Cellular/Molecular

A Calcium- and Calmodulin-Dependent Kinase Iα/Microtubule Affinity Regulating Kinase 2 Signaling Cascade Mediates Calcium-Dependent Neurite Outgrowth

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Calcium is a critical regulator of neuronal differentiation and neurite outgrowth during development, as well as synaptic plasticity in adulthood. Calcium- and calmodulin-dependent kinase I (CaMKI) can regulate neurite outgrowth; however, the signal transduction cascades that lead to its physiological effects have not yet been elucidated. CaMKIα was therefore used as bait in a yeast two-hybrid assay and microtubule affinity regulating kinase 2 (MARK2)/Par-1b was identified as an interacting partner of CaMKI in three independent screens. The interaction between CaMKI and MARK2 was confirmed *in vitro* and *in vivo* by coimmunoprecipitation. CaMKI binds MARK2 within its kinase domain, but only if it is activated by calcium and calmodulin. Expression of CaMKI and MARK2 in Neuro-2A (N2a) cells and in primary hippocampal neurons promotes neurite outgrowth, an effect dependent on the catalytic activities of these enzymes. In addition, decreasing MARK2 activity blocks the ability of the calcium ionophore ionomycin to promote neurite outgrowth. Finally, CaMKI phosphorylates MARK2 on novel sites within its kinase domain. Mutation of these phosphorylation sites decreases both MARK2 kinase activity and its ability to promote neurite outgrowth. Interaction of MARK2 with CaMKI results in a novel, calcium-dependent pathway that plays an important role in neuronal differentiation.

Key words: **calcium; protein-protein interaction; CaMKI; microtubule affinity regulating kinase 2; calcium and calmodulin-dependent protein kinase I; MARK2**

Introduction

Calcium is a critical regulator of neuronal differentiation, neurite outgrowth and synaptic plasticity, making it an essential second messenger involved in neuronal remodeling (for review, see Berridge, 1998). Identification of the signaling molecules that mediate these effects of calcium will be critical in understanding the pathways underlying both neuronal development and adult neuronal plasticity. Calcium- and calmodulin-dependent kinase I (CaMKI) is a serine/threonine kinase that belongs to the superfamily of calcium-dependent kinases that includes CaMKII, CaMKIV, and CaMK-kinase (CaMKK) (for review, see Soderling, 1999; Hook and Means, 2001). The most abundant CaMKI isoform, CaMKIα, is expressed throughout the CNS, with highest expression in the frontal cortex (Picciotto et al., 1993). Significant levels of CaMKIα are present in both developing and adult CNS, but mRNA level peaks between postnatal day 1 (P1) and P7 (Sawamura et al., 1996). CaMKIα is cytoplasmically localized by a nuclear export signal (NES) within its regulatory domain (Stedman et al., 2004). This pattern of expression suggests that CaMKIα may regulate cytoplasmic processes, such as neuronal differentiation or neurite extension, which are critical during CNS development.

The ability of CaMKI to promote cellular differentiation has been described in both hematopoietic (Lawson et al., 1999) and neuronal (Schmitt et al., 2004) cell types. CaMKI regulates axonal extension and growth cone motility in primary neurons (Wayman et al., 2004), induces differentiation of NG108 neuroblastoma cells (Schmitt et al., 2004) and promotes dendritic branching in hippocampal neurons (Wayman et al., 2004). In the current study, we identified a novel protein–protein interaction involving CaMKIα that is important for mediating the effects of CaMKI on regulation of neuronal differentiation. Microtubule affinity regulating kinase 2 (MARK2), a regulator of the microtubular cytoskeleton (Drewes et al., 1997; Ebneth et al., 1999; Drewes, 2004), was identified as a novel binding partner of CaMKI both *in vitro* and *in vivo*. Biochemical studies and functional studies demonstrated that MARK2 is phosphorylated and activated by CaMKI, and that this novel protein–protein interaction is important for neurite outgrowth in a neuroblastoma cell line and in primary hippocampal neurons. Overall, these studies identify MARK2 as a critical effector immediately downstream of CaMKI signaling that promotes neuronal differentiation.

Materials and Methods

Plasmid construction. CaMKI fragments were subcloned into *Bam*HI and *Nco*I sites of a modified yeast expression vector pAS2 (Fromont-Racine et

Received July 14, 2006; accepted March 7, 2007.

This work was supported by National Institutes of Health Grants DA00436, DA10455, DA10044, and AA15632. MARK2 antiserum was a generous gift from Dr. S. McConnell (Stanford University, Palo Alto, CA). We thank Annette Kolar for expert help and advice with hippocampal primary culture.

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DOI:10.1523/JNEUROSCI.0725-07.2007

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al., 1997). For mammalian cell expression, CaMKI was subcloned into *Eco*RI and *Bam*HI sites of pCMV-myc and *Bgl*II and *Kpn*I sites of pEGFPC1 vectors (Clontech, Mountain View, CA). A constitutively active (Yokokura et al., 1995), cytosolic form of CaMKI (CaMKI-293NES) was generated by introducing nucleotides encoding the nuclear export sequence (AVVRHMRKLQL), which is normally found in the deleted domain (Stedman et al., 2004), into one of the oligonucleotides used to amplify the fragment by PCR before subcloning the N-terminal 293 aa into pEGFPC1 vector. A kinase-dead form of CaMKI was generated by mutating the catalytic lysine in the ATP-binding domain to alanine (K49A). MARK2 was subcloned from embryonic day 18 (E18) rat brain mRNA (RNAqueous kit; Ambion, Austin, TX). cDNA was synthesized by reverse transcriptase-PCR (Stratagene, La Jolla, CA) and used to amplify the MARK2 open reading frame. The amplified MARK2 product was cloned into the PCR II-TOPO vector (Invitrogen, Eugene, OR) and then subcloned into the *Kpn*I site of the pCMV-myc vector. Single amino-acid substitutions were introduced into constructs using the QuickChange Mutagenesis Kit (Stratagene) according to the manufacturer's instructions. All constructs were confirmed by sequencing by the Keck Facility at Yale University School of Medicine (New Haven, CT).

Yeast two-hybrid screen. Yeast two-hybrid screens were performed using CG1945 and Y187 yeast strains (Clontech). Galactosidase-4 (Gal4)-CaMKI fusion constructs (see Fig. 1*A*) were generated by subcloning CaMKI into a modified pAS2 vector. Screen 1 used a bait consisting of the first 321 N-terminal amino acids of CaMKI. For screen 2, the first 50 aa were truncated to generate a catalytically inactive protein. For screen 3, CaMKI was truncated at phenylalanine 293, right before its regulatory and CaM binding domains generating a constitutively active kinase (Yokokura et al., 1995). A kinase dead (K49A) form of this construct was used to reduce toxicity in yeast (Rasmussen, 2000). No screens were successful with full-length CaMKI because of poor mating efficiency. A random/oligonucleotide-dT rat brain cDNA library cloned in fusion with the Gal4 activation domain of pACT2 vector was used for all screens (Flajolet et al., 2003).

Using a lithium acetate protocol, baits were transformed into the CG1945 yeast strain. Expression of constructs was verified by immunoblotting after SDS-PAGE with an antibody against the Gal4 DNA binding domain (Clontech). Constructs were tested for self-activation of the *His3* reporter gene in the absence of prey by plating transformed yeast on selective media. Because some self-activation was detected with the 1–293 K49A construct, it was grown in the presence of 7.5 mM 3AT (3-amino-1,2,4-triazole), which completely abolished self-activation. The mating strategy was designed as described previously (Fromont-Racine et al., 1997). For each screen performed, 4×10^8 Y187 yeast transformed with the pACT2 adult rat brain cDNA library (Clontech) were thawed and mixed with CG1945 cells transformed with the bait construct. Yeast were incubated on rich media for 4 h at 30°C, after which they were collected and plated on selective media (−lysine/tryptophan/histidine). Controls were plated to determine parental cell viability and mating efficiency. Yeast were allowed to grow for 72 h at 30°C before His+ cells were scored and an X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) overlay assay was performed. Positive clones expressing both reporter genes [*LacZ* (β-galactosidase), *His*] were isolated on selective medium and DNA yeast extracts were used to PCR amplify prey plasmid inserts. Sequence homology was determined using basic local alignment search tools from NCBI (Bethesda, MD). Clones of interest were cotransformed with bait into yeast to confirm the specificity of interactions.

GST pull down of an isolated MARK2 domain. Both full-length (1–374 aa) and truncated, constitutively active (1–293 aa) forms of glutathione S-transferase (GST)-CaMKI fusion proteins were purified from BL21 *Escherichia coli* and immobilized on GST beads. The MARK2 domain isolated in the yeast two-hybrid screen was amplified directly from the yeast expression vector using a sense primer containing the T7 RNA polymerase promoter. The product was subsequently transcribed and translated in the presence of S^{35} methionine using a rabbit reticulocyte-coupled, *in vitro* transcription/translation system (Promega, Madison, WI). Immobilized CaMKI was incubated with translated MARK2 domain in binding buffer [50 mM PBS, pH 7.4, 100 mM NaCl, 0.5% Triton X-100, protease inhibitor mixture from Roche (Basel, Switzerland)] at 4°C overnight. Glutathione agarose was washed three times in binding buffer for 5 min at 4°C, and proteins were resuspended in 1× SDS loading buffer, resolved on 10% SDS-PAGE, transferred onto nitrocellulose membrane, and developed at −80°C for 24 h on Kodak (Rochester, NY) Superfilm in the presence of an enhancer screen.

