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Protein Kinase A Rescues Microtubule Affinity-regulating Kinase 2-induced Microtubule Instability and Neurite Disruption by Phosphorylating Serine 409*

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Si-Si Deng[‡1], Le-Yu Wu[§1], Ya-Chao Wang[¶], Peng-Rong Cao[‡], Lei Xu[‡], Qian-Ru Li[‡], Meng Liu[‡], Lun Zhang[‡], Yue-Jing Jiang[‡], Xiao-Yu Yang[§], Sheng-Nan Sun[‖], Min-jia Tan[‖], Min Qian[¶], Yi Zang[‡2], Linyin Feng[§3], and Jia Li[‡¶4]

From the [‡]National Center for Drug Screening, State Key Laboratory of Drug Research, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, 189 Guo Shoujing Road, Shanghai 201203, China, [§]Department of Neuropharmacology, CAS Key Laboratory of Receptor Research, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, 555 Zu Chong Zhi Road, Shanghai 201203, China, [‖]The Chemical Proteomics Center and State Key Laboratory of Drug Research, Shanghai Institute of Materia Medica , Chinese Academy of Sciences, 555 Zu Chong Zhi Road, Shanghai 201203, China, and [¶]School of Life Sciences, East China Normal University, 3663 North Zhongshan Road, Shanghai 200062, China

Background: MARK2 and PKA are both involved in the regulation of microtubule stability.
Results: PKA significantly inhibits MARK2 by phosphorylating serine 409 and rescues MARK2-induced microtubule instability and neurite disruption.
Conclusion: PKA functions as an upstream inhibitor of MARK2 by directly interacting and phosphorylating it.
Significance: The results identify a novel interaction between PKA and MARK2, providing new insight into the signal networks of both.

Microtubule affinity-regulating kinase 2 (MARK2)/PAR-1b and protein kinase A (PKA) are both involved in the regulation of microtubule stability and neurite outgrowth, but whether a direct cross-talk exists between them remains unclear. Here, we found the disruption of microtubule and neurite outgrowth induced by MARK2 overexpression was blocked by active PKA. The interaction between PKA and MARK2 was confirmed by coimmunoprecipitation and immunocytochemistry both *in vitro* and *in vivo*. PKA was found to inhibit MARK2 kinase activity by phosphorylating a novel site, serine 409. PKA could not reverse the microtubule disruption effect induced by a serine 409 to alanine (Ala) mutant of MARK2 (MARK2 S409A). In contrast, mutation of MARK2 serine 409 to glutamic acid (Glu) (MARK2 S409E) did not affect microtubule stability and neurite outgrowth. We propose that PKA functions as an upstream inhibitor of MARK2 in regulating microtubule stability and neurite outgrowth by directly interacting and phosphorylating MARK2.

Microtubules, a key component of the cytoskeleton, are composed of polymerized α- and β-tubulin dimers. Their dynamic polymerization is important for cell division, cellular morphology and polarity, and intracellular trafficking (1, 2). Microtubule tracks are covered with microtubule-associated proteins (MAPs),[5] which contribute to their stability and thus to the maintenance of cell shape and neurite outgrowth. The best-studied members of the MAPs are the ubiquitous MAP4 and the neuronal MAPs Tau (largely axonal) and MAP2 (largely dendritic) (3, 4).

Certain kinases are particularly efficient in phosphorylating and detaching MAPs from microtubules. One of the best examples is the microtubule affinity-regulating kinase 2 (MARK2)/PAR-1b, which phosphorylates the Lys-*X*-Gly-Ser (K*X*GS) motifs in the repeat domains of MAP4, MAP2, or Tau (5–8). The overexpression of MARK2 leads to microtubule breakdown and cell death in CHO cells (7, 9). The ectopic expression of MARK2 causes the phosphorylation of Tau at Ser-262 and a loss of axons, whereas knockdown of MARK2 expression with small interfering RNAs induces the formation of multiple axon-like neurites and promotes axon outgrowth in hippocampal neurons (10).

