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

Received for publication, February 22, 2000, and in revised form, April 6, 2000 Published, JBC Papers in Press, April 7, 2000, DOI 10.1074/jbc.M001471200

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Calcium influx through the N-methyl-D-aspartate (NMDA)-type glutamate receptor and activation of calcium/calmodulin-dependent kinase II (CaMKII) are critical events in certain forms of synaptic plasticity. We have previously shown that autophosphorylation of CaMKII induces high-affinity binding to the NR2B subunit of the NMDA receptor (Strack, S., and Colbran, R. J. (1998) J. Biol. Chem. 273, 20689–20692). Here, we show that residues 1290–1309 in the cytosolic tail of NR2B are critical for CaMKII binding and identify by site-directed mutagenesis several key residues (Lys1292, Leu1296, Arg1299, Arg1300, Gln1301, and Ser1303). Phosphorylation of NR2B at Ser1302 by CaMKII inhibits binding and promotes slow dissociation of preformed CaMKII-NR2B complexes. Peptide competition studies imply a role for the CaMKII catalytic domain, but not the substrate-binding pocket, in the association with NR2B. However, analysis of monomeric CaMKII mutants indicates that the holoenzyme structure may also be important for stable association with NR2B. Residues 1260–1316 of NR2B are sufficient to direct the subcellular localization of CaMKII in intact cells and to confer dynamic regulation by calcium influx. Furthermore, mutation of residues in the CaMKII-binding domain in full-length NR2B bidirectionally modulates colocalization with CaMKII after NMDA receptor activation, suggesting a dynamic model for the translocation of CaMKII to postsynaptic targets.

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-T286CaMKII) 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 isoform 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 AflAI and NdeI sites, allowing for single-step ligation of the mutagenized fragment into the full-length fusion expression vector.
NR2B cDNA (in a cytomegalovirus promoter-driven mammalian expression vector). Point mutants were generated by PCR using Phusion 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 (BgIII). 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 SfII and NdeI and fill in/religation. GST-NR2B fusion proteins were bacterially expressed and purified on glutathione-agarose 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 pME185 mammalian expression vector (chimeric simian virus 40/retrovirus (SRα) promoter-driven; DNAX).

Mitochondrion-targeting Protein (MTP)—The basis of this multidomain fusion protein is the mammalian green fluorescent protein (GFP) expression vector pEGFP-N1 (CLONTECH). The GST coding sequence was amplified from the murine CaMKIIα cDNA with a sense primer with a BamHI site and an antisense primer with a HindIII site and in-frame stop codon. The GST coding sequence was ligated into pEGFP-N1, whose EcoRI and BamHI sites had previously been removed by fill in/religation, to create a GST-GFP fusion plasmid.

Oligonucleotides encoding a mitochondrial-targeting sequence, the 15 amino-terminal amino acids of hexokinase I (16), and a Myc epitope tag were ligated into N-terminal multiple cloning sites (MCS), 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 allowed the expression of proteins of the correct size, which are similar to those of the wild type. Sequences of all constructs were verified using an ABI 3100 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/ pmol) to a stoichiometry of 0.1–0.4 mol/mol and desalted as described (11). Aliquots (50 μl) were 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]T-286eCaMKIIα by overlay (11, 12), a concentration close to the Kd of binding to wild-type NR2B (12). Bound [32P]T-286eCaMKIIα was quantified by a PhosphorImager (Molecular Dynamics, Inc.) and normalized to GST protein (Sigma) and iodinated secondary (Amersham Pharmacia Biotech) antibodies on duplicate blots.