Cell culture. Human embryonic kidney (HEK) 293T cells (American Type Culture Collection, Manassas, VA) were maintained in DMEM high-glucose medium supplemented with 10% FBS in the presence of penicillin/streptomycin (Invitrogen). N2a cells (American Type Culture Collection) were maintained in Minimum Essential Media (MEM) supplemented with 0.1% MEM nonessential amino acids/10% FBS in the presence of penicillin/streptomycin (Invitrogen). N2a cells were differentiated by 24 h serum withdrawal as described (Pignatelli et al., 1999), or by a 48 h treatment with 1.5 μM ionomycin (Sigma, St. Louis, MO).

Antibody-mediated pull-down assays. HEK 293T cells were grown to 80% confluency and transfected using Fugene6 according to the manufacturer's instructions (Roche). MARK2–669 was generated by inserting a stop codon before the C-terminal kinase-associated domain (KA1). MARK2–369 was generated by inserting a stop codon before the spacer region. MARK2–326 was truncated before the ubiquitin-associated domain, right after the putative membrane-associated domain (Timm et al., 2003). MARK2–305 was truncated outside the kinase domain (see Fig. 1*B*). 48 h after transfection, cells were washed with PBS and lysed in cold radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 50 mM Tris-HCl pH 7.4, 0.5% Triton X-100) containing protease (Roche) and phosphatase inhibitors (Sigma). Myc-tagged proteins were immunoprecipitated by incubation with myc-agarose (Santa Cruz Biotechnology, Santa Cruz, CA) for 1.5 h at 4°C and washed three times in RIPA buffer. Immobilized myc-MARK2 was incubated for 3 h at 4°C with 30 ng of GST-tagged CaMKI in RIPA buffer containing either 1.5 mM $CaCl_2$ and 1 μM calmodulin or 1 mM EGTA. Complexes were washed three times with cold RIPA buffer, resuspended in 1× SDS loading buffer and resolved by SDS-PAGE (10% acrylamide). GST-tagged CaMKI was visualized using an anti-GST antibody (Santa Cruz Biotechnology). Efficiency of initial immunoprecipitation was verified by comparison with 20% of the immobilized myc-MARK2 loaded on a separate gel and probed with an anti-myc antibody (Santa Cruz Biotechnology). In these experiments, myc-agarose immunoprecipitation of untransfected cells served as a negative control. All experiments were replicated two to five times.

Coimmunoprecipitation studies. HEK 293T cells were grown to 80% confluency and cotransfected with myc-tagged MARK2 constructs and green fluorescent protein (GFP)-tagged CaMKI-293NES constructs. Forty-eight hours after transfection, cells were washed with PBS and lysed in cold RIPA buffer (150 mM NaCl, 50 mM Tris-HCl pH 7.4, 0.5% Triton X-100) containing 1.5 mM $CaCl_2$, protease (Roche) and phosphatase inhibitors (Sigma). Myc-tagged proteins were immunoprecipitated with myc-agarose (Santa Cruz Biotechnology) for 1.5 h at 4°C. Immune complexes were washed three times with cold RIPA buffer, resuspended in 1× SDS loading buffer, and resolved by SDS-PAGE. Myc- and GFP-tagged proteins were visualized using anti-myc (Santa Cruz Biotechnology) or anti-GFP (Clontech) antibodies; endogenous CaMKI was visualized with CC77 antibody at 1/1,000 dilution (Picciotto et al., 1993).

For coimmunoprecipitation of endogenous proteins, E18 mouse brain was lysed in RIPA buffer supplemented with 1.5 mM $CaCl_2$, protease inhibitors (Roche), and phosphatase inhibitors (Sigma). Tissue was homogenized and centrifuged for 15 min at 14,000 rpm at 4°C. The lysate was subsequently incubated for 1 h with either MARK2 antiserum (Dr. S. McConnell, Stanford University, Palo Alto, CA) or nonimmune immunoglobulin (Sigma) as a negative control, followed by incubation with protein A. Protein complexes were washed three times in cold RIPA buffer, resolved by SDS-PAGE, and visualized using anti-CaMKI (CC77) and anti-MARK2 antibodies.

N2a cell neurite outgrowth. N2a cells were seeded at low density (250,000 per 30 mm well) on glass coverslips (Warner Instruments, Hamden, CT) pretreated with 0.5 mg/ml poly-L-lysine (Peptides International, Louisville, KY) for 30 min at room temperature (RT) and 4 μg/ml mouse laminin (Invitrogen) at 37°C for 6 h. Cells were allowed to

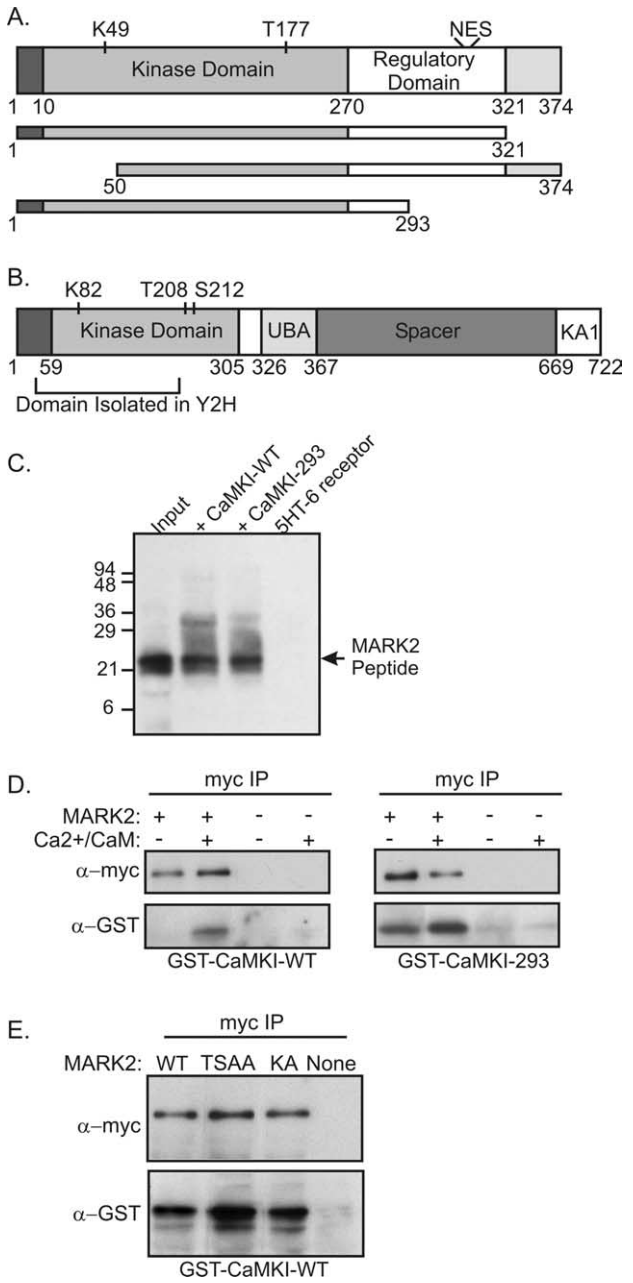

Figure 1. CaMKI and MARK2 interact in a calcium- and calmodulin-dependent manner. ***A***, Structure of CaMKI constructs used in the yeast two-hybrid screens. K49 is essential for ATP binding. Phosphorylation of T177 is required for activation of the kinase. The regulatory region contains overlapping autoinhibitory and CaM-binding domains, as well as a nuclear export sequence. ***B***, Structure of MARK2 with the domain isolated during the yeast two-hybrid screen indicated. K82 is essential for ATP binding. Phosphorylation of T208 and S212 are required for activation of the kinase. UBA, Ubiquitin-associated domain. ***C***, GST pull-down assay between full-length or truncated CaMKI and MARK2 peptide synthesized directly from the plasmid isolated during the screen. Lane 1, *In vitro* transcribed/translated MARK2 (20% of total); lane 2, MARK2 bound to full-length CaMKI; lane 3, MARK2 bound to truncated CaMKI; lane 4, MARK2 incubated with a 50 aa portion of the 5HT-6 receptor. ***D***, Myc-MARK2 was expressed in HEK 293T cells and immunoprecipitated with anti-myc antibody. Full-length (GST-CaMKI-WT) and truncated, constitutively active (GST-CaMKI-293) CaMKI were incubated with immobilized full-length myc-MARK2. As a negative control, CaMKI was incubated with myc-agarose incubated with cell lysate lacking MARK2-Myc overexpression. Anti-myc (α-myc) and anti-GST (α-GST) immunoblots show interaction between immunoprecipitated myc-MARK2 and WT-GST-CaMKI (left) or 293-GST-CaMKI (right) in the presence of Ca^{2+}/CaM (+) or EGTA (−). ***E***, Anti-myc and anti-GST immunoblots show interaction between immunoprecipitated myc-MARK2 constructs [MARK2-WT (WT), MARK2-TSAA (TSAA), MARK2-KA (KA)] and wild-type GST-CaMKI in the presence of Ca^{2+}. None, No myc-MARK2 transfected.

adhere for 12 h and were then transfected with various CaMKI and MARK2 constructs using FUGENE6 (Roche). For a subset of experiments, cells were maintained under differentiating conditions. Subsequently, cells were fixed with 4% paraformaldehyde (PFA) in PBS for 15 min at RT. Transfected cells were imaged by an experimenter blinded to condition. On average 60–80 cells from at least four individual transfections were analyzed. Cell processes were traced using NIH Image J software. Processes equal to or >2 cell diameters in length were considered neurites (Biernat et al., 2002).