In addition to MARK2, protein kinase A (PKA), a cyclic AMP (cAMP)-dependent protein kinase is implicated in regulating microtubule stability and cell polarity in various biological contexts. Forskolin, a PKA agonist, attenuates microtubule disassembly induced by the anti-microtubule agent nocodazole, suggesting a critical role for PKA activity in the stabilization of the microtubule cytoskeleton in endothelial cells (11). Further-

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[1] Both authors contributed equally to this work.
[2] To whom correspondence may be addressed. Tel.: 86-21-50801552; E-mail: yzang@simm.ac.cn.
[3] To whom correspondence may be addressed. Tel.: 86-21-50806810; Fax: 86-21-50806810; E-mail: lyfeng@simm.ac.cn.
[4] To whom correspondence may be addressed: 189 Guo Shoujing Road, Shanghai 201203, China. Tel.: 86-21-50801552; E-mail: jli@simm.ac.cn.

[5] The abbreviations used are: MAP, microtubule-associated protein; MARK2/PAR-1b, microtubule affinity-regulating kinase 2; PKA, protein kinase A; AMARA, AMARAASAAALARRR (Ala-Met-Ala-Arg-Ala-Ala-Ser-Ala-Ala-Ala-Leu-Ala-Arg-Arg-Arg); PKAc, PKA catalytic subunit; EGFP, enhanced GFP; pEGFP-MARK2 WT, wild-type MARK2 with enhanced GFP appended to its N terminus; HA-PKAc, PKA catalytic subunit with an HA-YPYDVPDYA tag at its N terminus; LKB1, liver kinase B1; TLNB, total length of neurite branches; AD, Alzheimer disease; CREB, cAMP-response element-binding protein.

TABLE 1

Summary of recombinant plasmids, expressed proteins, and primers used for cloning

The oligonucleotides for primers are displayed from 5′ to 3′. The bold italic bases indicate the mutation of serine 409 to alanine or glutamic acid.

Plasmid	Protein expressed	Primer sequences
pET28b-MARK2	Wild type (1–788)	CGCGTCGACATTCGGGGCCGCAACTCAGC (forward) ATTGCGGCCGCTTAAAGCTTCAGCTCGTTGGCTA (reverse)
pcDNA3.1-MARK2	Wild type (1–788)	AACGGTACCGACCATGGGCATTCGGGGCCGCAACTCAA (forward) AGAGCGGCCGCAAGCTTCAGCTCGTTGGCT (reverse)
pcDNA3.0-PKA	α (1–351)	TATAATATCGCGGATCCGGCAACGCCGCCGCCAA (forward) CGCACTCGCTACCGCTCGAGCTAAAACTCAGTAAACTCCTT (reverse)
pET28b/pcDNA3.1/pEGFP-MARK2 S409A	S409A (1–788)	GCCAATCCCAAGCAGCGGCGCTTC***GCG***GACCAGGCAGCTGGTCCTGCC (forward) GGCAGGACCAGCTGCCTGGTC***CGC***GAAGCGCCGCTGCTTGGGATTGGC (reverse)
pET28b/pcDNA3.1/pEGFP-MARK2 S409E	S409E (1–788)	GCCAATCCCAAGCAGCGGCGCTTC***GAG***GACCAGGCAGCTGGTCCTGCC (forward) GGCAGGACCAGCTGCCTGGTC***CTC***GAAGCGCCGCTGCTTGGGATTGGC (reverse)

more, cAMP promotes neurite outgrowth and extension through PKA in cultured rat motor neurons (12).

Because both MARK2 and PKA are involved in the regulation of microtubule stability and neurite outgrowth, we sought to determine whether a direct interaction exists between MARK2 and PKA. Here, we showed that PKA is a novel regulator of MARK2 and that PKA counteracts the activity of MARK2 in microtubule stability and neurite outgrowth.

MATERIALS AND METHODS

Antibodies—The antibodies used for immunostaining or immunoblotting analysis were bought from Millipore (Tau1, MARK2), Abcam Inc. (α-tubulin), Cell Signaling Technology (myc-tag, HA tag, PKA catalytic subunit (PKAc), and phospho-PKA substrate (RR*X*(S*/T*))), Wallac (horseradish peroxidase-labeled anti-rabbit or anti-mouse secondary antibody), and Invitrogen (phospho-Tau (Ser-262), Alexa 568-, and CF633-conjugated secondary antibodies).

