

Substrate-selective and Calcium-independent Activation of CaMKII by α -Actinin^{*S}

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Background: Synaptic signaling is modulated by protein-protein interactions involving receptor ion channels, cytoskeletal proteins, and protein kinases.

Results: α -Actinin enhances CaMKII signaling to GluN2B-NMDARs, but interferes with CaMKII signaling to GluA1-AMPA receptors.

Conclusion: CaMKII actions on key synaptic targets can be differentially modulated by a Ca^{2+} -independent interaction with α -actinin.

Significance: These findings provide new molecular insights into the synaptic mechanisms underlying learning and memory.

Protein-protein interactions are thought to modulate the efficiency and specificity of Ca^{2+} /calmodulin (CaM)-dependent protein kinase II (CaMKII) signaling in specific subcellular compartments. Here we show that the F-actin-binding protein α -actinin targets CaMKII α to F-actin in cells by binding to the CaMKII regulatory domain, mimicking CaM. The interaction with α -actinin is blocked by CaMKII autophosphorylation at Thr-306, but not by autophosphorylation at Thr-305, whereas autophosphorylation at either site blocks Ca^{2+} /CaM binding. The binding of α -actinin to CaMKII is Ca^{2+} -independent and activates the phosphorylation of a subset of substrates *in vitro*. In intact cells, α -actinin selectively stabilizes CaMKII association with GluN2B-containing glutamate receptors and enhances phosphorylation of Ser-1303 in GluN2B, but inhibits CaMKII phosphorylation of Ser-831 in glutamate receptor GluA1 subunits by competing for activation by Ca^{2+} /CaM. These data show that Ca^{2+} -independent binding of α -actinin to CaMKII differentially modulates the phosphorylation of physiological targets that play key roles in long-term synaptic plasticity.

Transient changes in intracellular Ca^{2+} concentrations can be decoded by the ubiquitous Ca^{2+} /calmodulin (CaM)³-dependent protein kinase II (CaMKII) to elicit diverse physiological

responses. Binding of Ca^{2+} /CaM activates CaMKII and Thr-286 autophosphorylation, creating an autonomously active form of CaMKII that is a molecular memory of transient Ca^{2+} signals. Dissociation of Ca^{2+} /CaM allows for rapid autophosphorylation at Thr-305 or Thr-306 (Thr-305/6), blocking re-association of Ca^{2+} /CaM such that CaMKII is desensitized to subsequent Ca^{2+} signals (reviewed in Refs. 1–4). These autophosphorylation reactions are key for CaMKII-dependent modulation of synaptic glutamate receptors and other neuronal functions related to long-term synaptic plasticity, learning, and memory (5–8).

As for many signaling proteins, precise targeting of CaMKII is crucial for its actions (9, 10). Neuronal Ca^{2+} signals can be tightly compartmentalized in dendritic spines (11), F-actin-rich synaptic structures that contain postsynaptic densities (PSDs) enriched in glutamate receptors and other signaling proteins, including CaMKII α . CaMKII α appears to be initially cytosolic, but Ca^{2+} /CaM binding initiates translocation to dendritic spines; a recent study suggests that only a small fraction of CaMKII in spines is tightly associated with PSDs (12). Retention of CaMKII in the spines is modulated by autophosphorylation; Thr-286 autophosphorylation stabilizes spine localization, whereas Thr-305/6 autophosphorylation promotes CaMKII dissociation from spines (13). *N*-Methyl-D-Aspartate (NMDA)-type glutamate receptor (NMDAR) GluN2B subunits are the most widely recognized binding partner for CaMKII; this interaction modulates CaMKII activity and is important for synaptic plasticity and memory (14–19). GluN2B selectively binds activated CaMKII, perhaps contributing to CaMKII translocation and retention in spines. However, PSDs contain ~20-fold more CaMKII holoenzymes than NMDAR subunits (20), suggesting that direct interactions with NMDARs account for only a fraction of spine-localized CaMKII holoenzymes (12). Thus, the overall dynamics of CaMKII targeting presumably reflect integration of NMDAR interactions with CaMKII binding to several other proteins (1). Indeed, CaMKII can simultaneously interact with α -actinin, GluN2B, and densin (21). Importantly, pharmacological disruption of F-actin interferes with synaptic clustering of CaMKII α and actin-binding proteins such as α -actinin without changes in NMDAR localiza-

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³ The abbreviations used are: CaM, calmodulin. CaMKII, Ca^{2+} /calmodulin-dependent protein kinase II. NMDAR, *N*-methyl D-Aspartate receptor. PSD, postsynaptic density. AMPAR, α -amino-3-hydroxyl-5-methyl-4-isoxazole-propionic acid receptor. CaMKAP, CaMKII-anchoring protein. CTD, C-terminal domain.

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tion (22, 23). Synaptic CaMKII targeting therefore depends on an intact F-actin cytoskeleton.

The four isoforms of α -actinin (1–4) link various signaling molecules to the cytoskeleton by virtue of conserved modular protein domains: an N-terminal F-actin-binding domain, four spectrin-like repeat domains (responsible for antiparallel dimerization), an EF hand domain (that binds divalent cations only in α -actinin-1 and α -actinin-4), and a C-terminal domain (CTD) (24, 25). For example, α -actinin links NMDAR GluN1 and GluN2B subunits to the actin cytoskeleton and modulates NMDAR activity by diverse mechanisms (26–30). CaMKII also associates with multiple α -actinin isoforms in brain (21, 31, 32). Our previous studies defined the CTD of α -actinin-2 (A2-CTD; residues 819–894) as the minimal CaMKII binding domain (21). *In vitro*, A2-CTD interaction with CaMKII is Ca²⁺-independent, competitive with Ca²⁺/CaM, and disrupted by Thr-305/6 autophosphorylation (18). However, the mechanism and functional consequences of CaMKII interaction with full-length α -actinin are poorly understood. Here, we show that α -actinin-2 mimics CaM in binding to the CaMKII regulatory domain, and targets CaMKII to the actin cytoskeleton. *In vitro* and intact cell studies show that α -actinin-2 is a Ca²⁺-independent, substrate-selective CaMKII activator, which also enhances CaMKII interaction with NMDARs containing GluN2B subunits.