Immunofluorescence. Mouse anti-myc antibody (Clontech), rabbit and mouse anti-β III tubulin (Sigma), rabbit anti-CaMKI (CC77), and rabbit anti-MARK2 antibodies were used for fluorescent immunocytochemistry. After culture on glass coverslips, cells were washed once with PBS and fixed with 4% PFA for 15 min at RT After fixation, cells were permeabilized in 0.1% Triton X-100 in PBS for 8 min, blocked in 4% normal goat serum (Vector Laboratories, Burlingame, CA), and incubated for 1 h at RT each in primary antibodies and fluorescent secondary antibodies (Invitrogen) diluted to optimized concentration in blocking solution. Before and after the secondary antibody incubations cells were washed three times for 10 min in PBS. After staining, coverslips were mounted with aqueous mounting medium (Biomeda, Foster City, CA) on precleaned microscope slides (Fisher Scientific, Houston, TX).

Primary rat hippocampal culture. Rat hippocampal neurons were prepared as described (Brewer et al., 1993). Cells were plated at high density on coverslips (Warner Instruments) precoated with 0.1% (w/v) poly-L-lysine (Peptides International), in Neurobasal medium supplemented with B-27 (Invitrogen), 0.5 mM glutamine, and 5% fetal calf serum. Four hours after plating, media was replaced with serum-free Neurobasal media supplemented with B-27 and 0.5 mM glutamine. Neurons were transfected with CaMKI and MARK2 constructs after 3 d *in vitro* using LipofectAmine 2000 (Invitrogen). Twenty-four hours after transfection, cells were fixed with 4% PFA, immunostained, and analyzed using fluorescent microscopy by an observer blinded to condition. On average, 25 cells from 6 to 10 transfections per condition were analyzed. The length of the primary neurite was measured using Image J software from NIH. Length was presented as a fraction of the GFP control.

In vitro kinase assays. GST-CaMKI was purified from *E. coli* and assayed as described (Picciotto et al., 1993). CaMKK was partially purified from mouse cortex. Briefly, adult mouse cortex was homogenized in lysis buffer [50 mM Tris pH 7.5, 10 mM KCl, 0.1 mM EDTA, 1 mM DTT, protease inhibitors (Roche), phosphatase inhibitors (Sigma)]. The lysate was centrifuged for 15 min at 14,000 rpm, mixed with 0.33% ammonium sulfate, and centrifuged again to precipitate large proteins. Supernatant was incubated with calmodulin-agarose (Upstate Biotechnology, Lake Placid, NY) in the presence of 1.5 mM $CaCl_2$ for 1.5 h at 4°C. Agarose was subsequently washed twice in buffer A supplemented with $CaCl_2$ then eluted with lysis buffer containing 1 mM EGTA.

For *in vitro* kinase reactions, HEK 293T cells were grown until 80% confluent in 100 mm dishes then transfected with myc-MARK2 constructs. 48 h after transfection, cells were lysed in RIPA buffer and lysates incubated for 1.5 h at 4°C with myc-agarose (Santa Cruz Biotechnology). Immobilized myc-MARK2 was washed three times in RIPA buffer and resuspended in kinase buffer (50 mM Tris pH 7.6, 1 mM $CaCl_2$, 10 mM $MgCl_2$, 1 μM CaM, 0.5 mM DTT, and 1.5 μCi/ml γ-^{32}P-ATP). CaMKI (1 μg) was added to each reaction and MARK2 served as a potential substrate. For a subset of reactions, CaMKI was preactivated by incubation with CaMKK for 30 min at 30°C. Kinase assays were stopped with the addition of 5× SDS-loading dye. Proteins were resolved by SDS-PAGE, transferred onto nitrocellulose membrane, and visualized by autoradiography. Efficiency of immunoprecipitation was verified by comparison to one-fifth of each initial kinase assay probed with anti-myc antibody (Santa Cruz Biotechnology).

To determine the kinetics of phosphorylation, a GST-MARK2 construct truncated at amino acid 326 was expressed in and purified from *E. coli*. Phosphorylation with activated CaMKI was conducted as described above. To identify phosphorylation sites, point mutations were introduced into the MARK2 construct as described above. In addition, a phosphorylated sample was analyzed by mass spectrometry to identify phosphopeptides (McGill Proteomics Facility, McGill University, Montreal, Quebec, Canada).

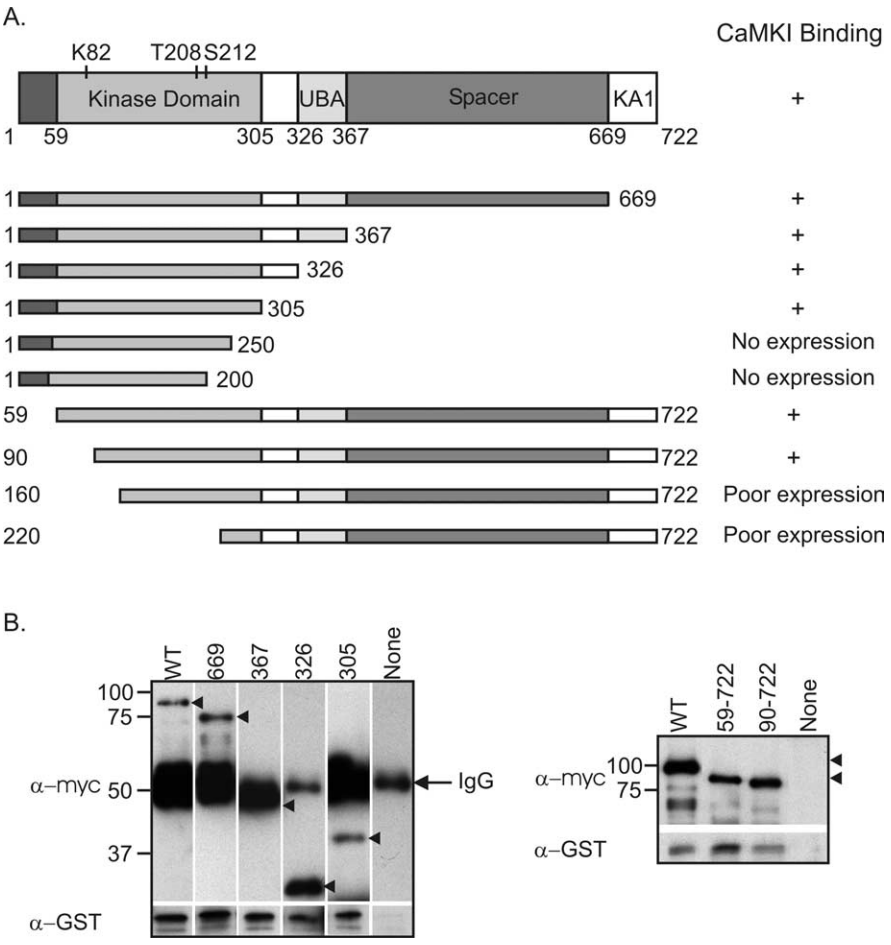

Figure 2. CaMKI interacts with the kinase domain of MARK2. ***A***. MARK2 constructs generated for pull-down experiments to map the domain of interaction. +, Interaction was detected, and protein was highly expressed; poor expression, a band of the correct size was detected but protein levels were low; no expression, no protein could be detected from this construct. Truncations within the kinase domain (at amino acids 250 or 205) did not produce a protein detectable by Western blot, likely because of disrupted secondary structure and subsequent degradation (Benton et al., 2002). ***B***, GST-CaMKI was incubated with various myc-MARK2 constructs immunoprecipitated from HEK 293T cells in the presence of Ca^{2+} and calmodulin and immobilized on myc-agarose. Anti-myc (α-myc) and anti-GST (α-GST) immunoblots show interaction between CaMKI and MARK2 proteins containing the kinase domain. Except for MARK2–305, all constructs that expressed detectable proteins migrated at the expected size by SDS-PAGE. MARK2–305 migrated more slowly than predicted. A construct truncated at amino acid 309 also migrated more slowly than predicted (data not shown). Arrowheads indicate where each MARK2 protein migrated by SDS-PAGE. None, No myc-MARK2 transfected.

For MARK2-tau kinase assays, myc-MARK2 constructs were expressed in HEK 293T cells. 48 h after transfection, cells were lysed in RIPA buffer and lysates were incubated for 1.5 h at 4°C with myc-agarose (Santa Cruz Biotechnology). Immobilized myc-MARK2 was washed three times in RIPA buffer and resuspended in MARK2 kinase buffer (50 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 2 mM EGTA, 0.5 mM PMSF, 0.5 mM dithiothreitol). The substrate in the reaction was 100 μM tau repeat 1 (TR1) peptide and assays were performed for 30 min at 30°C as described previously (Biernat et al., 2002). Kinase reactions were stopped with 15% acetic acid. One half of the reaction was spotted on p81 paper and the other half was resolved by SDS-PAGE to determine the amount of kinase present (measured by immunoblotting with anti-myc antibody). Mutant kinase activities were normalized to that of wild-type MARK2.

