Mechanism and Regulation of Calcium/Calmodulin-dependent Protein Kinase II Targeting to the NR2B Subunit of the N-Methyl-D-aspartate Receptor*

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CaMKII is a family of ubiquitous, calcium/calmodulin-dependent kinases with broad substrate specificity (1). The α and β isoforms are especially abundant in brain, constituting as much as 2% of total protein in the hippocampus (2). There is now overwhelming evidence that CaMKII is central to the mechanism of hippocampal, NMDA receptor-dependent long-term potentiation (LTP), a widely studied cellular model of learning and memory. Reduction of CaMKII activity by pharmacological or genetic means impairs LTP (3), whereas injecting or overexpressing CaMKII increases synaptic strength, which occludes and is occluded by electrically induced LTP (4, 5). Crucial to its function in LTP and spatial learning (6, 7), CaMKII undergoes rapid autophosphorylation following NMDA receptor-mediated calcium influx at a specific residue in its autoregulatory domain (Thr286 in the α isoform of CaMKII). This autophosphorylation renders the kinase calcium-independent and has been proposed as a form of molecular memory (8). In support, recent in vitro studies show that CaMKII autophosphorylation permits integration of oscillating calcium signals (9).

We have recently demonstrated a second role for Thr286 autophosphorylation, namely in promoting translocation of CaMKII to postsynaptic densities (PSDs) (10), cytoskeletal scaffolds for the neurotransmitter receptor, ion channels, and their regulators. The search for proteins that target Thr286-autophosphorylated CaMKII (P-T286/CaMKII) to the PSD initially identified a 190-kDa binding activity (11), corresponding in size to the highly PSD-enriched NR2A and NR2B subunits of the NMDA receptor. Indeed, we recently showed that NR2B is a binding protein for P-T286/CaMKII and isolated a CaMKII-NMDA receptor complex from PSDs (12). Subsequently, other laboratories implicated NR2A and NR2B (13, 14) and NR1 and NR2B (15) as CaMKII-binding proteins. In this report, we identify amino acids critical for CaMKII binding in NR2B and investigate the regulation of CaMKII targeting to NR2B in vitro and in cells.

EXPERIMENTAL PROCEDURES

Materials

The phospho-Thr286-specific CaMKII antibody was a generous gift from Said Goueli (Promega). The recombinant γ1 isofrom of the protein phosphatase 1 catalytic subunit was generously provided by Dr. E. Lee (New York Medical College, Valhalla, NY). Sources of other materials are indicated below.

Construction, Mutagenesis, and Expression of cDNAs

NR2B Mutants—Fragments of the rat NR2B cDNA were amplified by polymerase chain reaction (PCR) with primers incorporating 5′-BamHI and 3′-EcoRI sites and subcloned into pGEX-2T (Amersham Pharmacia Biotech) for expression of glutathione S-transferase (GST) fusion proteins. All NR2B mutants were generated in the context of the NR2B(1260–1339) sequence, which includes the core CaMKII-binding domain (see Fig. 2) flanked by AluNI and Ndel sites, allowing for single-step ligation of the mutated fragment into the full-length

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NR2B cDNA (in a cytomegalovirus promoter-driven mammalian expression vector). Point mutants were generated by PCR using Ffl Turbo polymerase (Stratagene) and sense and antisense primers harboring the mutation as well as diagnostic silent restriction sites according to instructions supplied with the QuickChange kit (Stratagene). A cassette-based approach was used for the construction of internal deletion mutants and the B2A mutant. After disruption of the 3'-EcoRI site used for subcloning the NR2B-(1260–1339) fragment, unique silent restriction sites were introduced by PCR (see above) at the following NR2B amino acids: 1286–1288 (EcoRI), 1297–1298 (HindIII), and 1308–1310 (BglII). Mutagenic sense and antisense primers with compatible overhangs were ligated into cassettes generated by cutting the NR2B construct with two of the three restriction enzymes. Since NR2B-(1310–1339) includes at least one in vitro phosphorylation site for CaMKII in addition to Ser1303 (data not shown), GST-NR2B mutants analyzed for Ser1303 phosphorylation were truncated to NR2B-(1260–1316) by digestion with SphI and NdeI and fill in/religation. GST-NR2B fusion proteins were bacterially expressed and purified on glutathione-Sepharose according to standard protocols.

**CaMKII** Mutants—The CaMKII-(1–420) truncation mutant was PCR-amplified from the murine CaMKIIα cDNA with a sense primer incorporating a BamHI site and an antisense primer containing an EcoRI site and in-frame stop codon. The Δ380–420 internal deletion mutant was generated by three-step “loop-out” PCR utilizing primers spanning the deletion. Mutagenic cDNAs were ligated into the pVL1393 baculovirus transfer vector (Invitrogen). Sf9 cells were infected with recombinant baculovirus, and protein was expressed and purified by calmodulin-agarse affinity chromatography as described (11). Point mutants of murine CaMKIIα were generated by PCR as described for NR2B mutants and subcloned into the pME18S mammalian expression vector (chimeric simian virus 40/reovirus (SRa) promoter-driven; DNAX). 