EXPERIMENTAL PROCEDURES

Bacterial and Mammalian Expression Vectors—The cDNA encoding full-length (FL) α -actinin-2 (A2) (894 amino acids) in pCDNA3.1(+) was a gift from Dr. A. Beggs (Harvard). The GluA1 construct was provided by Dr. T. Soderling (Oregon Health Sciences University). Other mammalian expression vectors were previously described; full-length A2 with an N-terminal HA-tag and untagged CaMKII α (33, 34); GluN1, GluN2A, and GluN2B (35). Expression of GST-A2-CTD, His₆-A2-CTD, and His₆-densin was described (21, 36). Site-directed mutagenesis was confirmed by sequencing. All constructs were purified using Maxi-Prep kits (Invitrogen, Carlsbad, CA).

Structural Alignment—The structure of CaM bound to CaMKII regulatory domain (PDB:1CM1) was aligned with that of α -actinin-2 bound to the titin z-repeat (1H8B) using the align command of PyMOL (DeLano Scientific LLC).

CaMKII Autophosphorylation—CaMKII α (WT, T305A, or T306A) were purified from baculovirus-infected Sf9 cells and sequentially autophosphorylated essentially as described (37). Briefly, CaMKII α (5 μ M subunit) was incubated on ice for 90 s with 50 mM HEPES, pH 7.5, 10 mM magnesium acetate, 1.5 mM CaCl₂, 10 μ M CaM, 2 mM DTT, and 500 μ M ATP. After 90 s, 4 mM EGTA was added and reaction continued for 2 min at 30 °C before termination using 25 mM EDTA. The efficacy of autophosphorylation of each protein was verified by assessing the impact on Ca²⁺/CaM binding (see “Results”).

GST Co-sedimentation Assays—Purified GST-fusion proteins or GST alone (\approx 250 nM full-length protein), CaMKII (WT or mutated) (\approx 250 nM subunit; sequentially autophosphorylated, or non-phosphorylated, as indicated) and glutathione-agarose (Sigma; 30 μ l packed resin) were incubated at 4 °C for 2 h in 0.5 ml of PD buffer (50 mM Tris-HCl, pH 7.5, 200 mM

NaCl, 0.5% (v/v) Triton X-100). After centrifugation, beads were washed with PD buffer and analyzed by immunoblotting. CaMKII bound to GST proteins was quantified using Image J from scans of Ponceau-S-stained membranes, immunoblotted membranes, or linearly exposed x-ray films, and normalized to the amount of recovered GST fusion protein (detected by Ponceau-S stain). Any background binding to GST alone was subtracted.

CaMKII Activation Assay—Purified CaMKII α (10 nM) was incubated with indicated concentrations of CaM or His₆-A2-CTD in presence of 50 mM HEPES pH 7.5, 1 mg/ml bovine serum albumin, 10 mM magnesium acetate, 0.5 mM CaCl₂ or 1 mM EGTA, 1 mM DTT and a model peptide substrate (AC-2, syntide-2, or NR2B; 100 μ M each). Reactions were started by adding [γ -³²P]ATP (0.4 mM; 500–1000 cpm/pmol) and incubated at 30 °C for 10 min. Aliquots were spotted on phosphocellulose paper (Whatman P81), washed, and analyzed by liquid scintillation counting (38).

Cell Culture and Transfection—HEK293 cells maintained at 37 °C in 5% CO₂ in modified Eagle’s medium (MEM) containing 10% fetal bovine serum (Invitrogen, Carlsbad, CA), penicillin-streptomycin (Sigma) and 2 mM glutamate (Sigma) were transfected using Fugene (Roche, Indianapolis, IN) or PolyJet (SignaGen Laboratories, Gaithersburg, MD) (3 μ l/ μ g DNA). Cells expressing NMDAR subunits were cultured with 1 mM (2R)-amino-5-phosphonovaleric acid; (2R)-amino-5-phosphonopentanoate (AP-5) (Ascent Scientific) or 500 μ M AP-5 plus 5 mM MgCl₂.

HEK293 Cell Lysis and Immunoprecipitation—Cell extracts were made 24–48 h after transfection (38). NaCl (150 mM final) was added to supernatants, and equal aliquots were incubated overnight at 4 °C with CaMKII IgG or normal IgG (2 μ g each; goat). GammaBind Plus-Sepharose (Amersham Biosciences, Piscataway, NJ) (30 μ l, 1:1 slurry) was added and incubations were continued for 2 h at 4 °C. Resin was washed \geq 4 times with Buffer B (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% (v/v) Triton X-100) before analysis by immunoblotting.

Immunofluorescence Co-localization—HEK293 cells expressing TT305/6AA-CaMKII α with or without WT or Y861R HA- α -actinin-2 were fixed 24 h after transfection, labeled with mouse CaMKII α and goat HA antibody (Bethyl Labs). F-actin was detected using phalloidin conjugated to Alexafluor-647 (Invitrogen; 1:500, mixed with primary antibodies). Methods for cell imaging and intensity correlation analysis were described previously (33).

Immunoprecipitation from Mouse Brain Extracts—Mouse forebrains were homogenized in low-ionic strength buffer (38), and supernatants were immunoprecipitated using goat CaMKII α (2 μ g) or rabbit α -actinin H-300 (Santa Cruz Biotechnology) (8 μ g) antibodies, or an IgG control.