Results

MARK2 is a novel binding partner of CaMKI

Yeast two-hybrid screens were conducted to identify novel binding partners for CaMKIα (for summary, see supplemental Table 1, available at www.jneurosci.org as supplemental material). Three truncated CaMKIα polypeptides, all containing the kinase domain, were used as bait to screen a rat brain cDNA library (Fig. 1*A*). MARK2/Par-1b (also known as an ELKL motif kinase) was the only candidate interacting protein found using all three baits. MARK2 is a serine/threonine kinase that can regulate cell shape and polarity by phosphorylation of microtubule-associated proteins (MAPs) (Drewes et al., 1997; Ebneth et al., 1999; Drewes, 2004). A number of mammalian MARK isoforms that share a similar overall structure have been identified (MARK1, MARK2, MARK3, MARK4) (for review, see Drewes, 2004). These isoforms all contain a variable N-terminal domain, catalytic domain, ubiquitin-associated domain, spacer domain, and a C-terminal KA1 [Fig. 1*B*, adapted from Timm et al. (2003)].

To confirm the specificity of the interaction between the two proteins, bait and prey constructs were rescued from original double positive clones, amplified in *E. coli*, sequenced, and retransformed into yeast. Both *His* and *LacZ* reporter genes were activated after cotransformation of CaMKIα and MARK2 plasmids, but were not activated after transformation of the corresponding empty plasmids (data not shown). Based on the intensity of color, MARK2–CaMKI interactions were scored as moderate to strong. The interaction between CaMKI and MARK2 was also demonstrated using pull-down assays between purified GST-CaMKI and the *in vitro* transcribed/translated MARK2 domain isolated from the yeast two-hybrid screen (Fig. 1*C*). The 20 kDa radiolabeled product, corresponding to MARK2, bound to immobilized GST-CaMKI, but failed to bind to the negative control, a 50 aa portion encompassing the intracellular loop of the 5HT-6 receptor (Fig. 1*C*).

Calcium/calmodulin-dependent activation of CaMKI is required for interaction with MARK2

Experiments using full-length and truncated proteins were conducted to characterize the interaction between GST-CaMKIα and myc-MARK2. The potential role of calcium and calmodulin (Ca/CaM) were also investigated. Binding between the full-length proteins was dependent on Ca/CaM (Fig. 1*D*). Consistent with the idea that CaMKI must be in an active conformation to bind to MARK2, constitutively active GST-CaMKI interacted with myc-MARK2 in a calcium-independent manner. The catalytic activity of MARK2 did not influence CaMKI binding because two inactive MARK2 constructs, one with regulatory phosphorylation sites mutated (myc-MARK2-TSAA) and one with the ATP binding site mutated (myc-MARK2-KA) (Drewes et al., 1997), bound full-length GST-CaMKI (plus Ca/CaM) at least as efficiently as wild-type MARK2 (myc-MARK2-WT) (Fig. 1*E*).

Assays were also performed to identify the region of MARK2 that interacted with CaMKI (Fig. 2*A*,*B*). The first 305 aa of

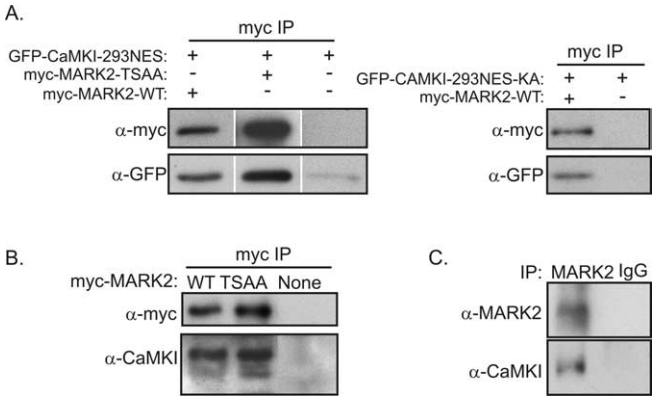

Figure 3. Coimmunoprecipitation of CaMKI and MARK2. ***A***, myc-MARK2-WT, myc-MARK2-TSAA, GFP-CaMKI-293NES, and GFP-CaMKI-293NES-KA were expressed alone or together in HEK 293T cells as indicated. Cells were lysed and myc-tagged proteins were immunoprecipitated by incubation with myc-agarose. After washing, proteins were resolved by SDS-PAGE, and anti-myc antibody and anti-GFP antibodies were used for immunoblotting. GFP-CaMKI and myc-MARK2 expression were verified on a separate gel (data not shown). Cells overexpressing GFP-CaMKI alone served as a negative control. ***B***, Endogenous CaMKI coimmunoprecipitated with myc-MARK2 constructs [myc-MARK2-WT (WT), myc-MARK2-TSAA (TSAA)] expressed in HEK 293T cells. Myc-tagged proteins were immunoprecipitated with myc-agarose and CaMKI was detected by immunoblotting with anti-CaMKI antibody. ***C***, Coimmunoprecipitation of endogenous proteins from E18 mouse brain. Anti-MARK2 antibody or control IgG was used for immunoprecipitation. MARK2 and CaMKI were detected by immunoblotting with specific antibodies. All immunoprecipitation studies were performed in the presence of 1.5 mM $CaCl_2$.

MARK2 comprising its catalytic domain were sufficient to bind wild-type GST-CaMKI in the presence of Ca/CaM. myc-MARK2 constructs lacking either the first 59 or the first 89 aa bound to GST-CaMKI. Thus, amino acids 90–305 of MARK2 are sufficient for CaMKI binding.

Coimmunoprecipitation between CaMKI and MARK2

Coimmunoprecipitation studies were conducted to determine whether a CaMKI–MARK2 complex could be identified *in vivo*. Because full-length CaMKI required activation to bind MARK2 *in vitro*, initial studies were performed using the constitutively active, cytosolic form of CaMKI (GFP-CaMKI-293NES). After coexpression in HEK 293T cells, both wild-type and inactive (TSAA) forms of myc-MARK2 bound GFP-CaMKI-293NES (Fig. 3*A*). Moreover, a kinase-dead form of CaMKI (GFP-CaMKI-293NES-KA) also bound myc-MARK2 (Fig. 3*A*), indicating that although an active conformation of CaMKI is necessary for binding, kinase activity is not required.

HEK 293T cells express CaMKI but very little MARK2 (data not shown). We therefore determined whether over-expressed myc-MARK2 could form a complex with endogenous CaMKI. myc-MARK2-transfected cells were lysed in the presence of calcium to ensure activation of CaMKI. Both active and inactive myc-MARK2 constructs bound endogenous CaMKI (Fig. 3*B*), whereas no CaMKI was immunoprecipitated with lysate of non-transfected cells.

Both CaMKI and MARK2 are expressed at high levels in the developing CNS (Sawamura et al., 1996; Drewes et al., 1997). A stable complex of CaMKI and MARK2 was immunoprecipitated from E18 mouse brain in the presence of calcium (Fig. 3*C*), demonstrating that endogenous CaMKI and MARK2 can form a complex.

CaMKI phosphorylates MARK2 on novel sites

Because both CaMKI and MARK2 are protein kinases, we hypothesized that MARK2 might be a CaMKI substrate or vice versa. Initial studies indicated that active CaMKI phosphorylated MARK2, but wild-type MARK2 did not phosphorylate CaMKI (data not shown). Subsequent studies therefore focused only on analysis of MARK2 as a substrate for CaMKI. Myc-MARK2-KA was used as substrate in these studies to prevent autophosphorylation. GST-CaMKI was preactivated by phosphorylation with CaMKK, which is essential for full-activation of the kinase and efficient phosphorylation of other substrates (Matsushita and Nairn, 1998; Suizu et al., 2002; Qin et al., 2003). Activated GST-CaMKI phosphorylated myc-MARK2-KA, whereas no significant ^{32}P incorporation occurred after addition of CaMKK alone or without addition of any kinases (Fig. 4*A*). Mutation of residues normally phosphorylated within the activation loop of MARK2 (MARK2-TSAA) or truncation before the ubiquitin-associated domain (MARK2–1-326) resulted in significant phosphorylation by GST-CaMKI (Fig. 4*B*,*C*), suggesting that phosphorylation of MARK2 occurs within the kinase domain on site(s) different from those in the activation loop. Kinetic analysis indicated that CaMKI phosphorylated both wild-type GST-MARK2–1-326 and the MARK2-TSAA mutant with K_m values of 0.26 μM, indicating that MARK2 is a kinetically favorable CaMKI substrate.

Tryptic digestion of MARK2 phosphorylated by CaMKI, followed by mass spectrometry, identified a peptide containing threonine 294 (T294) that was phosphorylated (Fig. 4*D*). This sequence does not match a canonical consensus for CaMKI phosphorylation (Lee and Edelman, 1994), although there is an arginine residue at the −2 position and a lysine at the −3 position. Notably, MARK3/C-TAK1 (Cdc25C-associated kinase 1) is phosphorylated by Pim1, a member of the CaMK kinase subfamily, on three residues within the kinase domain (Bachmann et al., 2004). We therefore aligned this sequence with the corresponding region of other MARK isoforms and found that these residues are conserved (Fig. 4*D*,*E*). Moreover, MARK2 contains an additional serine within this peptide. We then mutated the three serine residues in this domain of MARK2 (MARK2-S91–93A), as well as T294, in the background of the MARK2-TSAA protein. MARK2-S91–93A was phosphorylated to a lesser degree than MARK2-TSAA (Fig. 4*F*,*G*), suggesting that CaMKI could phosphorylate at least one of these residues. Surprisingly, MARK2-T294A was phosphorylated as efficiently as MARK2-TSAA, whereas phosphorylation of the quadruple mutant (MARK2-S91–93A, T294A) was greatly reduced (Fig. 4*F*,*G*).