**Mitochondrion-targeting Protein (MTP)—** The basis of this multidayon fusion protein is the mammalian green fluorescent protein (GFP) expression vector pEGFP-N1 (CLONTECH). The GST coding sequence including the C-terminal multiple cloning site, but excluding the stop 3′-endonuclease (16), and a Myc epitope had previously been removed by fill in/religation, to create a GST-GFP fusion. The basis of this multidayon fusion protein is the mammalian green fluorescent protein (GFP) expression vector pEGFP-N1 (CLONTECH). The GST coding sequence including the C-terminal multiple cloning site, but excluding the stop 3′-endonuclease (16), and a Myc epitope had previously been removed by fill in/religation, to create a GST-GFP fusion. The basis of this multidayon fusion protein is the mammalian green fluorescent protein (GFP) expression vector pEGFP-N1 (CLONTECH). The GST coding sequence including the C-terminal multiple cloning site, but excluding the stop 3′-endonuclease (16), and a Myc epitope had previously been removed by fill in/religation, to create a GST-GFP fusion. The basis of this multidayon fusion protein is the mammalian green fluorescent protein (GFP) expression vector pEGFP-N1 (CLONTECH). The GST coding sequence including the C-terminal multiple cloning site, but excluding the stop 3′-endonuclease (16), and a Myc epitope had previously been removed by fill in/religation, to create a GST-GFP fusion.

Oligonucleotides encoding a mitochondrion-targeting sequence, the 15 amino-terminal amino acids of hexokinase I (16), and a Myc epitope tag were ligated into N-terminal Nhel and BglII sites, resulting in a hexokinase-Myc-GST-GFP fusion cDNA. Wild-type or mutant CaMKII-binding domains in the context of NR2B-(1260–1316) were ligated into BamHI and EcoRI sites between GST and GFP coding sequences. The resulting hexokinase-Myc-GST-NR2B-GFP fusion plasmid resulted in the expression of a 60-kDa protein and mitochondrial-localized GFP fluorescence (see Fig. 7A), demonstrating that the protein was expressed intact in cells. Transfection of plasmids encoding CaMKII and NR2B into COS cells resulted in the expression of proteins of the correct size, levels, and similar to those of the wild type. Sequences of all constructs were verified using an ABI 310 fluorescence sequencer at Center for Molecular Neuroscience, Vanderbilt University Medical Center.

**Overlay Analysis of NR2B Mutants**

Recombinant murine CaMKIIα was autophosphorylated at Thr286 in the presence of calcium/calmodulin and [γ-32P]ATP (20–40,000 cpm/pmole) to a stoichiometry of 0.1–0.4 mol/mol and desalted as described (11). A 50 μl (50 μg/ml) fraction was used to express GST-NR2B-(1260–1339) wild-type and mutant proteins, and lysates (20 μg/lane) separated by SDS-polyacrylamide gel electrophoresis and blotted onto nitrocellulose were analyzed for binding of 200 nm [32P]CaMKII by overlay (11, 12), a concentration close to the K0 for binding to wild-type NR2B (12). Bound [32P]CaMKII was quantified by a PhosphorImager (Molecular Dynamics, Inc.) after the protein amount, detected by GST primary (Sigma) and iodinated secondary (Amersham Pharmacia Biotech) antibodies on duplicate blots.

**Microtiter Plate Solution Binding** This solution binding assay is a modification of the N[3H]-coated microtiter plate assay previously described (12) using GST fusion proteins adsorbed to glutathione-coated 96-well plates (Pierce) as the binding surface. Briefly, plates were adsorbed for 2–16 h with GST fusion proteins at room temperature or 4 °C (25 μg/ml, 200 μl/well, ~50% of binding capacity) in wash buffer (5 mg/ml bovine serum albumin, 200 mM NaCl, 50 mM Tris, pH 7.5, 0.1% Tween 20, 5 mM β-mercaptoethanol, and 0.1 mM EDTA). After extensive washes, 200 μl/well [32P]-T286 CaMKIIa diluted in wash buffer was added to allow binding to the tethered fusion protein for 2 h at room temperature, followed by 10–12 more washes. Bound [32P]-T286 CaMKII was solubilized in 1% SDS, 0.2 mM NaOH, and 10 mM EDTA and quantified by liquid scintillation counting. Non-specific binding to GST alone (5–20% of the total, same as wash buffer without GST) was subtracted from total binding to obtain specific binding.