Immunoblotting—SDS-polyacrylamide gels were transferred to nylon-backed nitrocellulose membranes (38). Primary antibodies included: CaMKII α (Affinity BioReagents, Golden, CO); HA (Vanderbilt Monoclonal antibody core, ICA5); GluN2B, GluN2A, and GluN1 (BD Biosciences); phospho-Ser1303 GluN2B (Millipore/Upstate 07–398); α -actinin (7A4, Abcam); total GluA1 (Abcam ab31232); phospho-Ser831 GluA1 (Phospho Solutions p1160–831). Secondary antibodies conjugated



FIGURE 1. Ca²⁺-independent activation of CaMKII by α -actinin-2. *A*, ClustalW2 alignment of the C-terminal domain (CTD) of human α -actinin-2 (A2; residues 819–894) with the CTDs of α -actinin-1, -3, and -4 (A1, A3, A4) and full-length human CaM (CM). Identical and similar residues are highlighted in black and gray, respectively. Asterisks indicate residues targeted for mutagenesis in α -actinin-2. *B*, concentration-dependent effects of CaM and His₆-A2-CTD on CaMKII phosphorylation of autocamtide-2 in the presence of CaCl₂ (+Ca; black/dashed lines) or EGTA (–Ca; gray/solid) (mean \pm S.E., $n = 3$). *C*, Ca²⁺-independent activation of CaMKII by His₆-A2-CTD (14 μ M) is detected with autocamtide-2 (AC2) and GluN2B-1290–1309, but not syntide-2. Data plotted as a percentage of maximum activation by Ca²⁺/CaM (mean \pm S.E., $n = 3$).

to alkaline phosphatase (Jackson ImmunoResearch, West Grove, PA), horseradish peroxidase (Promega or Santa Cruz), or infrared dyes (LiCor Biosciences, Lincoln, NE) were used for development with NBT/BCIP, enhanced chemiluminescence (Perkin-Elmer, Waltham, MA), or an Odyssey system (LiCor), respectively.

Statistics—Data were analyzed by one-way or two-way ANOVA with the specified post-hoc tests. CaMKII activation curves were fitted to one-site binding curves using GraphPad Prism.

RESULTS

Substrate-selective Activation of CaMKII by α -Actinin—The minimal CaMKII-binding A2-CTD (residues 819–894) (21) is highly conserved in all α -actinin isoforms and is similar to the C-terminal domain (lobe) of CaM (residues 73–149) (Fig. 1A and supplemental Fig. S1). Since the C-terminal lobe of CaM is sufficient to partially activate CaMKII in the presence of Ca²⁺ (39), we tested the effects of a His₆-tagged A2-CTD on CaMKII activity. We detected a concentration-dependent activation of purified CaMKII α using a model peptide substrate, autocamtide-2 (AC-2). This activation was Ca²⁺-independent (–Ca²⁺: $V_{\max} 1.5 \pm 0.2 \mu\text{mol/min/mg}$, $K_a 8.6 \pm 1.6 \mu\text{M}$; +Ca²⁺: $V_{\max} 1.3 \pm 0.2 \mu\text{mol/min/mg}$, $K_a 6.7 \pm 1.7 \mu\text{M}$), whereas CaM is a Ca²⁺-dependent activator (+Ca²⁺: $V_{\max} 6.1 \pm 0.1 \mu\text{mol/min/mg}$, $K_a 97 \pm 17 \text{ nM}$) (Fig. 1B). Moreover, Ca²⁺/CaM stimulated the phosphorylation of all substrates tested, whereas CaMKII activation by the A2-CTD was detected with AC-2 and GluN2B-1290–1309, but not with syntide-2 (Fig. 1C). Thus, the A2-CTD is a Ca²⁺-independent and substrate-selective activator of CaMKII.

Identification of CaMKII-binding Determinants in α -Actinin-2—We hypothesized that the different affinities and maximal activation by CaM and A2-CTD reflect distinct interactions with the α -helical CaMKII regulatory domain (residues 293–310). The N- and C-terminal lobes of full-length CaM interact with both sides of this helix (40) (supplemental Fig. S2A). Inter-

estingly, the A2-CTD interacts with a single face of a regulatory domain helix in the giant muscle protein kinase, titin (41) (supplemental Fig. S2B). Given the amino acid sequence similarity between the A2-CTD and the C-lobe of CaM (Fig. 1A), we aligned these two structures (supplemental Fig. S2C). The alignment (RMS 2.97 Å) predicts a hydrophobic interface between one face of the CaMKII regulatory domain helix and a concave binding “pocket” in A2-CTD formed by Ser-834, Leu-854, Tyr-861, Leu-888, and other residues (Fig. 2A). To test this alignment as a model for CaMKII-binding to the A2-CTD, we introduced charge in the predicted hydrophobic binding pocket. S834R, L854R, and Y861R mutations completely abrogated CaMKII binding to GST-A2-CTD, and the L888R mutation reduced binding by $\approx 70\%$ (Fig. 2B). However, none of these mutations affected binding of GST-A2-CTD to the densin PDZ domain in parallel experiments (supplemental Fig. S3A). Additional mutations reduced hydrophobicity without introducing charge; L854A and Y861A mutations completely abrogated CaMKII binding (like L854R and Y861R mutations), but S834A mutation had a minimal effect (supplemental Fig. S3B). In combination, these findings support our model for CaMKII interaction with the hydrophobic binding pocket in the CTD of α -actinin-2.

Determinants in CaMKII for Binding to α -Actinin—Ca²⁺-independent autophosphorylation blocks CaMKII binding to both GST-A2-CTD and Ca²⁺/CaM, and this loss of binding can be prevented by mutation of both Thr-305 and Thr-306 in CaMKII α to Ala (21, 37). Our alignment model indicates that the Thr-306 side chain is buried in the A2-CTD interaction site, whereas the Thr-305 side chain is solvent exposed (Fig. 2C), suggesting that phosphorylation at Thr-306, but not Thr-305, should disrupt binding. Autophosphorylation of wild type (WT) CaMKII α can occur at both Thr-305 and Thr-306, whereas T305A- and T306A-CaMKII α mutants can be autophosphorylated at only one residue (Thr-306 and Thr-305, respectively) (37). Indeed, Ca²⁺-independent autophosphory-