Plasmids—The pET-28b expression vector was purchased from Novagen. The vectors for transfection, pcDNA3.1, pcDNA3.0, and pEGFP-N1, were purchased from Invitrogen. pEGFP-MARK2 wild type (MARK2 WT) was a kind gift from Dr. Zhen-Ge Luo (Shanghai Institutes for Biological Sciences). The pET15b-PKAc was a gift from Dr. Susan Taylor (University of California, San Diego, CA). The primers for construction of all recombinant plasmids are listed in Table 1.

Cell Culture and Transfection—HEK293 cells were maintained routinely in DMEM (Invitrogen) supplemented with 10% bovine growth serum (HyClone, Logan, UT) and 100 units/ml each of penicillin and streptomycin. Lipofectamine™ 2000 (Invitrogen) was used for transfection as directed by the manufacturer.

The hippocampi of embryonic-day-18 rat embryos were digested with 0.125% trypsin-EDTA for 15 min at 37 °C followed by trituration with pipettes in the plating medium (DMEM with 10% FBS). Dissociated neurons were transfected using a nucleofector device (Amaxa Nucleofector II). Control and transfected neurons were plated onto coverslips coated with poly-D-lysine (0.1 mg/ml). After culturing for 4 h, the medium was changed to neuronal culture medium (neurobasal media containing 1% glutamine and 2% B27).

Immunocytochemistry—All cell samples were seeded on 12-mm poly-L-lysine-coated glass coverslips for immunofluorescence analysis. The treated cells were washed with PBS, fixed in 4% paraformaldehyde at 4 °C for 30 min, and incubated with 0.1% Triton X-100 in PBS for 10 min. After blocking with 10% goat serum in PBS at room temperature for 1 h, the cells were incubated with primary antibodies at 4 °C overnight and subsequently with the appropriate Alexa 555- or CF633-conjugated secondary antibody. Coverslips were mounted, and the cells were examined under a confocal microscope (Olympus, Okoya, Japan). For the quantitative analysis, images were automatically taken by a laser-based confocal high content system (Opera, PerkinElmer Life Sciences) by 40× objective magnification. Disrupted microtubule networks were quantified and plotted by an image data analysis system (Columbus, PerkinElmer Life Sciences). Data the mean ± S.E. from three independent experiments.

Coimmunoprecipitation—HEK293 cells at 80% confluency were cotransfected with myc-tagged MARK2 WT and HA-tagged PKAc constructs using Lipofectamine 2000 reagent for 48 h. Cells were washed with PBS and lysed in cold radioimmune precipitation assay buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.4, and 0.5% Triton X-100) containing a protease inhibitor mixture (Roche Applied Science), 1 mM PMSF, and 1 mM Na_3VO_4. For the coimmunoprecipitation of endogenous proteins, E18 mouse brain was lysed in radioimmune precipitation assay buffer as described above. Lysates were clarified by centrifugation at 12,000 × *g*. Cell lysates were preincubated with myc-tagged antibody or HA-tagged antibody overnight at 4 °C and then immunoprecipitated with protein A-agarose (Sigma) for 4 h at 4 °C. The beads were washed with ice-cold PBS, an appropriate volume of loading buffer was added to the beads, the beads were boiled, and the proteins were then analyzed by Western blot.

Recombinant Protein Expression and Purification—The *Escherichia coli*-containing recombinants pET28b-MARK2 WT, pET28b-MARK2 S409A (the mutant of MARK2 that Ser-409 changed to Ala), pET28b-MARK2 S409E (the mutant of MARK2 that Ser-409 changed to Glu), and pET28b-PKAc were induced with 0.1 mM isopropyl-1-thio-β-D-galactopyranoside for 16 h at 22 °C. The cells were harvested, washed, and resuspended in lysis buffer (50 mM sodium phosphate, pH 7.5, 100 mM NaCl, 1 mM DTT, and 1 mM PMSF for MARK2 WT, S409A, and S409E; 50 mM sodium phosphate, pH 8.0, 100 mM NaCl, 1 mM DTT, 1 mM PMSF for PKAc) with 1% Triton X-100 and sonicated on ice. After centrifugation, the supernatant was loaded onto nickel-nitrilotriacetic acid-agarose. The nonspecifically bound proteins were washed with wash buffer (50 mM sodium phosphate, pH 7.5, 100 mM NaCl, 50 mM imidazole, and 1 mM DTT for MARK2 WT, S409A, and S409E; 50 mM sodium