**CaMKII-NR2B Dissociation Assays**

[32P]-T286 CaMKIIa and GST-NR2B-(1260–1316) wild-type or S130A fusion protein were incubated (30 min, 4 °C) at 1–2 μM each in binding buffer (200 mM NaCl, 50 mM Tris, pH 7.5, 0.25 mg/ml bovine serum albumin, 0.1% Triton X-100, 1 mM dithiothreitol, and 1 mM EDTA). After addition of 0.1 volume of a 50% glutathione-agarose slurry and 15 min of continued incubation, CaMKII-NR2B complexes were recovered by brief centrifugation and washing in binding buffer. The agarose slurry was resuspended in detergent buffer (0.5 ml of binding buffer with NaCl concentration reduced to 100 μM to permit efficient phosphorylation or dephosphorylation) supplemented with Mg-ATP or protein phosphatase as described in the figure legends and rotated at 25 or 30 °C. Aliquots were removed at the indicated time points and analyzed for soluble and glutathione-agarose-bound CaMKII by immunoblotting and/or autoradiography. [32P]-T286 CaMKIIa deletion was quantified by adjusting aliquots to 20% (w/v) trichloroacetic acid and liquid scintillation counting the supernatant after high-speed microcentrifugation.

**HEK293 Cell Colocalization**

HEK293 cells were seeded on coverslips (no. 1) in 35-mm dishes, transfected at 40–70% confluency with a total of 4–6 μg/dish DNA (2 μg CaMKIIα expression plasmid plus either 2 μg GST plasmid or 2 μg of each NR1a and NR2B cytomegalovirus promoter plasmids) using TransIT-LT1 transfection reagent (Pansera) according to the manufacturer's instructions, and grown for 48 h in minimal essential medium with 10% fetal bovine serum and 1 mM glutamine. In experiments with MTP, dishes were either immediately fixed for immunofluorescence analysis or first incubated for variable amounts of time with 2 μg calcium ionophore A23187 (Sigma) in growth medium. When NMDA receptor subunits were transfected, the growth medium was supplemented with the NMDA receptor antagonist 2-amino-5-phosphonovaleric acid (APV; 1 mM), and cells were washed and incubated in Mg2+-free Hanks' balanced saline buffered with 20 mM Hepes, pH 7.5, containing 2 mM CaCl2 and either 50 μM APV or NMDA/Glycine (100/10 μM). In contrast, in prior to fixation, cultures were treated with 50 μM glycine/methanol for 10–15 min at 25 °C and processed for immunofluorescence using 1:500 antibody dilutions of goat anti-CaMKII antibody (11) and either mouse anti-Myc tag or rabbit anti-NR2A/B (Chemicon International, Inc.) antibody as described (12, 17). Cultures were randomized and coded prior to sampling digital images on a confocal microscope, so the degree of colocalization between signals for CaMKII and MTP or NR2B could be estimated blindly. Cells (15–25/dish) were assigned a colocalization score from 0 to 4: 0, mutual exclusion; 1, coincidental overlap; 2 and 3, increasing degrees of colocalization; and 4, complete overlap of labels (12).

**RESULTS**

CaMKII binding to NR2B is mediated by 50 amino acids (positions 1260–1309) in the NR2B C terminus (12). To investigate whether CaMKII binding to intact PSDs and to NR2B occur by similar mechanisms, co-sedimentation binding experiments (10) were performed in which [32P]-T286 CaMKIIα was allowed to interact with isolated native PSDs in the presence of increasing concentrations of NR2B-(1260–1309) fused to GST. GST-NR2B-(1260–1309) potently (IC50 50 nM) inhibited co-sedimentation of CaMKII with PSDs (Fig. 1B). Inhibition was specific to NR2B because a GST fusion protein with the corresponding region of NR2A, which does not bind appreciably to CaMKII (12), had no effect. Although it is possible that NR2B allosterically interferes with the CaMKII/PSD association, we consider it more likely that a single domain in CaMKII interacts with both PSDs and NR2B since the two binding events share a dependence on CaMKII autophosphorylation and have similar affinities (10, 12). The 30% residual binding observed at
the highest NR2B concentrations could either be nonspecific (there is no meaningful blank for this assay) or reflect CaMKII associating with PSDs via a separate mechanism.

Analysis of the CaMKII-binding Domain in NR2B—To further elucidate the molecular determinants for CaMKII binding in NR2B, we initially narrowed down the CaMKII-binding domain by constructing a series of overlapping GST fusion proteins. Binding of [32P-T286]CaMKIIα by overlay depended on the presence of NR2B residues 1290–1309 (Fig. 2A), which thus constitute the “core” CaMKII-binding domain. However, we cannot formally rule out redundant stabilizing effects of NR2B residues 1260–1289 and 1310–1339, even though deleting either flanking region by itself had little effect on CaMKII binding. To further define the core domain, small internal deletion mutants were analyzed in the context of NR2B-(1260–1339). Whereas deletion of residues C-terminal of Ser1303 (A1304–1307 and A1306–1309) had a modest to no effect, incremental deletions of N-terminal amino acids from positions 1291 to 1296 reduced CaMKII binding by up to 75% (Fig. 2B).