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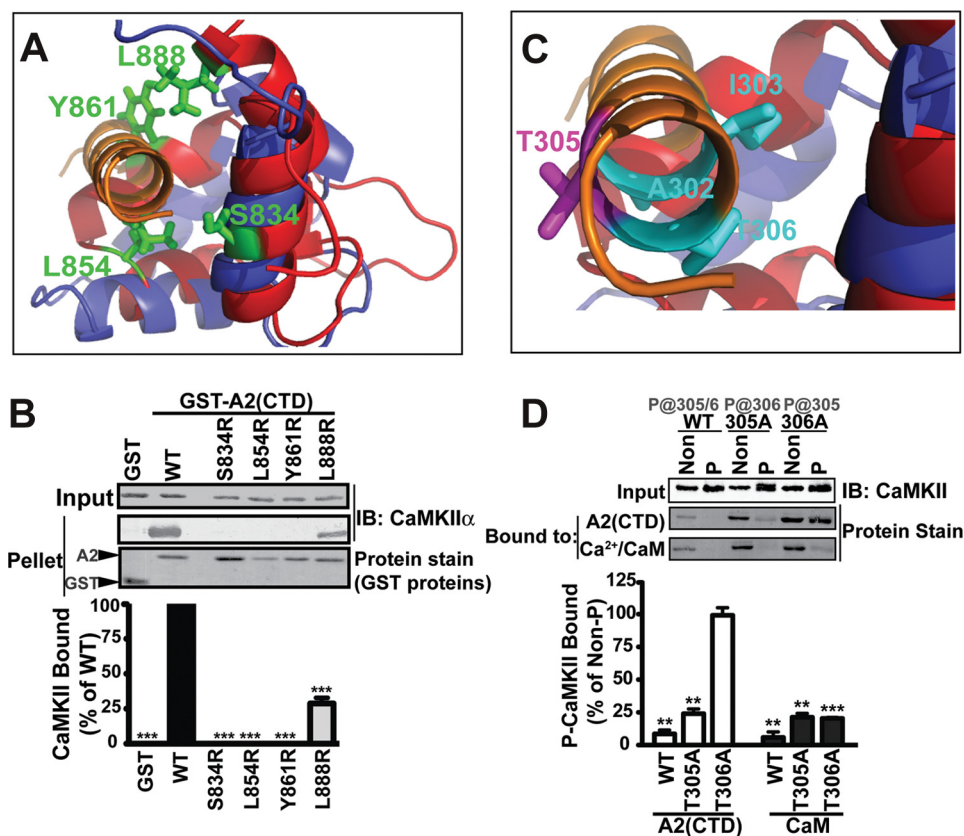


FIGURE 2. α -Actinin mimics CaM in binding to the CaMKII regulatory domain. *A* and *B*, structural alignment of the A2-CTD (red) with the C-terminal lobe of CaM (blue) bound to CaMKII (from supplemental Fig. S2C). Residues in α -actinin-2 highlighted in green were mutated to arginine. Introduction of charge in the predicted hydrophobic binding pocket disrupted CaMKII α binding, as seen by protein staining for GST proteins and immunoblotting (IB) for CaMKII. Binding is expressed as a percentage of binding to WT (mean \pm S.E., $n = 4$). ***, $p < 0.0001$ versus WT; 1-way ANOVA, Bonferroni's post-test). *C*, close-up of CaMKII regulatory domain helix showing residues in CaMKII targeted for mutagenesis (cyan or magenta). *D*, effects of sequential autophosphorylation of WT, T305A-, or T306A-CaMKII α (P) on binding to Ca²⁺/CaM or GST-A2-CTD. Levels of CaMKII in inputs were compared by immunoblot (top). Isolated complexes were analyzed by protein staining. Binding of autophosphorylated CaMKIIs was expressed as a percentage of binding of corresponding non-phosphorylated control CaMKII (mean \pm S.E., $n = 3$. Two-way ANOVA, $p < 0.0001$. **, $p < 0.001$, ***, $p < 0.0001$ versus 100% by post-hoc 1-column t test).

lation reduced binding of WT, T305A-, and T306A-CaMKII α to Ca²⁺/CaM-agarose by $\geq 80\%$ (Fig. 2D), suggesting that the remaining native Thr residue in both mutated kinases was effectively autophosphorylated. In contrast, Ca²⁺-independent autophosphorylation had no effect on the binding of purified T306A-CaMKII α to GST-A2-CTD, but reduced the binding of purified WT and T305A-CaMKII α to GST-A2-CTD by $\geq 80\%$ (Fig. 2D). Thus, CaMKII α binding to α -actinin is sensitive to autophosphorylation at Thr-306, but not at Thr-305, consistent with our model for α -actinin binding to one face of the CaMKII regulatory domain helix.

To identify additional interaction determinants, we tested the binding of GST-A2-CTD to additional CaMKII α mutants that had been expressed in HEK293 cells. Initial studies suggested that basal autophosphorylation of WT CaMKII α at Thr-305/6 in HEK293 cells (supplemental Fig. S4A) suppressed interactions with both A2-CTD and Ca²⁺/CaM (supplemental Fig. S4B). Moreover, we found that levels of Thr-305/6 phosphorylation were sensitive to mutations within the CaMKII regulatory domain (data not shown). Thus, we analyzed the effects of regulatory domain mutations of CaMKII on binding to the A2-CTD on a background of a T305A/T306A double mutant kinase (TT305/6AA-CaMKII α) to avoid possible indirect effects. A302R and I303R mutations had similar effects to

reduce or block the binding of TT305/6AA-CaMKII α to either A2-CTD or Ca²⁺/CaM (supplemental Fig. S4C), consistent with the hydrophobic interactions predicted by our alignment model. In contrast, Thr-310 in CaMKII α contacts only the N-lobe of CaM (40), and a T310D mutation disrupted binding to Ca²⁺/CaM, with no apparent effect on binding to A2-CTD (supplemental Fig. S4B). In combination, these findings show that α -actinin interacts with a subset of residues in the CaMKII α regulatory domain used for interaction with Ca²⁺/CaM.