phosphate, pH 8.0, 100 mM NaCl, 50 mM imidazole, and 1 mM DTT for PKAc). The target proteins (MARK2 WT, S409A, S409E, and PKAc) were eluted with an elution buffer (50 mM sodium phosphate, pH 7.5, 200 mM imidazole, and 1 mM DTT for MARK2 WT, S409A, and S409E; 50 mM sodium phosphate, pH 8.0, 200 mM imidazole, and 1 mM DTT for PKAc) and dialyzed with desalting buffer (50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM DTT, and 1 mM EDTA for MARK2 WT, S409A, and S409E; 50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 1 mM DTT, and 1 mM EDTA for PKAc) at 4 °C to remove imidazole. Recombinant liver kinase B1 (LKB1) protein was bought from Carna Biosciences.

Kinase Assay of MARK2 in Vitro—Recombinant MARK2 WT or MARK2 S409A (400 nM) were incubated with or without PKAc (400 nM) at 30 °C for 4 h in 50 mM HEPES, pH 7.5, 10 mM $MgCl_2$, 1 mM EGTA, 0.01% Brij-35, and 100 μM ATP. The samples (MARK2 WT with or without PKAc and MARK2 S409A with or without PKAc and MARK2 S409E) were then incubated for a further 30 min at 30 °C by the addition of 20 nM LKB1 protein. Analyses of the activities of MARK2 proteins were carried out in typical assay conditions of a 50-μl reaction mixture containing 50 mM HEPES, pH 7.5, 10 mM $MgCl_2$, 1 mM EGTA, 0.01% Brij-35, 100 μM ATP (0.4 μCi of [γ-^{33}P]ATP per reaction), and 100 μM AMARA peptide as described previously (13). The reaction was initiated by the addition of MARK2 proteins, incubated at 30 °C for 30 min, and terminated by the addition of 50 μl of 1% H_3PO_4. Particulate matter was then transferred to phosphocellulose pH plates (Millipore) and washed 4 times with 0.1% H_3PO_4. Radioactivity that had been incorporated in the AMARA peptide was determined by liquid scintillation counting in a Wallac MicroBeta plate counter. Background radioactivity estimated from reactions conducted without enzymes was subtracted from sample radioactivities. All reactions were repeated in three independent experiments.

Immunoblotting Analysis—The samples were loaded on 10% (w/v) SDS-PAGE and immunoblotted using the indicated primary antibodies. The bands were visualized using HRP-conjugated secondary antibody and ECL substrate (Thermo) and analyzed with Bio-Rad ChemiDoc System(Bio-Rad) and Image laboratory software.

Nano-HPLC-MS/MS Analysis—The tryptic peptides generated by tryptic in-gel digestion were dissolved in solvent A (0.1% formic acid, 2% acetonitrile, 98% H_2O). Samples were then injected onto a manually packed reversed phase C18 column (150 mm × 79 μm, 3-μm particle size, Dikma, China) coupled to Easy nLC (Thermo Fisher Scientific, Waltham, MA). Peptides were eluted from 8% to 80% solvent B (0.1% formic acid in 90% acetonitrile and 10% H_2O) in solvent A (0.1% formic acid in 2% acetonitrile and 98% H_2O) with a 90-min gradient at a flow rate of 300 nl/min. The eluent was analyzed by using Q Exactive mass spectrometer. For full MS analysis, the scan range was m/z 350–1300 with a resolution of 70,000. The 10 most intense ions in each full MS spectrum were sequentially fragmented by higher energy collision dissociation with a normalized collision energy of 28%. The dynamic exclusion duration was set to be 30 s, and the isolation window was 2.0 m/z.

Analyses of Mass Spectrometric Data—The raw files were analyzed by Mascot software (Version 2.3) against the UniProt human database to identify the phosphorylation sites. For mascot search, we specified the parameters including carbomidomethyl (C) as the fixed modification and acetylation (protein N-term), oxidation (M), and phosphorylation (ST) as variable modifications. Other parameters were as follows: mass error for parent ion mass was ±10 ppm with fragment ion as ± 0.02 Da, and the enzyme was trypsin with 2 maximum missing cleavages.