We next investigated whether phosphorylation of MARK2 might regulate its activity. MARK2 proteins in which phosphorylation sites were mutated were therefore tested for their ability to phosphorylate the tau peptide TR1, which contains a known MARK2 substrate site (Biernat et al., 2002). Comparable levels of each myc-MARK2 mutant were expressed in HEK 293T cells and, after immunoprecipitation, were assayed for kinase activity (Fig. 4*H*). Mutation of serine 92 to alanine (S92A) or S91, S92 and S93 to alanine (S91–93A) resulted in decreased MARK2 activity. In contrast, mutation of S93 alone had no effect on MARK2 activity. It is important to note that, because MARK2 is immunoprecipitated from cells, we cannot rule out the relatively unlikely possibility that the differences in activity are caused by coimmunoprecipitated binding partners of each mutant form of MARK2.

Calcium-dependent neurite outgrowth in N2a cells is regulated by a CaMKI–MARK2 cascade

Both CaMKI and MARK2 have been implicated in regulating neuronal differentiation and polarity (Biernat et al., 2002; Cohen et al., 2004; Suzuki et al., 2004). In particular, both CaMKI and

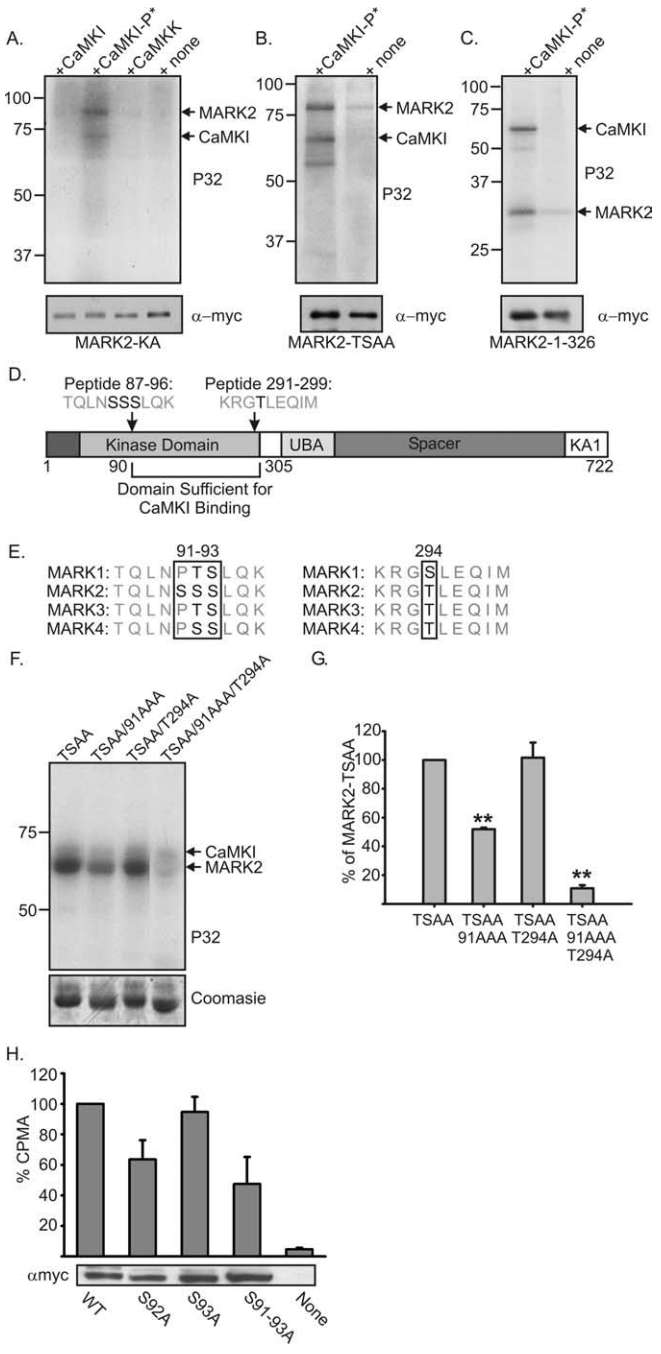

Figure 4. MARK2 is a novel CaMKI substrate. ***A–C***, HEK 293T cells were transfected with myc-MARK2 constructs. After incubation with myc-agarose, immobilized myc-MARK2 was incubated with GST-CaMKI, GST-CaMKI preactivated by incubation with CaMKK in the presence of $\gamma[^{32}P]$ATP (CaMKI-P*), or CaMKK alone. Proteins were resolved by SDS-PAGE, transferred onto nitrocellulose membrane, and visualized by autoradiography. Efficiency of immunoprecipitation was verified by comparison to one fifth of each initial kinase assay loaded on a separate Western blot and probed with an anti-myc antibody (α-myc in panels below each immunoprecipitation). Kinase assays used the following substrates: immunoprecipitated myc-MARK2-KA (***A***); myc-MARK2-TSAA (in which T208 and S212 were mutated to alanine residues; ***B***); truncated MARK2 containing amino acids 1–326 (***C***). ***D***, MARK2 structure showing the two potential phosphorylated peptides within the domain sufficient for CaMKI binding. Residues 87–96 of all MARK isoforms contain potential Pim-1 phosphorylation sites (identified previously for MARK3). Residues 291–299 with a site phosphorylated at T294 was identified as a phosphopeptide by mass spectrometry. ***E***, Alignment of putative phosphorylation sites across MARK isoforms, with sites mutated in MARK2 kinase assays highlighted in bold. ***F***, Kinase assays using GST-MARK2-TSAA-1–326 as substrate or mutant GST-MARK2 proteins in which S91, S92, and S93 (91AAA), T294 (T294A), or S91, S92, S93 plus T294 were mutated to alanine as substrates. Proteins were separated by SDS-PAGE and analyzed by autoradiography. The positions of CaMKI (top band) and MARK2 (lower band) are indicated. The bottom shows levels of MARK2 proteins as detected by Coomassie stain to verify equal loading. ***G***, Quantitative analysis of ^{32}P incorporation into MARK2 mutants presented as a percentage of MARK2-TSAA phosphorylation. Kinase reactions were repeated two to four times per mutant construct. $^{**}p < 0.01$ in one-way ANOVA, least significant difference *post hoc* test. Error bars show SEM. ***H***, WT and mutated (S92A, S93A, and S91–93A) forms of MARK2 were expressed in HEK 293T cells, MARK2 was immunoprecipitated using anti-Myc antibody, and phosphorylation of tau peptide (100 μM) was measured *in vitro*. All reactions were normalized to MARK2-WT. Error bars show SEM. A representative blot of immunoprecipitated MARK2 constructs is shown below the graph.

MARK2 have been shown to be involved in the regulation of neurite formation (Biernat et al., 2002; Wayman et al., 2004). Thus, we hypothesized that the two kinases could be part of the same signaling cascade that regulates neuronal differentiation.

MARK2 can regulate neurite formation in the neuroblastoma cell line N2a (Biernat et al., 2002). Transfection of MARK2-WT or CaMKI-293NES alone, but not wild-type CaMKI, resulted in a significant increase in neurite outgrowth when compared with GFP control and required no additional stimuli, such as serum withdrawal (data not shown). Serum withdrawal resulted in a significant increase in neurite formation in GFP transfected cells (from ~12 to ~25%, data not shown). This effect was further increased by expression of MARK2-WT or CaMKI-293NES (Fig. 5*A*). MARK2-TSAA is a dominant-negative form of the kinase, based on it ability to block differentiation of N2a cells induced by retinoic acid (Biernat et al., 2002). Similarly, mutation of the catalytic lysine in CaMKI lacking the Ca/CaM binding domain results in a dominant-negative form of the kinase based on its ability to inhibit neurite length and number of primary processes in cerebellar and hippocampal neurons (Wayman et al., 2004). As expected, transfection of N2a cells with MARK2-TSAA suppressed the neurite outgrowth induced by serum withdrawal. However, CaMKI-293NES-KA had no significant effect on neurite outgrowth in N2a cells (Fig. 5*A*).

The results of the studies described above indicate that activation of CaMKI is required for the interaction with MARK2 and CaMKI phosphorylates MARK2. CaMKI is therefore likely upstream of MARK2. Consistent with this model, cotransfection of dominant-negative MARK2 with CaMKI-293NES abolished the increase in neurite outgrowth produced by constitutively active CaMKI alone (Fig. 5*B*). Kinase-dead CaMKI (CaMKI-293NES-KA) also significantly decreased neurite outgrowth triggered by MARK2-WT expression (Fig. 5*B*). It is possible that dominant-negative CaMKI blocked the effects of MARK2 because MARK2 must be activated by endogenous CaMKI to exert its effects on neurite outgrowth. Consistent with this idea, the increased neurite outgrowth caused by overexpression of a partially active form of MARK2-T208E (MARK2-TE) (Timm et al., 2003) in N2a cells was not blocked by dominant negative CaMKI-293NES-KA (Fig. 5*C*).

CaMKI is normally activated by Ca/CaM binding and subsequent phosphorylation by CaMKK (Yokokura et al., 1995; Matsushita and Nairn, 1998). Inhibition of CaMKK with STO-609 (Tokumitsu et al., 2002) significantly reduced neurite formation induced by MARK2-WT (Fig. 6*A*), suggesting that CaMKK activity is necessary for over-expressed MARK2 to stimulate neurite outgrowth. In contrast, constitutively active MARK2 (MARK2-TE) was insensitive to STO-609 (Fig. 6*A*), suggesting that CaMKK lies upstream of MARK2 and that activity of CaMKK and CaMKI are necessary for MARK2 function. STO-609 alone did not have an effect on neurite outgrowth at the concentration used.