CaMKII Association with the CTD in α -Actinin-2 Dimers—Our *in vitro* studies with the A2-CTD constructs suggest that α -actinin has the potential to profoundly affect CaMKII activity and/or localization in cells. However, CaMKII interactions with full-length, dimeric α -actinins have not been directly characterized. Therefore, we co-expressed full-length HA-tagged α -actinin-2 (HA-actinin-2) with CaMKII α in HEK293 cells. HA-actinin-2 was readily detected in CaMKII immune complexes isolated from lysates of cells expressing WT or TT305/6AA-CaMKII α , but mutation of Thr-305 and Thr-306 to Ala substantially enhanced the interaction (supplemental Fig. S4A). Subsequent studies used TT305/6AA-CaMKII α to avoid potential confounding effects of variations in basal phosphorylation at Thr-305/6 (see above). Importantly,

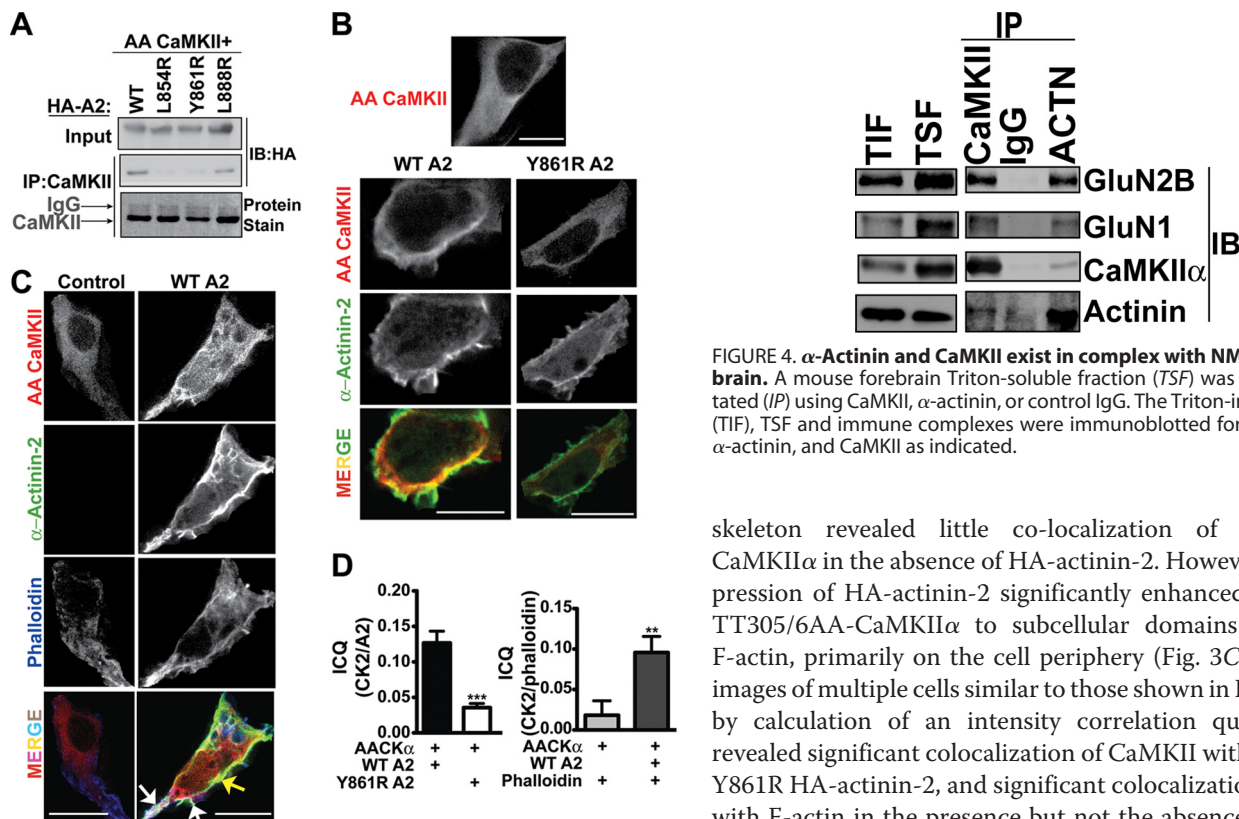


FIGURE 3. CaMKII α is targeted to F-actin by the α -actinin-2 CTD in cells. A, CaMKII immune complexes isolated from extracts of HEK293 cells co-expressing TT305/6AA-CaMKII α with full-length HA-actinin-2 (HA-A2; WT or mutated) were analyzed by protein stain for CaMKII and immunoblotting for HA. B, immunofluorescence microscopy of HEK293 cells expressing TT305/6AA-CaMKII α (AA-CaMKII) alone (top) or with HA-actinin-2 (WT or Y861R; green). C, localization of AA-CaMKII and F-actin (phalloidin staining; blue) in the absence and presence of WT HA-actinin-2 (green). Arrows indicate colocalization of HA-A2 and AA-CaMKII alone (yellow) or HA-A2, AA-CaMKII and phalloidin (white). D, intensity correlation quotient (ICQ) analysis of colocalization between AA-CaMKII and WT or Y861R HA-actinin-2 (left) or AA-CaMKII and phalloidin in the presence and absence of WT HA-actinin-2 (right) ($n = 8$ cells; ***, $p < 0.0001$ versus WT by t test; **, $p < 0.005$ versus control by t test, $n = 6$ cells)

L854R or Y861R mutations in the CTD that prevent CaMKII binding *in vitro* (Fig. 2B) substantially reduced the amount of HA-actinin-2 that co-immunoprecipitated with TT305/6AA-CaMKII α (Fig. 3A). Thus, the CTD of full-length α -actinin-2 appears to be critical for the association of CaMKII in intact cells.