Microtiter Plate Solution Binding

This solution binding assay is a modification of the N2+–coated microtiter plate assay previously described (12) using GST fusion proteins adsorbed to glutathione-coated 96-well plates (Fisher) 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 μM well, ~50% of binding capacity) in wash buffer (5 mM 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]-T-286eCaMKIIα diluted in wash buffer was allowed to bind to the tethered fusion protein for 2 h at room temperature, followed by 10–12 more washes. Bound [32P]-T-286eCaMKIIα 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]-T-286eCaMKIIα and GST-NR2B-(1260–1316) wild-type or S1303A 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 distilled water (0.5 ml of binding buffer with NaCl concentration reduced to 100 mM 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-agarse-bound CaMKII by immunoblotting and/or autoradiography. [32P]-T-286eCaMKIIα-dephosphorylation 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% confluence with a total of 4–6 μg/dish DNA (2 μg CaMKII expression plasmid plus either 2 μg GFP plasmid or 2 μg of each NR1a and NR2B cytomegalovirus promoter plasmids) using TransIT-LT1 transfection reagent (Panvera) 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 or first incubated for variable amounts of time with 2 μM 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 Heps, pH 7.5, containing 2 mM CaCl2 and either 50 μM APV or NMDA/glycine (100/10 μM) in the presence of either MTP or a control plasmid. Cells were fixed to permit co-localization analysis. Nonspecific binding to GST alone (5–20% of the total, same as wash buffer without GST) was subtracted from total binding to obtain specific binding.

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]-T-286eCaMKIIα 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. 1A). Inhibition was specific to NR2B because a GST fusion protein with the corresponding region of NR2A, which does not bind appreciably to CaMKII, 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]-T286CaMKIIα 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 (Δ1304–1307 and Δ1306–1309) 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) (10) 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 Ser¹³⁰⁰ in NR2B (18). Homology-scanning or “reversal” mutations were generated to probe the role of residues unique to NR2B. Replacing Lys²⁵⁸ or Arg¹²⁹⁸ with corresponding NR2A residues (K1292Q or R1299N) compromised binding by 30–40% and by 50% in the double mutant. Changing four additional residues to their NR2A counterparts (A1290Q, Q1291F, R1295K, and N1296L) in the B2A mutant led to a 65% reduction in CaMKII binding.

NR2B residues conserved in NR2A were also subjected to mutational analysis. Mutation of the phosphorylation site Ser¹³⁰⁰ to Ala had little effect, whereas introduction of a negatively charged (S1303D) or a hydrophobic side chain (S1303L) severely interfered with the CaMKII interaction (75–80% reduction). NR2B Leu¹²⁹⁸ and Arg¹³⁰⁰ align with the predicted CaMKII substrate recognition motif (V/L)(X)XX(S/T) (19). Replacing Arg¹³⁰⁰ with Glu or Gln diminished binding by >85%, and the L1298A mutation almost completely obliterated the interaction with CaMKII, similar to the R1300Q/S1303D double mutant. As expected, the conservative substitution mutant L1298I displayed CaMKII binding indistinguishable from the wild-type. In agreement with White et al. (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]-T286CaMKIIα (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). CaMKII-PST complex was recovered by centrifugation through a sucrose cushion, and bound CaMKIIα was quantified by liquid scintillation counting. Data are representative of three 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 [γ-32P]-T286CaMKIIα similarly 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 Thr²⁸⁶/³⁰⁶ in the absence of Ca²⁺/calmodulin (data not shown), in agreement with previous results demonstrating that interaction with NR2B requires autophosphorylation at the autonomy site, Thr²⁸⁶ (12).

The finding that NR2B amino acids important for the interaction with CaMKII include the substrate recognition motif (Ser¹³⁰³, Arg¹³⁰⁶, and Leu¹²⁸⁹) prompted us to examine whether peptide substrates are effective competitors for the CaMKII autophosphorylation site in glycogen synthase, at concentrations of up to 400 μM. Two mutations in the C-terminal domain required phosphorylation of 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 Ser¹³⁰³ phosphorylation not only inhibited initial association of CaMKII, but also dissociated 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 Ser¹³⁰³ 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 Ser¹³⁰³ phosphorylation, as opposed to continued, calcium/calmodulin-independent autophosphorylation of CaMKII.