Since CaMKII binding to NR2A-(1255–1298) is 10 times weaker than to NR2B-(1260–1309) and NR2B-(1260–1339) by overlay (Fig. 2B) (12), nonconserved amino acids in NR2B must play a critical role in the high-affinity interaction with CaMKII. Interestingly, nine amino acids within the core CaMKII-binding domain of NR2B (positions 1290–1309) are identical in NR2A-(1279–1298) (Fig. 2B), including residues surrounding a high-affinity CaMKII phosphorylation site at Ser1303 in NR2B (18). Homology-scanning or “reversal” mutations were generated to probe the role of residues unique to NR2B. Replacing Lys1292 or Arg1299 with the corresponding residues in the NR2A sequence (reversal mutants), and mutations of the CaMKII phosphorylation site Ser1303 (18) and more N-terminal residues that are part of the consensus CaMKII phosphorylation site (Arg1300 and Leu1299) (19), who noted a preference of CaMKII for substrates containing Gln at position 2, replacing Gln1301 with Ala in NR2B reduced CaMKII binding by 50%.

FIG. 1. Inhibition of CaMKII binding to PSDs by NR2B. [32P-T286]CaMKIIα (100 nM) was incubated with purified PSDs (100 μg/ml) in the presence of the indicated concentrations of GST-NR2A-(1255–1298) or GST-NR2B-(1260–1309). CaMKIIPSD complexes were recovered by centrifugation through a sucrose cushion, and bound CaMKIIα was quantified by liquid scintillation counting. Data are representative of three similar experiments.

FIG. 2. Identification of critical amino acids in the CaMKII-binding domain of NR2B. A, the NR2B fragments indicated in the bar diagram were expressed as GST fusion proteins and assayed for binding of [32P-T286]CaMKIIα (200 nM) by overlay. Data were normalized to GST-NR2B-(1260–1339) and are plotted as means ± S.E. of three to four independent experiments. The region of NR2B identified as indispensable for CaMKII binding (core domain, residues 1290–1309) is indicated by hatching. B, the indicated mutations (boxed in black) in the context of GST-NR2B-(1260–1339) were analyzed by [32P-T286]CaMKIIα overlay. Binding to GST-NR2A-(1255–1298) wild-type (w.t.) fusion protein, which contains the region homologous to the core CaMKII-binding domain in NR2B (identical residues are shown in gray), is shown for comparison. Mutations are grouped into internal (Int.) deletion mutants, mutants in which NR2B amino acids were replaced with corresponding residues in the NR2A sequence (reversal mutants), and mutations of the CaMKII phosphorylation site Ser1303 (18) and more N-terminal residues that are part of the consensus CaMKII phosphorylation site (Arg1300 and Leu1299) (19). Plotted are means ± S.E. of three to six experiments expressed as percent binding to GST-NR2B-(1260–1339) wild-type fusion protein. C, the indicated GST-NR2B-(1260–1316) fusion proteins were adsorbed to glutathione-coated 96-well plates (5 μg/well) and incubated with the indicated concentrations of [32P-T286]CaMKIIα; bound ligand was quantified by liquid scintillation counting. Data are representative of four similar experiments.
To verify that critical mutations affect CaMKII binding, as opposed to the ability of fusion proteins to renature on the blot prior to CaMKII overlay, solution-phase binding assays were performed with native GST-NR2B fusion proteins. Whereas the S1303A mutant bound \([^{32}P]-T286\)CaMKII similar to the wild type, the R1300Q, R1300E, and B2A mutants were severely binding-impaired (Fig. 2C and data not shown), confirming results from overlay experiments. None of the NR2B mutants displayed specific binding to CaMKII autophosphorylated at Thr\(^{305/306}\) in the absence of Ca\(^2+\)/calmodulin (data not shown), in agreement with previous results demonstrating that interaction with NR2B requires autophosphorylation at the autonomy site, Thr\(^{286}\) (12).

The finding that NR2B amino acids important for the interaction with CaMKII include the substrate recognition motif (Ser\(^{1303}\), Arg\(^{1300}\), and Leu\(^{1299}\)) prompted us to examine whether peptide substrates are effective competitors for the CaMKII/NR2B interaction. Syntide-2, derived from a CaMKII autoregulatory domain surrounding Thr\(^{286}\) in CaMKII, was an effective competitor, with an IC\(_{50}\) of 10 \(\mu\)M similar to the \(K_m\) for phosphorylation (21). A possible explanation for this dramatic difference in inhibitory potency is provided by an alignment of the two peptides with the corresponding sequence in NR2B, revealing more extensive homology of NR2B to autocamtide-2 compared with syntide-2 N-terminal of the phosphorylated residue (Fig. 3A). In particular, autocamtide-2 contains residues corresponding to the mutation-sensitive NR2B residues Gln\(^{1303}\) and Arg\(^{1299}\), but syntide-2 does not.

**Modulation of the CaMKII/NR2B Interaction**—Since our mutational analysis implicated an important role for NR2B Ser\(^{1303}\), the effect of Ser\(^{1303}\) phosphorylation on CaMKII binding was investigated. In agreement with Omkumar et al. (18), GST-NR2B-(1260–1309), but not GST-NR2B-(1260–1299), could be phosphorylated by CaMKII to \(-1\) mol/mol stoichiometry (Fig. 4). Furthermore, mutating Ser\(^{1303}\) to Ala in the context of GST-NR2B-(1260–1316) completely abrogated phosphorylation (data not shown). Stoichiometric phosphorylation of GST-NR2B-(1260–1309) reduced CaMKII interaction by overlay by 59 ± 6% \((n = 3)\) (Fig. 4).