The cellular localization of WT/mutated HA-actinin-2 and TT305/6AA-CaMKII α were also compared using immunofluorescence microscopy. In the absence of HA-actinin-2, TT305/6AA-CaMKII α filled the cell cytoplasm relatively uniformly (Fig. 3B). Full length HA-actinin-2 was targeted to the sub-membrane cortical cytoskeleton (Fig. 3B), as shown previously (42). In the presence of co-expressed WT HA-actinin-2, the distribution of TT305/6AA-CaMKII α became non-uniform. Moreover, there was a partial, but significant, co-localization of WT HA-actinin-2 with TT305/6AA-CaMKII α , as revealed by yellow coloration in an overlay of images displayed in the red and green channels, respectively (Fig. 3B). Importantly, colocalization was disrupted by Y861R mutation of HA-actinin-2. Additional studies using phalloidin to label the F-actin cyto-

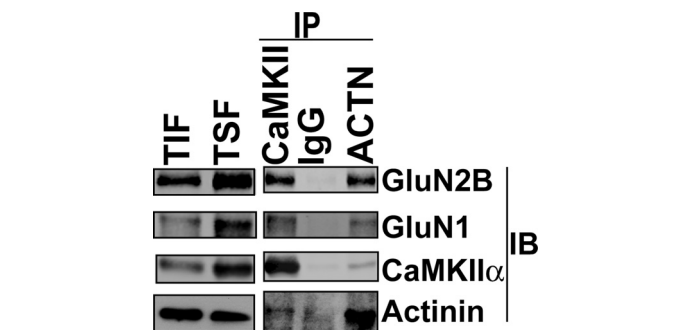


FIGURE 4. α -Actinin and CaMKII exist in complex with NMDARs in mouse brain. A mouse forebrain Triton-soluble fraction (TSF) was immunoprecipitated (IP) using CaMKII, α -actinin, or control IgG. The Triton-insoluble fraction (TIF), TSF and immune complexes were immunoblotted for GluN2B, GluN1, α -actinin, and CaMKII as indicated.

skeleton revealed little co-localization of TT305/6AA-CaMKII α in the absence of HA-actinin-2. However, the co-expression of HA-actinin-2 significantly enhanced targeting of TT305/6AA-CaMKII α to subcellular domains enriched in F-actin, primarily on the cell periphery (Fig. 3C). Analysis of images of multiple cells similar to those shown in Fig. 3, B and C by calculation of an intensity correlation quotient (ICQ) revealed significant colocalization of CaMKII with WT but not Y861R HA-actinin-2, and significant colocalization of CaMKII with F-actin in the presence but not the absence of WT HA-actinin-2 (Fig. 3D). Taken together, these data show that CaMKII α is targeted to the F-actin cytoskeleton by binding to the CTD of full-length α -actinin-2 dimers in intact cells.

α -Actinin Modulation of CaMKII Interactions with NMDARs in Cells—Previous studies showed that CaMKII associates with multiple α -actinin isoforms in brain (21, 31, 32). We expanded these analyses to demonstrate that these complexes also contain NMDAR GluN1 and GluN2B subunits (Fig. 4). To determine the role of α -actinin-2 in formation of this complex, we co-expressed CaMKII α phosphorylation site mutants with GluN1 and GluN2B in HEK293 cells, with or without HA-actinin-2. We again used TT305/6AA-CaMKII α to avoid potential confounding effects of varying levels of Thr-305/6 phosphorylation (see above). Both NMDAR subunits associate with TT305/6AA-CaMKII α in the absence of co-expressed HA-actinin-2 (Fig. 5A). This association was partially due to basal Thr-286 phosphorylation because additional mutation of Thr-286 to Ala (T286A/TT305/6AA-CaMKII α) resulted in a significant ≈ 4 -fold reduction in levels of co-precipitated NMDAR subunits (Fig. 5A). Co-expression of HA-actinin-2 significantly enhanced the associations of GluN1 and GluN2B with TT305/6AA-CaMKII α by ≈ 1.8 -fold, and with T286A/TT305/6AA-CaMKII α by ≈ 4 -fold, relative to corresponding levels in the absence of HA-actinin-2 (Fig. 5A). Indeed, co-expression of HA-actinin-2 largely rescued the deficit in association of GluN2B-NMDARs that was induced by T286A mutation. In contrast, there was a trend ($p = 0.09$) for HA-actinin-2 to decrease the association of GluN2A-NMDARs with TT305/6AA-CaMKII α (Fig. 5B). Collectively, these data show that α -actinin-2 specifically enhances CaMKII α targeting to GluN2B-NMDARs.

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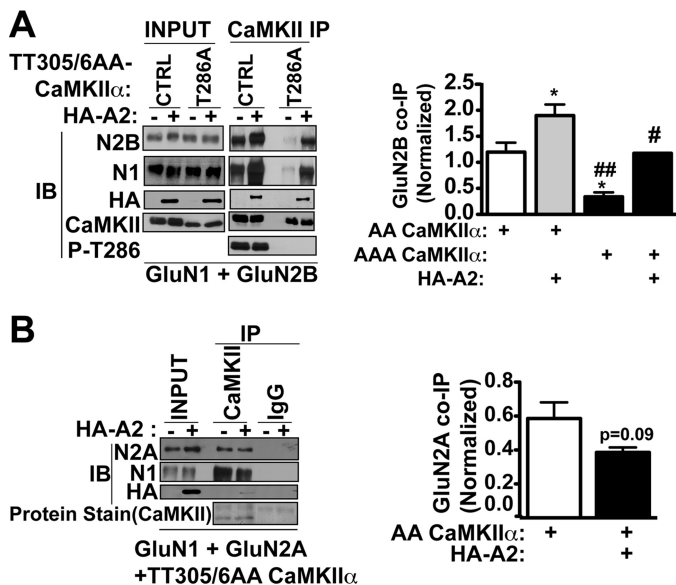


FIGURE 5. α -Actinin-2 selectively enhances CaMKII association with GluN2B-NMDARs. *A*, CaMKII was immunoprecipitated from extracts of HEK293 cells (input) expressing GluN1, GluN2B, and CaMKII α (TT305/6AA or T286A/TT305/6AA; AA-CaMKII α or AAA-CaMKII α , respectively) with or without HA- α -actinin-2. Input and CaMKII immune complexes were immunoblotted for GluN1, GluN2B, HA, CaMKII, and phospho-Thr286 (P-T286). Levels of co-immunoprecipitated GluN2B were normalized to input and plotted as mean \pm S.E. ($n = 5$). *, $p < 0.05$ versus GluN1 + GluN2B + AACK. ##, $p < 0.01$ versus GluN1 + GluN2B + AACK + HA-A2. #, $p < 0.05$ versus GluN1 + GluN2B + AACK. *B*, similar to *A*, except GluN2B is replaced with GluN2A.