Autophosphorylation of CaMKII at Thr²⁸⁶ 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 Thr²⁸⁶. Incubation of kinase-NR2B complexes with a 2 μg/ml concentration of the catalytic subunit of protein phosphatase 1 resulted in >70% dephosphorylation of [γ-32P]-T286CaMKIIα in 2 h, measured as release of trichloroacetic acid-soluble radioactivity (Fig. 5C), as well as a decrease in immunoreactivity with a phospho-Thr²⁸⁶-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 μg/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 Thr²⁸⁶ 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...
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 synthetic peptide substrate (wild-type: $K_m = 12.3$ μM, $K_{cat} = 324$ min$^{-1}$; 1–420: $K_m = 13.2$ μM, $K_{cat} = 236$ min$^{-1}$; and Δ380–420, $K_m = 14.8$ μM, $K_{cat} = 348$ 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: $S_{0.5} = 0.80 \pm 0.20 \mu M$, $K_{ca} = 9.0 \pm 3.0$ min$^{-1}$, $K_{cat}/S_{0.5} = 11.3$ min$^{-1}$ $\mu M^{-1}$; and Δ380–420: $S_{0.5} = 1.28 \pm 0.25 \mu M$, $K_{cat} = 10.8 \pm 2.4$ min$^{-1}$, $K_{cat}/S_{0.5} = 8.4$ 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 autoposphorylation-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 autoposphorylation detected with a phospho-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 $[^{32}P]$-T286(CaMKII)$\alpha$ mutant 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, with NR2B, but not with NR2A-containing NMDA receptors. 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 colocalization was observed after 2 and 5 min of ionophore treatment, returning to base line after 15 min (Fig. 7B). The R1300Q mutant of NR2B-(1260–1316), which is binding-defective in vitro (Fig. 2, B and C), was not able to target CaMKII to mitochondria at any time point.

These data are consistent with the following interpretation: NR2B Ser$^{1303}$ is substantially phosphorylated in HEK293 cells under basal conditions, and CaMKII autoprophorylation levels are insufficient for targeting to Ser$^{1303}$-phosphorylated NR2B, but sufficient for promoting colocalization with the non-phosphorylatable S1303A mutant. Calcium mobilization increases CaMKII autophosphorylation, allowing it to transiently overcome the inhibitory effect of Ser$^{1303}$ phosphorylation. Alternative, more complex scenarios involving, for example, 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 $[^{32}P]$-T286(CaMKII)$\alpha$ 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 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.

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2 Since double-reciprocal plots consistently yielded nonlinear relationships between $V$ and $S$, true $K_a$ values could not be calculated. Instead, the substrate concentration corresponding to half-maximal velocity ($S_{0.5}$) was obtained by curve-fitting with Hill equations.
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 R1300Q, 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\textsubscript{1303} are clearly important to the interaction, including several that are not conserved in the corresponding region of NR2A (e.g., Lys\textsubscript{1292} and Arg\textsubscript{1299}). However, the residues most sensitive to single point mutations (Ser\textsubscript{1303}, Arg\textsubscript{1300}, and Leu\textsubscript{1298}) are key determinants of a consensus CaMKII phosphorylation site (I/L)XRX(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 on Thr\textsubscript{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 Ser\textsubscript{1303} comparably to the wild-type kinase are unable to form stable complexes with GST-NR2B (Fig. 6).

