaMKIIα (15). In contrast, densin-180 does not bind to the monomeric CaMKIIα (1–420) (Fig. 3). Previous studies using yeast two-hybrid approaches have shown that residues 1–316 of CaMKIIα are incapable of binding to densin-180, and that residues 317–478 are sufficient for binding (15). These data suggest two hypotheses: 1) essential components of the densin-180 interaction domain lie within residues 421–478 of CaMKIIα; 2) holoenzyme structure is required for association of densin-180 with a domain within residues 317–420. Additional experiments will be required to resolve this issue.

Interactions of CaMKII with individual CaMKAPs have been suggested to play a role in targeting CaMKII within dendritic spines (reviewed in Refs. 4, 7, and 9). In particular, the dynamic regulation of CaMKII interactions with NR2B seems to parallel the transient translocation of CaMKII to PSDs in neurons stimulated electrically, or by glutamate (35, 36). However, under some conditions CaMKII translocation to synapses is associated with the loss of synaptic NMDA receptors (37). The CaMKAPs also may functionally modulate CaMKII association with glutathione-agarose co sedimentation assays (Fig. 6). Thus, it is not clear whether a similar potentiation will occur in cells.

CaMKIIα is highly abundant in neurons (e.g. 1–2% of total hippocampal protein), greater than might be expected for a strictly enzymatic role (47, 48). Thus, nucleation of protein complexes by CaMKII holoenzymes may play one or more structural roles in dendritic spines. A variety of scaffolding proteins such as PSD-95 and CASK participate in multivalent interactions with cytoskeletal, receptor, and signaling proteins that are essential for proper targeting of receptor and signal transduction complexes (reviewed in Ref. 49). Recent work shows that isolated PSDs contain similar numbers of PSD-95 molecules and CaMKII holoenzymes (50). Interestingly, CASK contains an inactive kinase domain homologous to the catalytic domain of CaMKII that may have evolved from an ancestral calmodulin-dependent kinase to lose catalytic function while retaining the ability participate in protein-protein interactions (51, 52). Because CaMKII interactions with CaMKAPs are dynamically modulated (44), we propose that CaMKII may function as an autoregulated structural component, contributing to the reorganization of postsynaptic protein complexes depending on its activation state. These dynamic processes may play a role in the morphological changes in postsynaptic densities and dendritic spines following induction of synaptic plasticity (43, 53, 54), the ordered clustering of CaMKII in “towers” within the PSD (55), and/or the synaptic insertion of AMPA receptors (1). Further investigation of these structural roles will require selective manipulation of these interactions while preserving the catalytic function of the kinase.

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Multivalent CaMKII Interactions

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