Statistics—Data are presented as the means ± S.E. from three independent experiments. Significance was analyzed using a two-tailed unpaired Student's t test. A value of $p < 0.05$ (*) was considered statistically significant, a value of $p < 0.01$ (**) was considered statistically highly significant, and a value of $p < 0.001$ (***) was considered statistically extremely significant.

RESULTS

PKA Rescues the Microtubule Disruption Caused by MARK2 Overexpression in HEK293 Cells—MARK2 induces microtubule disruption by phosphorylating MAPs (7). Here, HEK293 cells were fixed and stained with antibodies against α-tubulin to visualize microtubules. pEGFP-MARK2 WT (wild-type MARK2 with enhanced green fluorescent protein appended to its N terminus) caused microtubule disruption and cell shrinkage in 60% of the transfected cell (Fig. 1, A, *e-h*, and *C*), which is extremely significant in contrast with the extended shape and intact microtubule network observed in the control cells (Fig. 1, A, *a–d*, and *C*) ($p < 0.001$) as reported previously (14). When HEK293 cells were cotransfected with HA-PKAc (PKA catalytic subunit with an HA-YPYDVPDYA tag at its N terminus) and EGFP-MARK2 WT, the shrinking cells with disrupted microtubule significantly decreased by 41% compared with the cells transfected only with MARK2 WT ($p = 0.003$) (Fig. 1, *A*, *i–l*, and *C*), whereas the overexpression of PKAc did not influence the protein amount of the transfected EGFP-MARK (Fig. 1*B*). HEK293 cells transfected with PKAc alone presented an intact microtubule network and normal morphology. The p value is 0.23 compared with the control cells. (Fig. 1, *D* and *E*).

MARK2 Interacts with PKA in HEK293 Cells—Coimmunoprecipitation experiments were conducted to clarify the interaction between PKA and MARK2. After being coexpressed with myc-MARK2 WT in HEK293 cells and immunoprecipitated by anti-myc antibody, PKAc was detected in the MARK2 immune complex (Fig. 2*A*). MARK2 was also coimmunoprecipitated and detected using anti-HA antibody (Fig. 2*B*), further demonstrating the interaction between PKA and MARK2.

PKA Phosphorylates MARK2 at the RRX(S/T) Motif—To explore how PKA inhibited the microtubule disruption caused by MARK2, we investigated whether PKA was able to phosphorylate and inhibit MARK2 kinase activity. Interestingly, after treatment with forskolin, immunoprecipitated endogenous MARK2 was phosphorylated, as determined by immunoblotting against the specific phospho-PKA substrate antibody at the optimal PKA substrate motif Arg-Arg-*X*-Ser/Thr (RR*X*(S*/T*)) (15, 16). This phosphorylation was totally blocked by pretreatment with H89, a specific PKA antagonist. The well defined PKA substrate CREB was used as a positive control to confirm the reliability of the experiment (Fig. 3*A*).