α -Actinin-2 Differentially Modulates CaMKII Phosphorylation of Glutamate Receptors in Cells—The CTD of α -actinin-2 activates CaMKII phosphorylation of AC2 or GluN2B-1290-1309 in the presence or absence of Ca²⁺ (Fig. 1, *B* and *C*), but competes with CaM to inhibit Ca²⁺-dependent phosphorylation of other substrates such as syntide-2 (18). These data lead us to hypothesize that α -actinin modulates the innate substrate-selectivity of CaMKII in cells. Therefore, we compared the effects of expressing HA-actinin-2 on CaMKII phosphorylation of two physiologically important substrates; Ser-831 in AMPA-type glutamate receptor GluA1 subunits and Ser-1303 in NMDAR GluN2B subunits (43–45). Phosphorylation of Ser-831 in GluA1 in HEK293 cells was enhanced ≈ 3 -fold by co-expression of TT305/6AA-CaMKII α , but additional expression of HA-actinin-2 blocked this increase (Fig. 6*A*). In contrast, phosphorylation of Ser-1303 in GluN2B by TT305/6AA-CaMKII was significantly enhanced by co-expression of HA-actinin-2 (Fig. 6*B*). Thus, α -actinin-2 modifies the innate substrate selectivity of CaMKII in intact cells, favoring phosphorylation of GluN2B over GluA1.

DISCUSSION

CaMKII interactions with several synaptic proteins are dynamically regulated by CaMKII activation and autophosphorylation, but their specific contributions to overall targeting and modulation of kinase activity are poorly understood (see Introduction). The present findings show that α -actinin can function as a novel modulator of CaMKII targeting and kinase activity by mimicking CaM.

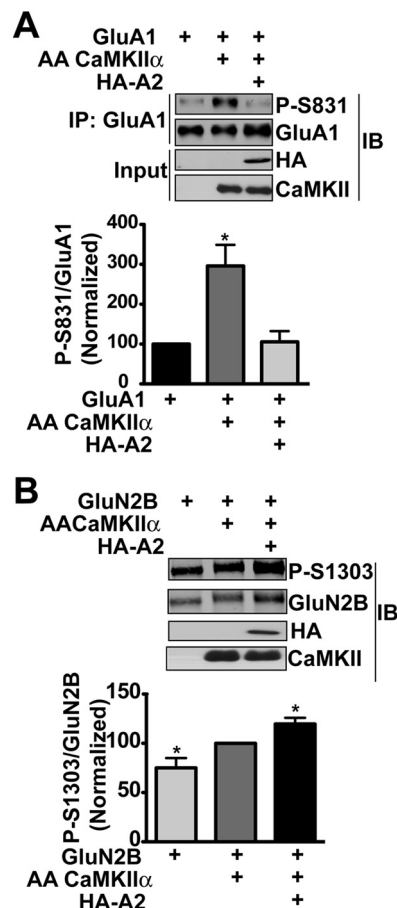


FIGURE 6. α -Actinin modulates phosphorylation of GluA1 and GluN2B by CaMKII. *A*, GluA1 was immunoprecipitated from lysates of HEK293 cells expressing GluA1 with or without TT305/6AA-CaMKII α and HA-actinin-2 (WT) and immunoblotted for total GluA1 and phospho-Ser831 (P-S831). *B*, lysates of HEK293 cells expressing GluN2B with or without TT305/6AA-CaMKII α and HA-actinin-2 were directly immunoblotted for total GluN2B, phospho-Ser1303 (P-S1303), CaMKII and HA as indicated. Graphs plot phospho-protein levels as a ratio to corresponding total protein after normalization to total GluA1 alone (*A*) or total GluN2B + AACK (*B*) (mean \pm S.E., $n = 4$, $p < 0.01$ by 1-way ANOVA with Bonferroni post-test).

α -Actinin as a Surrogate for CaM—In the presence of Ca²⁺, the two lobes of CaM wrap around the α -helical regulatory domain to activate CaMKII (40). However, each lobe of CaM can independently activate CaMKII, albeit with weaker affinity and to reduced levels (39, 46–48). The current data show that Ca²⁺-independent interaction of A2-CTD with one side of the regulatory domain helix can activate CaMKII (Fig. 1*B*). The weaker apparent affinity and partial activation by A2-CTD compared with full-length Ca²⁺/CaM (see “Results”) presumably results from occupation of a reduced surface area on the regulatory domain by the A2-CTD causing only partial displacement of the regulatory domain from the catalytic site. This may also account for the fact that activation by A2-CTD is selective for substrates with primary sequences resembling that surrounding the Thr-286 autophosphorylation site, such as GluN2B Ser-1303 and the AC-2 model peptide (Fig. 1*C*). These peptide substrates also mimic the regulatory domain in binding to a modulatory “T-site” on the catalytic domain (49), which can competitively displace the regulatory domain to help “open” the catalytic site for substrate and ATP binding (50, 51).

Thus, we posit that binding of α -actinin allows for peptide binding at the T-site, thereby selectively enhancing access of these substrates to the catalytic site. However, the regulatory domain is not displaced to the same extent as Ca²⁺/CaM, accounting for the reduced V_{max} . This cooperation may relate to a previous report that GluN2B can “trap” CaMKII in an autonomously active conformation in the absence of Thr-286 autophosphorylation (16). This mechanism may also explain why we have been unable to detect A2-CTD-dependent autophosphorylation at Thr-286 (data not shown), although we cannot exclude the possibility that the low binding affinity limits simultaneous activation of adjacent CaMKII subunits that is required for Thr-286 autophosphorylation.