To investigate whether NR2B Ser\(^{1303}\) phosphorylation not only inhibited initial association of CaMKII, but also could dissociate CaMKII previously bound to NR2B, release of CaMKII from CaMKII-GST-NR2B complexes was monitored with or without ATP. In the absence of ATP, CaMKII remained stably associated with GST-NR2B for \(>1\) h under these conditions (Fig. 5). Addition of ATP led to stoichiometric phosphorylation of NR2B Ser\(^{1303}\) by 5 min, revealed by the appearance of a lower mobility band (Figs. 4 and 5A, compare asterisks). Phosphorylation of NR2B was accompanied by dissociation of CaMKII, albeit incomplete and with a protracted time course (10% released after 75 min). No ATP-dependent release of CaMKII from complexes with the NR2B S1303A mutant was detected (Fig. 5B), demonstrating that this dissociation is a consequence of NR2B Ser\(^{1303}\) phosphorylation, as opposed to continued, calcium/calmodulin-independent autophosphorylation of CaMKII.

Autophosphorylation of CaMKII at Thr\(^{286}\) is required for high-affinity binding to PSDs and to NR2B (10, 12). We therefore investigated the reversibility of the CaMKII/NR2B interaction by dephosphorylation of Thr\(^{286}\). Incubation of kinase-NR2B complexes with a 2 \(\mu\)g/ml concentration of the catalytic subunit of protein phosphatase 1 resulted in \(>70%\) dephosphorylation of \([^{32}P]-T286\)CaMKIIa in 2 h, measured as release of trichloroacetic acid-soluble radioactivity (Fig. 5C), as well as a decrease in immunoreactivity with a phospho-Thr\(^{286}\)-specific CaMKII antibody (data not shown). Paralleling the time course of dephosphorylation, \(-20%\) of the CaMKII-NR2B complexes dissociated during this time period. Higher protein phosphatase 1 concentrations (10 \(\mu\)g/ml) led to release of up to 40% CaMKII under otherwise identical conditions (data not shown). Both dephosphorylation and dissociation were blocked by inhibiting protein phosphatase 1 with microcystin, demonstrating that Thr\(^{286}\) dephosphorylation promotes release of CaMKII from NR2B.

*Monomeric CaMKII Mutants Do Not Bind to NR2B*—In an effort to delineate domains in CaMKII important for the interaction with NR2B, we constructed deletion mutants of CaMKIIα. Two mutations in the C-terminal domain required...
by colorimetric immunoblotting, and release was quantified by densitometry
and dephosphorylation.

A

The absence or presence of 10 mM 

CaM with GST-NR2B-(1260–1316) (0.5

M) and Mg

ATP 

(autoph., autophosphorylation).

Moreover, a detailed comparison of GST-NR2B-(1260–1316) with comparable efficiency (Fig. 6B).

B

Dissociation of the CaMKII-NR2B complex by phosphorylation and dephosphorylation. A, a preformed complex of [32P-T286]CaMKII and GST-NR2B-(1260–1316) on glutathione-agarose was incubated at 30 °C with mixing with or without nonradioactive MgATP (0.5 mM). Aliquots were removed at the indicated times, and the agarose-associated and soluble [32P-T286]CaMKII enzymes were analyzed by SDS-polyacrylamide gel electrophoresis/autoradiography (upper panel). GST-NR2B in the agarose pellet was stained with Coomassie Blue (lower panel), revealing a gel mobility shift (asterisk) after 5 min in the presence of ATP. B, a complex of [32P-T286]CaMKII with either GST-NR2B-(1260–1316) wild-type (w.t.) or S1303A mutant fusion protein was analyzed for dissociation as described for A, except that CaMKII in the supernatant and glutathione-agarose pellet was detected by immunoblotting. C, shown is the dissociation of a [32P-T286]CaMKII-GST-NR2B-(1260–1316) wild-type complex in the presence of the protein phosphatase 1 (PP1) catalytic subunit (2 μg/ml) in the absence or presence of 10 μM microcystin-LR. CaMKII was detected by colorimetric immunoblotting, and release was quantified by densitometry. Dephosphorylation of [32P-T286]CaMKII was quantified in parallel as trichloroacetic acid-soluble radioactivity as percent of total. Data are representative of two to three similar experiments.