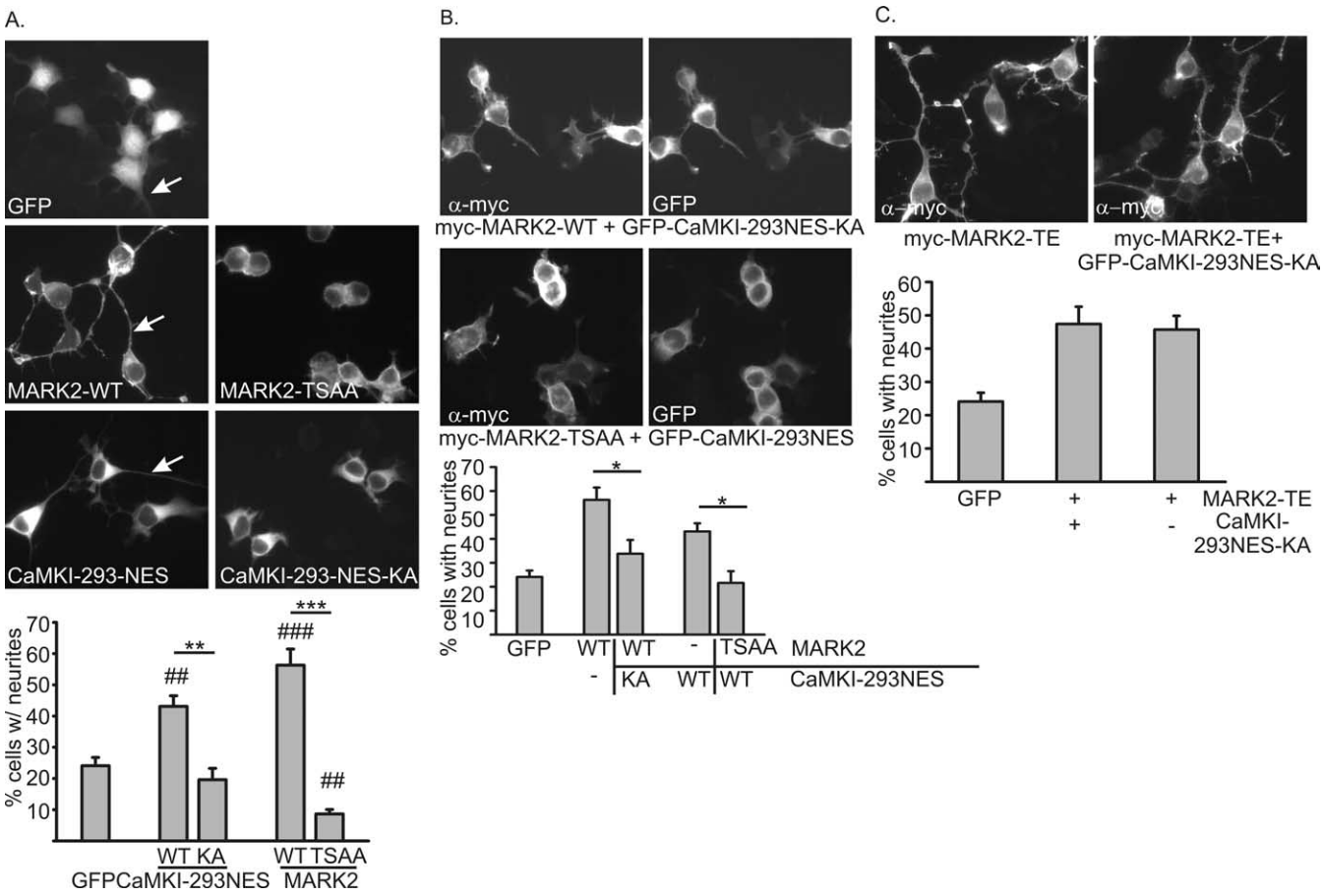

Figure 5. Catalytically active CaMKI and MARK2 augment neurite outgrowth in N2a cells. N2a cells were transfected with CaMKI and/or MARK2 constructs and differentiated in serum free media. After 24 h, cells were fixed and imaged. Neurites longer than two cell body diameters were counted. GFP or GFP-CaMKI expression was visualized by direct fluorescence and myc-MARK2 expression was visualized by immunofluorescence. ***A***, Neurite outgrowth after expression of WT CaMKI-293NES, MARK2, or kinase-dead constructs [CaMKI-293NES-KA (KA), MARK2-TSAA (TSAA)]. Cells were transfected with GFP alone as a control. An average of 80 cells from four to eight independent transfections were analyzed. Arrows point to representative processes measured in N2a cells. Arrows indicate cellular processes analyzed using ImageJ. ***B***, Neurite outgrowth after coexpression of wild-type MARK2 (WT) without or with kinase-dead CaMKI-293NES-KA (KA), or wild-type CaMKI-293NES (WT) without or with kinase dead MARK2-TSAA (TSAA). Cells were transfected with GFP alone as a control. An average of 120 cells from four independent transfections were analyzed. ***C***, Expression of constitutively active MARK2 (MARK2-TE) with or without kinase-dead CaMKI (CaMKI-293NES-KA). An average of 100 cells from five independent transfections were analyzed. Cells were transfected with GFP alone as a control. Error bars indicate SEM. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{\#\#}p < 0.01$ compared with GFP control; $^{\#\#\#}p < 0.001$ compared with GFP control; paired *t* test.

We also conducted neurite outgrowth experiments under conditions that mimic the environment in the developing nervous system to determine whether the CaMKI–MARK2 interaction might be important for calcium-dependent neurite outgrowth. N2a cells were differentiated by raising intracellular calcium via the calcium ionophore ionomycin (Wu et al., 1998). Ionomycin-induced differentiation was significantly inhibited in cells expressing dominant negative CaMKI-293NES-KA or MARK2-TSAA (Fig. 6*B*). There was also a trend toward a decrease in neuritogenesis in the presence of the CaMKK inhibitor STO-609, but the results did not reach statistical significance (Fig. 6*B*).

We then examined the effects of MARK2 mutants in which sites for CaMKI were mutated on neurite extension in N2a cells. Expression of MARK2-T294A induced neurite outgrowth to a similar level as MARK2-WT. In contrast, cells transfected with either MARK2-S91–93A or MARK2-S91–93A-T294A showed significantly less neurite outgrowth (Fig. 6*C*). Expression of mutant forms of MARK2 in which S91, S92, or S93 were individually mutated revealed that S93 had no effect, whereas mutation of either S91 or S92 to alanine decreased the ability of MARK2 to promote neurite outgrowth. These data suggest that phosphorylation of S91 and S92 may be critical for the ability of MARK2 to increase neurite outgrowth, likely by regulating the ability of MARK2 to phosphorylate down-stream substrates.

Previous studies had suggested that the ability of CaMKI to promote neurite outgrowth in NG108 cells was dependent on activation of extracellular signal-regulated kinase (ERK) (Schmitt et al., 2004). We therefore attempted to block CaMKI-induced neurite formation with the ERK kinase (MEK) inhibitor U0126 (1,4-diamino-2,3-dicyano-1,4-bis[2-amino-phenylthio]butadiene). However, this inhibitor had no effect on the ability of CaMKI to increase neurite formation in N2a cells either in the presence or the absence of serum (data not shown).

MARK2 and CaMKI signal together to regulate axonal extension in primary hippocampal neurons

We next used rat primary hippocampal cultures to study the effects of CaMKI and MARK2 on axonal outgrowth in nontransformed primary neurons. We first confirmed that CaMKI and MARK2 were normally expressed in hippocampal neurons by immunocytochemistry and by immunoblotting after SDS-PAGE of cell lysates (Fig. 7). Endogenous MARK2 runs at the same molecular weight as overexpressed MARK2 on a Western

blot (data not shown), confirming the specificity of the MARK antiserum and the expression of endogenous MARK2 in cultured hippocampal neurons from E18 embryo. At the time of transfection, hippocampal neurons exhibited a typical stage 4 neuronal morphology, with one long axon and multiple neurites (Fukata et al., 2002). Expression of CaMKI-293NES or MARK2-WT in hippocampal neurons resulted in an ~50% increase in axonal length when compared with the GFP control (Fig. 7*A*). This effect was dependent on the kinase activity of the proteins, because it was abolished when kinase-dead CaMKI (CaMKI-293NES-KA) or MARK2 (MARK-TSAA) were expressed in neurons (Fig. 8*A*). Moreover, CaMKI-293NES-KA significantly suppressed the axonal outgrowth observed in GFP-transfected cells, suggesting that endogenous CaMKI normally contributes to differentiation of hippocampal neurons.

Similar to observations in N2a cells, expression of MARK2-TSAA abolished the increase in axonal length induced by CaMKI-293NES (Fig. 8*B*), whereas expression of CaMKI-293NES-KA significantly decreased the increase in axonal length observed with MARK2-WT alone (Fig. 8*B*). Cotransfection of MARK2 and CaMKI-293NES did not result in an additional increase in process length (data not shown). Thus, as in the case of neurite formation in N2a cells, the activity of both kinases is necessary for regulation of axonal length in primary neurons.