Our studies using synthetic peptides to compete for the interaction provide some insight into the mechanism. Syntide-2, a “pure” peptide substrate based on a CaMKII phosphorylation site in glycogen synthase, is unable to compete for [P-T286]CaMKII binding to NR2B at 20 times its $K_m$ concentration, arguing that a typical enzyme/substrate interaction is not critical for stable binding. In contrast, and somewhat surprisingly, autocamtide-2, another peptide substrate for CaMKII based on the sequence surrounding the Thr\textsubscript{286} autophosphorylation site, potently competes for binding (Fig. 3B). Our interpretation of this observation rests on the previous demonstration that autocamtide-2 interacts with two distinct sites on the Thr\textsubscript{286}-autophosphorylated catalytic domain of CaMKII (31). The first site (site A) appears to be a conventional substrate-biding site. The second site (site B) is thought to be occupied by the Thr\textsubscript{286} autophosphorylation site within the CaMKII autoinhibitory domain in its non-phosphorylated state. Interestingly, a peptide corresponding to the autoinhibitory domain of CaMKII (residues 281–302) also potently inhibits the CaMKII/NR2B interaction (data not shown). Thus, our hypothesis is that autophosphorylation of CaMKII exposes site B for binding to NR2B. Although the high degree of identity between NR2B residues surrounding Ser\textsubscript{1303} and autocamtide-2 supports this idea, an indirect mechanism is also possible, in which site B occupancy (by autocamtide-2 or the autoinhibitory domain) allosterically occludes a third interaction site for NR2B. A recent mutagenesis study provides insights into the identity of residues that constitute site B and its location in the CaMKII catalytic domain (32), and it will be important to test whether site B mutations disrupt the CaMKII/NR2B interaction.

Although these peptide competitor studies implicate the catalytic domain in the interaction with NR2B, the monomeric CaMKII mutants containing fully functional catalytic domains are unable to form stable stoichiometric complexes with GST-NR2B, although weak interactions can be detected (Fig. 6). It is possible that monomeric mutants are missing amino acids that are making direct contacts with NR2B, in addition to intersubunit contacts in the dodecameric holoenzyme. However, we currently favor the interpretation that CaMKII oligomerization has a permissive role, increasing binding affinities by allowing for concerted binding of multiple catalytic domains in the CaMKII holoenzyme to multiple NR2B subunits. This model suggests an intriguing mechanism by which the CaMKII holoenzyme may be preferentially attracted to membranes with a threshold density of NR2B subunits, present in either the same tetrameric or pentameric NMDA receptor complex or in separate adjacent receptors.

A Dynamic Model for CaMKII/NR2B Association—Regardless of the mechanism of CaMKII/NR2B association, the cell transfection experiments demonstrate that NR2B sequences can efficiently redistribute CaMKII in cells. Moreover, the enhanced targeting of the NR2B S1303A mutant combined with the biochemical data provides strong evidence for a modulatory role of this serine residue. Thus, the CaMKII/NR2B interaction is inversely controlled by two phosphorylation events, i.e., enhanced by CaMKII Thr\textsubscript{286} autophosphorylation and inhibited by NR2B Ser\textsubscript{1303} phosphorylation. However, NR2B phosphorylated at Ser\textsubscript{1303} appears to retain residual affinity for CaMKII, as evidenced by overlay assays (Fig. 4) and by slow dissociation of CaMKII from NR2B after phosphorylation (Fig. 5) and as suggested by the activity-dependent targeting of CaMKII by the NR2B S1303D mutant (Fig. 7D). Furthermore, the extremely low turnover rate ($K_{off}$) of NR2B Ser\textsubscript{1303} phosphorylation by CaMKII (~10 min$^{-1}$ compared with 300 min$^{-1}$ for syntide-2) implies that CaMKII/NR2B complexes exist for several seconds before phosphorylated NR2B can dissociate, even in the presence of a large excess of non-phosphorylated NR2B.

Phosphatases add another layer of complexity to the regulation of the CaMKII/NR2B interaction. 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\textsubscript{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\textsubscript{286} and NR2B Ser\textsubscript{1303}. Whereas CaMKII Thr\textsubscript{286} is dephosphorylated by protein phosphatase type 1 or 2A depending on its subcellular localization (33), the identity of the NR2B Ser\textsubscript{1303} phosphatase is unknown.

Induction of LTP triggers persistent CaMKII autophosphorylation that is dependent on NMDA receptor activation (34–36). Synaptic activity also induces translocation of CaMKII to PSDs (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\textsubscript{1303}.
Acknowledgment—We thank M. Bass for invaluable technical assistance.

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