Fig. 5. Dissociation of the CaMKII-NR2B complex by phosphorylation and dephosphorylation. A, a preformed complex of [32P-T286]CaMKII and GST-NR2B-(1260–1316) on glutathione-agarose was incubated at 30 °C with mixing with or without nonradioactive MgATP (0.5 mM). Aliquots were removed at the indicated times, and the agarose-associated and soluble [32P-T286]CaMKII enzymes were analyzed by SDS-polyacrylamide gel electrophoresis/autoradiography (upper panel). GST-NR2B in the agarose pellet was stained with Coomassie Blue (lower panel), revealing a gel mobility shift (asterisk) after 5 min in the presence of ATP. B, a complex of [32P-T286]CaMKII with either GST-NR2B-(1260–1316) wild-type (w.t.) or S1303A mutant fusion protein was analyzed for dissociation as described for A, except that CaMKII in the supernatant and glutathione-agarose pellet was detected by immunoblotting. C, shown is the dissociation of a [32P-T286]CaMKII-GST-NR2B-(1260–1316) wild-type complex in the presence of the protein phosphatase 1 (PP1) catalytic subunit (2 μg/ml) in the absence or presence of 10 μM microcystin-LR. CaMKII was detected by colorimetric immunoblotting, and release was quantified by densitometry. Dephosphorylation of [32P-T286]CaMKII was quantified in parallel as trichloroacetic acid-soluble radioactivity as percent of total. Data are representative of two to three similar experiments.

for formation of a holoenzyme consisting of 10–12 subunits (22, 23) were expressed in insect cells. The 1–420 mutant lacks the C-terminal 58 amino acids, whereas the Δ380–420 mutant lacks 41 residues in the middle of the oligomerization domain (Fig. 6A). As expected, both deletions disrupt holoenzyme formation since the mutants migrated as monomers by gel filtration chromatography and sucrose gradient centrifugation (data not shown).

Both mutants underwent calcium/calmodulin-dependent autophosphorylation at Thr286 and attained levels of autonomous activity similar to those of the wild type (30–50% of calcium/calmodulin-dependent activity). However, whereas autophosphorylation of wild-type CaMKII is rapid (seconds), occurring between adjacent subunits of the same holoenzyme (24), maximal autophosphorylation of the monomeric mutants required high enzyme concentration and prolonged incubation (1–3 min) at 30 °C, consistent with an intermolecular reaction. Both mutants displayed normal catalytic activity toward substrate-2 peptide substrate (wild-type: \( K_{\text{cat}} = 12.3 \mu M, K_{\text{cat}} = 324 \) min\(^{-1}\); 1–420: \( K_{\text{cat}} = 13.2 \mu M, K_{\text{cat}} = 236 \) min\(^{-1}\); and Δ380–420, \( K_{\text{cat}} = 14.8 \mu M, K_{\text{cat}} = 348 \) min\(^{-1}\); \( n = 2–3 \)) and phosphorylated GST-NR2B-(1260–1316) with comparable efficiency (Fig. 6B). Moreover, a detailed comparison of GST-NR2B-(1260–1316)
phosphorylation kinetics failed to reveal significant differences between the wild type and the Δ380–420 mutant (wild-type: \( S_{0.5} = 0.80 \pm 0.20 \mu M, K_{cat} = 9.0 \pm 3.0 \text{ min}^{-1}, K_{M/S_{0.5}} = 11.3 \text{ min}^{-1} \mu M^{-1} \); and Δ380–420: \( S_{0.5} = 1.28 \pm 0.25 \mu M, K_{cat} = 10.8 \pm 2.4 \text{ min}^{-1}, K_{cat/S_{0.5}} = 8.4 \text{ min}^{-1} \mu M^{-1}; n = 3 \).)

Although CaMKII oligomerization mutants were catalytically normal, their ability to bind to NR2B was severely compromised. In qualitative GST co-sedimentation assays, GST-NR2B-(1260–1316) pulled down stoichiometric amounts of wild-type CaMKII in an autophosphorylation-dependent manner (Fig. 6C, inset), in agreement with previous data (12). In contrast, only small amounts of the 1–420 mutant were detected in the glutathione-agarose pellet, even though both kinases displayed similar levels of autophosphorylation detected with a phosphate-Thr^{286}-specific CaMKII antibody. Similar data were obtained with the Δ380–420 mutant; neither kinase bound to GST alone (data not shown). Glutathione microtiter plate assays confirmed these results, showing little or no binding of either monomeric \([32P]-T286\)CaMKII to GST-NR2B-coated wells (Fig. 6C).

**Regulation of CaMKII Targeting to NR2B in Cells**—In previous cell transfection studies, CaMKII was shown to colocalize with NR2B, but not with NR2A-containing NMDA receptors. This colocalization depended on receptor agonist treatment, calcium influx, and Thr^{286} autophosphorylation (12). In the present study, we set out to investigate whether the CaMKII-binding domain in NR2B identified in *in vitro* assays is sufficient to localize CaMKII in cells. To this end, a mitochondrion-targeted multidomain fusion protein (MTP) was constructed, containing 15 N-terminal amino acids of hexokinase I that bind to the outer mitochondrial membrane protein porin (16), other domains useful in detecting expression, and a multiple cloning site for insertion of additional sequences (Fig. 7A). MTPs with or without various NR2B inserts were coexpressed with CaMKII in HEK293 cells, and colocalization was assayed by immunofluorescence confocal microscopy (see “Experimental Procedures”). Expression of MTP without an NR2B insert did not affect CaMKII localization; the kinase remained diffusely cytosolic, as evidenced by low colocalization scores (Fig. 7B). Likewise, insertion of NR2B-(1260–1316) into MTP did not cause a redistribution of CaMKII to mitochondria. However, when a MTP containing the NR2B S1303A mutant fragment was expressed, CaMKII assumed a discrete mitochondrial localization, reflected in near-perfect colocalization scores.