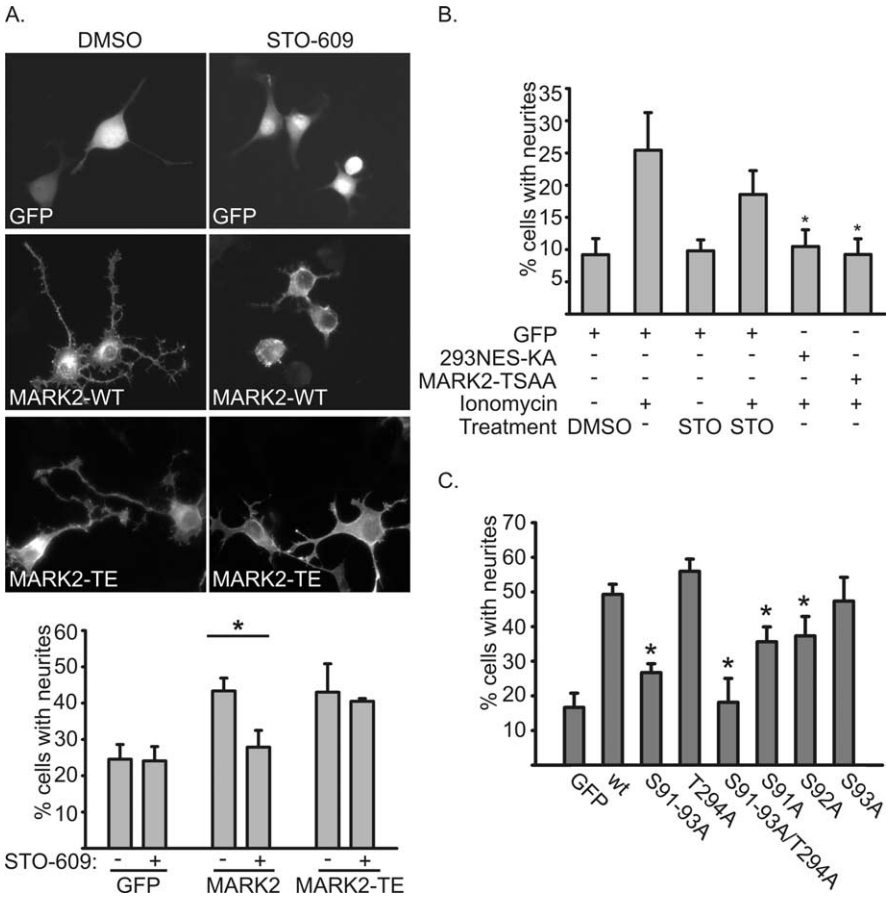

Figure 6. Involvement of CaMKI and MARK2 downstream of calcium-induced neurite outgrowth. ***A***, N2a cells were transfected with wild-type (MARK2-WT) or constitutively active MARK2 (MARK2-TE), and differentiated in the absence or presence of 1 μM STO-609, a CaMKK inhibitor, or vehicle. Cells were transfected with GFP alone as a control. An average of 60 cells from at least four independent transfections were analyzed. *$p < 0.05$ in paired *t* test. ***B***, Neurite outgrowth in N2a cells differentiated with 1.5 μM ionomycin and transfected with dominant negative CaMKI-293NES-KA or MARK2-TSAA, or treated with the CaMKK inhibitor STO-609. An average of 100 cells from at least four independent transfections were analyzed. *$p < 0.05$ in paired *t* test when compared with GFP-transfected cells differentiated with ionomycin. ***C***, Neurite outgrowth in N2a cells expressing wild-type and mutant MARK2 proteins in which sites phosphorylated by CaMKI were mutated to alanine residues (S91–93A, T294A, S91–93A/T294A, S91A, S92A, and S93A). Neurites longer than two cell body diameters were measured. An average of 60 cells from at least three independent transfections per mutant were analyzed. *$p < 0.05$ in paired *t* test compared with wild-type. Error bars show SEM.

Discussion

We have identified a novel pathway involving the calcium-regulated protein kinase CaMKI and the phylogenetically related kinase MARK2 that is involved in neurite outgrowth in both a neuroblastoma cell line and in primary hippocampal neurons. MARK2 is a newly identified CaMKI substrate, and is downstream of CaMKI in a signaling cascade regulating neuronal differentiation. CaMKI and MARK2 were coprecipitated from extracts of E18 mouse brain, suggesting that these kinases may signal together in the developing brain. The identification of the interaction of CaMKI and MARK2 provides a novel molecular mechanism that appears necessary for the ability of CaMKI to regulate calcium-dependent neuronal differentiation and neurite outgrowth.

Calcium is known to promote neuronal differentiation (Berridge, 1998), and is thought to be the critical messenger downstream of cell adhesion-induced neurite outgrowth (Dunican and Doherty, 2000). In the current studies, both neuroblastoma and primary hippocampal neurons were grown on adhesive substrates known to support outgrowth and increase intracellular calcium (Dunican and Doherty, 2000). Thus, the calcium-dependent interaction between CaMKI and MARK2 is an excellent candidate to mediate the effects of adhesion and cell-surface effectors on neurite outgrowth. CaMKI immunoreactivity is highly concentrated in axons around the area of highest microtubule density (Picciotto et al., 1995). The interaction between CaMKI and MARK2 may be important for recruiting CaMKI to this compartment. The observation that interaction between CaMKI and MARK2 was calcium-dependent demonstrates that the binding site for MARK2 is normally occluded by the autoinhibitory domain of CaMKI. This further implies that there may be a translocation of one or both proteins as a result of their interaction once calcium enters the cells to form an active complex.

The current study identifies novel phosphorylation sites within the kinase domain of MARK2 that are phosphorylated by CaMKI. Mutation of these phosphorylation sites decreases the ability of MARK2 to phosphorylate tau as well as its ability to promote neurite outgrowth; thus, CaMKI is likely to be an important effector coupling calcium entry to microtubule rearrangement by MARK2. The calcium dependence of the CaMKI–MARK2 interaction also raises the possibility that the interaction between CaMKI and MARK2 occurs as a result of a kinase-substrate interaction in the active site, and that calcium is required primarily to make the active site accessible.

The analogous serine residues in MARK3 (S90–92) are phos-

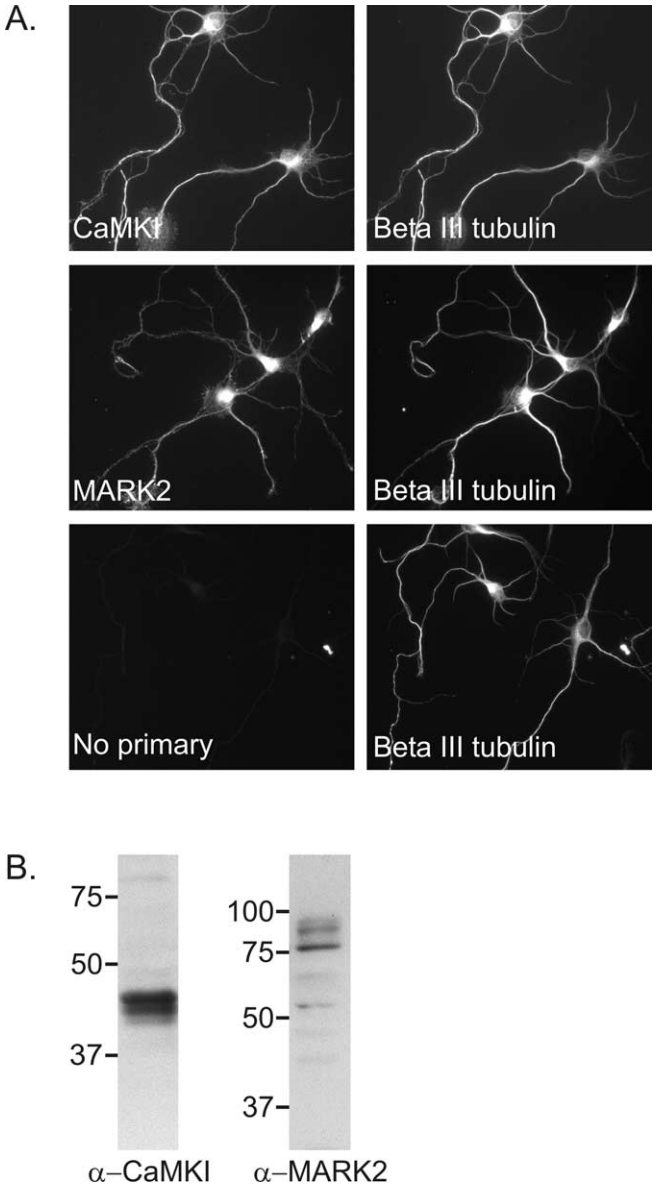

Figure 7. Expression of CaMKI and MARK2 in cultured hippocampal neurons. ***A***, ***B***, Endogenous expression of CaMKI and MARK2 in cultured primary hippocampal neurons was verified by immunocytochemistry (***A***) and immunoblotting (***B***). E18 hippocampal neurons were grown for 4 d, fixed, and costained for CaMKI or MARK2 and βIII tubulin. Both CaMKI and MARK2 show prominent expression in neuronal processes.

phorylated by Pim1, also a member of the CaMK superfamily (Bachmann et al., 2004). Mutation of these residues in MARK3 did not completely abolish Pim1 phosphorylation, suggesting the existence of an additional phosphorylation site. Similarly, mutation of T294 along with S90–92 was necessary to abolish MARK2 phosphorylation by CaMKI. The similarity in the primary structure of the MARK1–4 isoforms within this domain suggests that CaMKI may be able to phosphorylate other MARK family members in addition to MARK2. Whereas S92 is conserved across MARK isoforms, S91 is present only in MARK2, suggesting that this residue confers a unique level of regulation to MARK2 and may represent a specific interaction between CaMKI and MARK2 that does not occur with other MARK isoforms.