Role of Thr-305/Thr-306 Autophosphorylation—Most prior studies have treated Thr-305 and Thr-306 as functionally redundant because autophosphorylation at either Thr-305 or Thr-306 can block Ca²⁺/CaM binding (37). Thr-305 and Thr-306 are both strictly conserved among all CaMKII isoforms from *Hydra* to *Homo sapiens* (Fig. S5), perhaps suggesting that these sites have unique functional roles or that dual phosphorylation of both sites is functionally important. The current data favor the first possibility; unlike Thr-306 autophosphorylation, which prevents binding of both Ca²⁺/CaM and α -actinin, Thr-305 phosphorylation prevents binding of Ca²⁺/CaM, but not α -actinin (Fig. 2D). Unfortunately, the specific role of each site in controlling interactions with α -actinin is not recapitulated by T305D and T306D mutations, which individually disrupt CaMKII binding to both Ca²⁺/CaM and A2-CTD.⁴ Consequently disruption of CaMKII targeting to dendritic spines by a Thr-305 to Asp mutation (7, 13), may be due to loss of interaction with α -actinin, especially because Thr-305/6 phosphorylation appears to have relatively modest effects on direct interactions with GluN2B or other binding partners (21, 52). Moreover, the effects of mutating Thr-305 and Thr-306 to Ala (or Val) to remove both autophosphorylation sites and stabilize synaptic targeting, which appears to dictate the role of CaMKII in regulating synaptic strength and plasticity (5–7, 13), may result from stabilized interactions with Ca²⁺/CaM and/or α -actinin. Our data suggest that Thr-306 phosphorylation makes a specific contribution to modulating CaMKII that is mediated by disruption of α -actinin interactions and that is distinct from the effects of phosphorylation at Thr-305. Thus, it will be important to assess the specific phosphorylation at both Thr-305 and Thr-306 and the interactions with α -actinin to understand CaMKII regulation in cells.

CaMKII Targeting to Actin Cytoskeleton and NMDA-type Glutamate Receptors—Unlike certain CaMKII β splice variants (53–56), CaMKII α homomers are not directly targeted to F-actin (54). Nevertheless, postsynaptic localization of CaMKII α , the major isoform present in many forebrain neurons (57), is dependent on an intact F-actin cytoskeleton (23). This is consistent with the α -actinin-dependent targeting of CaMKII α to F-actin (Fig. 3) and the fact that CaMKII α associates with multiple α -actinin isoforms in brain (21, 31, 32) (Fig. 4). Thus, α -actinin plays an important role in targeting homomeric CaMKII α

holoenzymes that is independent of direct interactions between CaMKII and F-actin. However, a recent study from our laboratory indicates that at least one more F-actin-binding protein (spinophilin) is involved in CaMKII targeting to F-actin (58).

Activated CaMKII translocates to dendritic spines and binds NMDAR GluN2B subunits (16, 49, 59). CaMKII interactions with GluN2B are critical for synaptic plasticity, learning, and memory (14, 15, 17, 19). NMDAR GluN1 and GluN2B subunits, but not GluN2A, also interact with α -actinin (27), tethering NMDARs to the actin cytoskeleton to modulate channel activity (29, 30). Pharmacological destabilization of F-actin disrupts the colocalization of both α -actinin and CaMKII with synaptic NMDARs (22, 23). We found that α -actinin selectively stabilizes CaMKII targeting to GluN2B-NMDARs (Fig. 5A), apparently by the combined effects of activating CaMKII to interact directly with GluN2B and of bridging indirect interactions with GluN2B and/or GluN1. The trend for α -actinin to reduce CaMKII targeting to GluN2A-NMDARs may be explained by the lack of α -actinin interaction with GluN2A, the low affinity of direct CaMKII-GluN2A interactions, and/or competition between CaMKII and α -actinin for binding GluN1 (27, 59, 60). Thus, α -actinin may selectively target CaMKII to specific subpopulations of neuronal NMDARs.

Differential Modulation of Glutamate Receptor Phosphorylation—In addition to targeting CaMKII, the CTD of α -actinin has complex effects on CaMKII activity, not only *in vitro* (Fig. 1) but also toward physiologically relevant substrates in intact cells (Fig. 6). Phosphorylation of Ser-831 in GluA1 subunits increases the conductance of synaptic AMPARs during LTP (61, 62), and we found that α -actinin suppressed CaMKII phosphorylation of Ser-831 (Fig. 6A). GluA1 is considered to be a “pure” S-site substrate and α -actinin failed to directly activate S-site substrate phosphorylation (Fig. 1C). Therefore, we posit that α -actinin suppresses GluA1 phosphorylation by competing for Ca²⁺/CaM binding to the regulatory domain, in agreement with prior findings (18). In contrast, CaMKII is a negative-feedback modulator of Ca²⁺ influx via GluN2B-NMDARs (35) and α -actinin enhanced CaMKII phosphorylation of Ser-1303 in GluN2B NMDAR subunits (Fig. 6B). Interestingly, α -actinin can form a ternary complex with CaMKII and densin (21, 32), and densin contains a novel CaMKII inhibitory domain that effectively blocks phosphorylation of GluA1 but not GluN2B (38). Thus, densin and α -actinin may collaborate to target neuronal CaMKII, favoring phosphorylation of the associated GluN2B-NMDARs at the expense of GluA1-AMPA receptors. In this way, α -actinin may act as a PSD “phosphostat”, maintaining basal phosphorylation of a subset of CaMKII targets to control synaptic activity. It will be important to investigate the impact of α -actinin isoforms on the phosphorylation of CaMKII substrates at synapses and in other subcellular compartments.

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⁴ N. Jalan-Sakrikar, unpublished observations.

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