To investigate the calcium dependence of translocation, we examined the colocalization of CaMKII to the MTP-NR2B wild-type fusion protein at various times after addition of the calcium ionophore A23187 to the medium. Low but significant calcium-activated phosphatases can also be invoked to explain these results.

The question of whether amino acids identified as important in binding assays with NR2B fusion proteins are also important for the agonist-dependent translocation of CaMKII to NMDA receptor ion channels was addressed next. HEK293 cells were cotransfected with NR1, NR2B, and CaMKII, challenged for 15 min with co-agonists NMDA and glycine or the receptor antagonist APV, fixed, and analyzed for colocalization of CaMKII and NR2B. In agreement with previous results (12), NMDA/glycine treatment led to a 2-fold increase in the CaMKII/NR2B colocalization score compared with APV treatment (Fig. 7D). Changing six amino acids in full-length NR2B to the corresponding residues in NR2A (B2A mutant) completely abolishes the activity-induced increase in CaMKII colocalization, providing a molecular explanation for the inability of NR1/NR2A receptor activation to recruit CaMKII (12). Likewise, the single point mutant R1300E abrogated activity-dependent recruitment of CaMKII into NR2B patches. Intriguingly, but in complete agreement with the MTP data (Fig. 7B), significant colocalization of CaMKII with NR2B S1303A was apparent even in the absence of receptor activation, and optical overlap was further increased by NMDA/glycine treatment. These data further support the notion that NR2B Ser^{1303} phosphorylation is a negative feedback regulator of CaMKII binding. In apparent contrast to the *in vitro* binding experiments showing a 75% reduction of [32P]-T286CaMKII binding to the NR2B S1303D mutant (Fig. 2B), this mutation transferred into the full-length subunit did not appreciably affect colocalization scores compared with wild-type NR2B. A possible explanation for this discrepancy consistent with the data on mitochondrion-targeted NR2B is that Ser^{1303} is highly phosphorylated in cells.

A previous report suggested that NMDA-dependent translocation of GFP-CaMKII to PSDs in cultured hippocampal neurons does not require autophosphorylation, but that calcium/calmodulin binding to the kinase is sufficient (25). For this reason, we reexamined the autophosphorylation dependence of the CaMKII/NR2B colocalization in cells. A calmodulin binding-defective CaMKII mutant (A302R) (26), an ATP hydrolysis-defective, “kinase-dead” CaMKII mutant (K42R), and a non-phosphorylatable CaMKII mutant (T286A) were not able to translocate to NMDA receptor patches after receptor activation (Fig. 7D), confirming our previous result with the T286A mutant that translocation to NMDA receptors requires Thr^{286} autophosphorylation (12).

**DISCUSSION**

*The CaMKII-binding Domain in NR2B*—This report presents the first detailed characterization of a targeting domain for CaMKII, an abundant kinase important in learning and memory. The NR2B subunit of the NMDA receptor is unique in its CaMKII-targeting function since NR1 and NR2A alone cannot specify the subcellular localization of CaMKII (12), although other laboratories have previously reported CaMKII interaction with NR1 and NR2A in *in vitro* (13–15).

A relatively small region in NR2B including amino acids between positions 1290 and 1303 was shown to be critical for CaMKII targeting in intact cells. Data reported by Leonard *et al.* (15) imply the existence of two separate CaMKII-binding domains in the cytosolic tail of NR2B, one between residues 839 and 1120 and the other C-terminal of residue 1120 (presumably corresponding to the domain characterized herein). Our previous domain mapping experiments (12) are not consistent with the existence of the domain between residues 839 and 1120, and the transfection studies in this report demonstrate that residues surrounding the phosphorylation site Ser^{1303} in NR2B are both necessary and sufficient to direct CaMKII localization.
**Mechanism and Regulation of CaMKII Binding to NR2B**