MARK2-S92A, but not MARK2-S93A, had decreased ability to phosphorylate tau peptide, an effect that was also seen when S91–93 were mutated together. This paralleled the ability of these MARK2 proteins to regulate neurite outgrowth. It is interesting that mutation of T294 alone did not lead to decreased ATP incorporation into MARK2 but that mutation of T294 and S90–92 together were necessary to completely abolish MARK2 phosphorylation. Cooperativity between distant phosphorylation sites has been observed in a number of proteins (Larsson et al., 1997; Dumaz et al., 1999; Bauer et al., 2003); thus, there may be an interaction between phosphorylation at the T294 site and the S90–92 sites.

Like CaMKI, MARK2 has been shown to bind other proteins via its kinase domain. For example, the p21-like kinase PAK5 has been identified as an interacting partner of MARK2 in a yeast two-hybrid screen (Matenia et al., 2005). This interaction was found to be independent of the kinase activity of the proteins and was demonstrated to inhibit MARK2 via noncatalytic binding to its kinase domain. The *Drosophila* homolog of MARK2, par-1, can also bind 14-3-3 proteins via its kinase domain (Benton et al., 2002). Thus, in addition to the ability to activate MARK2 via phosphorylation, CaMKI may also potentially alter MARK2 kinase activity through its ability to bind to the kinase domain and compete with other proteins.

The data presented here suggest that the ability of CaMKI to phosphorylate MARK2 is an important effector for its ability to induce neurite outgrowth, but the pathways that are upstream and downstream of these kinases may differ across cell types. This may explain the somewhat different effects of MARK2 and CaMKI constructs on neurite outgrowth at baseline in N2a cells and primary hippocampal neurons. It seems likely that several pathways converge to activate MARK2. For example, MARK kinase, LKB1 kinase, Pim-1, and GSK-3 can all phosphorylate MARK2 within its kinase domain (Timm et al., 2003; Bachmann et al., 2004; Lizcano et al., 2004; Kosuga et al., 2005), suggesting that multiple kinases, which may be differentially expressed in N2a cells and hippocampal neurons, potentially converge on MARK2 to regulate its activity. Similarly, downstream effectors of CaMKI may differ between neuronal types. For example, in some cell types, CaMKI could signal predominantly through an alternative pathway, such as an ERK-dependent cascade described previously in NG108 cells (Schmitt et al., 2004). N2a cell differentiation was unaffected by the presence of MEK inhibitor in our studies (data not shown), whereas this pathway may be more important in hippocampal neurons (Wayman et al., 2004). MARK2 and CaMKI may differentially regulate ERK, resulting in convergence between these signaling pathways in some cell types.

Phylogenetically, MARK2 belongs to the AMP-activated protein kinase (AMPK)/sucrose nonfermenting 1 (Snf1) subfamily of the CaMK group (Hanks and Hunter, 1995; Drewes et al., 1997). MARK2 expression is prominent in brain, and, like CaMKI, is highest during development (Drewes et al., 1997). A number of studies have identified interactions between members of the superfamily of CaM kinases and the MARK family of kinases. In yeast, AMPK/Snf1 can be phosphorylated by pak1, which is homologous to mammalian CaMKKβ (Nath et al., 2003). CaMKKβ can itself complement the loss of other known Snf1-activating kinases in yeast and activate Snf1 (Hong et al., 2005). Members of the AMPK family of kinases can be activated by CaMKK in mammalian cells as well (Lizcano et al., 2004; Hawley et al., 2005; Hurley et al., 2005). However, these studies demonstrate AMPK phosphorylation within its activation loop, which corresponds to T208 in MARK2. Our data demonstrate clearly that this is not the site phosphorylated by CaMKI, suggesting that the current study has identified a novel link between the CaMK and MARK pathways.

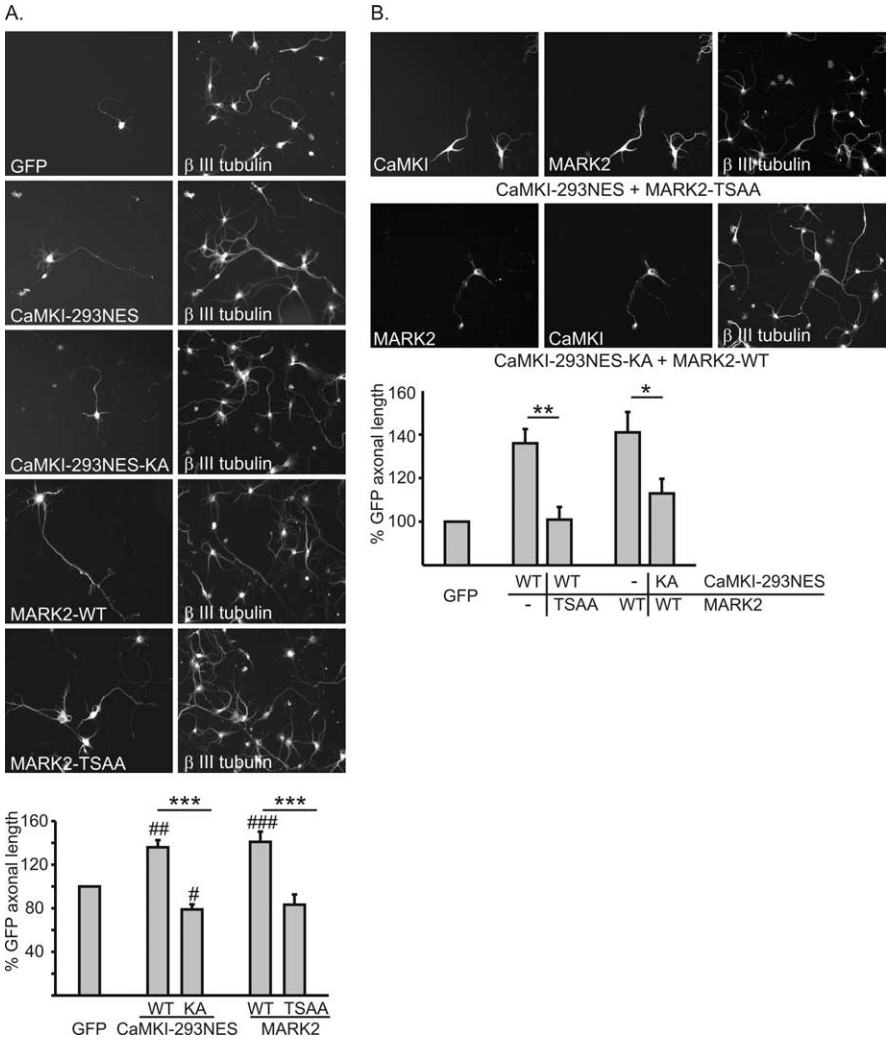

Figure 8. The kinase activity of CaMKI and MARK2 is required to increase axonal length in primary hippocampal neurons. Primary hippocampal neurons were transfected with GFP-CaMKI and myc-MARK2 constructs. Twenty-four hours after transfection, cells were fixed and stained for myc and β III tubulin. GFP was visualized via direct immunofluorescence. An average of 25 cells from six to 10 individual cultures were analyzed. Axonal length is presented as a percentage of the GFP control for each culture. Panels depict representative fields for each condition. ***A***, Expression of GFP-CaMKI-293NES (WT), kinase-dead GFP-CaMKI-293NES (KA), wild-type MARK2 (WT), or kinase-dead MARK2 (TSAA) in primary hippocampal neurons. Left, α-Myc; right, α-βIII tubulin. GFP-CaMKI was visualized via direct immunofluorescence. ***B***, Coexpression of CaMKI-293NES (WT) without or with kinase-dead MARK2 (TSAA), or wild-type MARK2 (WT) without or with kinase-dead CaMKI (KA) in primary hippocampal neurons. $^{\#}p < 0.05$; $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$ using one-way ANOVA with least significant difference (LSD) *post hoc* test when compared with GFP; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ in one-way ANOVA using LSD *post hoc* test. Error bars show SEM.

MARK2 plays a role in neuronal differentiation, migration, and transport via phosphorylation of MAPs (Biernat et al., 2002; Mandelkow et al., 2004; Schaar et al., 2004). Previous studies have indicated that tau phosphorylation was essential for the ability of MARK2 to promote retinoic acid-induced differentiation in N2a cells (Biernat et al., 2002). Thus, microtubule reorganization downstream of MARK2 signaling is likely to be critical for the ability of CaMKI to induce neurite outgrowth. Neurite initiation and axon elongation involve a complex interplay between the microtubule and actin cytoskeleton (for review, see Dehmelt and Halpain, 2004). MAPs are thought to play a key role in regulating actin and microtubule cross talk by serving not only as structural components of the cell, but also as signaling molecules (Dehmelt and Halpain, 2004). The current study provides new details of the signaling cascades upstream of MAP phosphorylation by MARK. Moreover, because CaMKI can induce actin filament reorganization via myosin II light chain phosphorylation in HeLa cells (Suizu et al., 2002), the interaction between CaMKI and MARK2 could provide a molecular mechanism connecting the actin and tubulin dynamics essential for neurite formation. In summary, the identification of a CaMKI–MARK2 interaction provides new details of CaMKI signaling cascades and identifies a novel molecular pathway important for neuronal differentiation. Future studies could explore the role of this cascade during neurodegeneration and adult neuronal plasticity, because these processes also involve calcium-mediated rearrangement of the cytoskeleton and are known to be mediated by members of these signaling families.

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