**FIG. 7.** Molecular determinants and regulation of CaMKII targeting to NR2B in cells. **A**, upper panel, diagram of a mitochondrion-targeted multidomain fusion protein (MTP) containing the following domains (not to scale): mitochondrion-targeting sequence (MTS; 15 N-terminal amino acids of hexokinase I), Myc epitope tag, GST, and GFP. A multiple cloning site between the GST and GFP domains allows for insertion of additional sequences, here NR2B-(1260–1316). Lower panel, green fluorescence image of a live primary astrocyte expressing MTP. **B**, HEK293 cells were cotransfected with CaMKII and MTP without insert or with an NR2B-(1260–1316) insert (wild-type (w.t.) or S1303A mutant) and analyzed for colocalization by immunofluorescence microscopy using anti-CaMKII antibodies (red) and anti-Myc tag antibodies (green). Regions of overlap appear yellow in the merged images of cells representative of each condition. Colocalization scores (subjective scale from 0 = mutual exclusion to 4 = perfect overlap) were assigned blindly and are listed as means ± S.E. (number of cells in parentheses) of two to three independent experiments. **C**, HEK293 cells cotransfected with CaMKII and the indicated MTP-NR2B-(1260–1316) fusion protein were treated for various times with 2 μM A23187 calcium ionophore and assayed for colocalization by immunofluorescence. The graph shows data from one experiment representative of three; sample cells are shown below. **D**, activity-dependent targeting of CaMKII to NMDA receptors. HEK293 cells were triply transfected with CaMKII wild-type or mutants), NR1, and NR2B (wild-type or mutants), treated for 15 min with control solution (200 μM APV) or solution containing receptor agonists (100 μM NMDA and 10 μM glycine), fixed, and assayed for immunofluorescence colocalization of CaMKII (red) and NR2B (green). The bar graph shows colocalization scores (mean ± S.E.) from the number of cells listed on top (two to six independent experiments). CaMKII colocalizes with NR2B S1303A, but not with NR2B R1300E, in discrete puncta (arrowheads, yellow in merged image), as shown in images of representative cells that received NMDA/glycine treatment on the right. Scale bars = 10 μm. *, significant increase (p < 0.0001) by two-tailed Student's t test compared with control (wild-type NR2B under control conditions).
Furthermore, we have not been able to demonstrate colocalization of CaMKII with MTPs containing residues 839–1120 of NR2B (data not shown).

Although the degree to which NR2B itself contributes to the interaction of CaMKII with PSDs is unclear, competition experiments suggest that the mechanisms of CaMKII binding to NR2B and to intact PSDs are similar (Fig. 1). An understanding of the targeting determinants in NR2B may thus aid in identifying additional CaMKII-targeting proteins involved in physiological and pathophysiological translocation of CaMKII to PSDs and other cytoskeletal structures (10, 25, 27–29).

**Mechanism of CaMKII Interaction with NR2B**—The mechanism of NR2B interaction with CaMKII has not been entirely resolved. Residues N-terminal to Ser\textsuperscript{1303} are clearly important to the interaction, including several that are not conserved in the corresponding region of NR2A (e.g., Lys\textsuperscript{1292} and Arg\textsuperscript{1299}). However, the residues most sensitive to single point mutations (Ser\textsuperscript{1303}, Arg\textsuperscript{1300}, and Leu\textsuperscript{1298}) are key determinants of a consensus CaMKII phosphorylation site (I/L)RX(S/T) (19). These data might be interpreted to suggest that CaMKII and NR2B interact via an enzyme/substrate mechanism. However, several pieces of data suggest that the interaction is more complex. For example, autophosphorylation at Thr\textsuperscript{286} is essential for interaction with NR2B, but substrate phosphorylation can be stimulated by calcium/calmodulin binding in the absence of autophosphorylation (e.g., in a T286A mutant (21, 30)).

Furthermore, monomeric CaMKII mutants that phosphorylate substrate phosphorylation site, potently competes for binding (Fig. 3)

**Phosphatases add another layer of complexity to the regulation of the CaMKII/NR2B association.** Although in vitro data suggest a negative role for protein phosphatases by returning CaMKII to its non-phosphorylated state (Fig. 5C), the in vivo situation is undoubtedly more complex since dephosphorylation of NR2B Ser\textsuperscript{1303} is expected to increase the affinity for CaMKII. Thus, the lifetime of the kinase-channel complex appears to be controlled by the balance of phosphatase activities toward CaMKII Thr\textsuperscript{286} and NR2B Ser\textsuperscript{1303}. Whereas CaMKII Thr\textsuperscript{286} is dephosphorylated by protein phosphatase type 1 or 2A depending on its subcellular localization (33), the identity of the NR2B Ser\textsuperscript{1303} phosphatase is unknown.

**Induction of LTP triggers persistent CaMKII autophosphorylation that is independent on NMDA receptor activation (34–36).**

**Somatic activity also induces translocation of CaMKII to PDSs (10, 25) and increases co-immunoprecipitation of NMDA receptor subunits with CaMKII (15).** Data presented here provide important insights into the molecular mechanism for the association of CaMKII with NMDA receptors. These studies also have implications for our understanding of CaMKII association with the PSD, although other proteins may be involved in addition to NR2B. The reversibility of the CaMKII/NR2B interaction by phosphorylation/dephosphorylation suggests that CaMKII targeted to the PSD structure may not remain permanently anchored to NR2B. Instead, NR2B could act to increase the local concentration of the kinase in the cytoskeletal lattice of the PSD, where it is more likely to be activated by subsequent calcium entry through the NMDA receptor. In addition, CaMKII released from NR2B may diffuse to other important postsynaptic substrates, such as the GluR1 glutamate receptor subunit (10, 36), to bring about long-term changes in synaptic efficacy (37). Future studies will also address whether CaMKII affects NMDA receptor activity, through either binding to the NR2B C terminus or phosphorylation of Ser\textsuperscript{1303}.
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