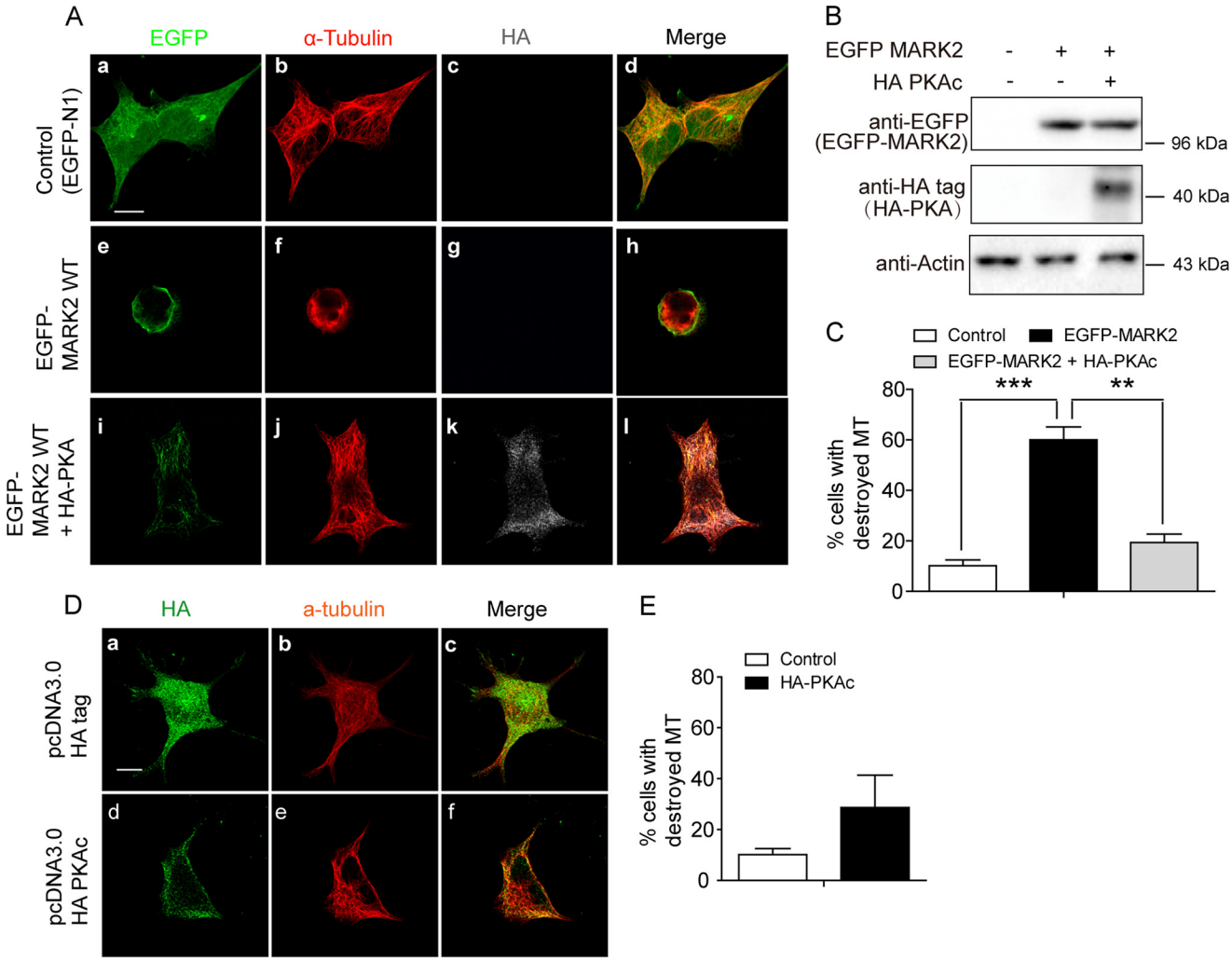

FIGURE 1. **PKA rescues the microtubule disruption caused by MARK2 overexpression in HEK293 cells.** *A*, *B*, and *C*, HEK293 cells transfected with the control plasmid, EGFP-MARK2 WT alone, or together with HA-PKAc were fixed and immunostained by α-tubulin antibody and CF568-conjugated secondary antibody (*red*, *b*, *f*, and *j*) and HA-tag antibody and CF633-conjugated secondary antibody (*gray*, *c*, *g*, and *k*). *A*, the microtubule network was visualized by a confocal microscopy. The *scale bar* represents 10 μm. *B*, Western blot analysis of the cell lysates directed against EGFP, HA, and endogenous protein actin. *C*, EGFP-positive cells (control, EGFP-MARK2 WT) or EGFP and HA-double-positive cells (EGFP-MARK2 WT+HA-PKAc) with disrupted microtubule networks were quantified and plotted by a high content system. Data are the mean ± S.E. from 3 independent experiments, and at least 100 EGFP-positive cells or EGFP and HA-double positive cells were analyzed for each transfection. **, $p < 0.005$; ***, $p < 0.001$. *MT*, microtubule. *D* and *E*, immunochemistry of HEK293 cells transfected with or without HA-PKAc. After 24 h, cells were fixed and immunostained for HA (*green*) and α-tubulin (*red*) to visualize the PKAc and microtubules, respectively. *D*, images were captured by a confocal microscopy. The *scale bar* represents 10 μm. *E*, for the quantitative analysis, HA-positive cells (control, HA-PKAc) with disrupted microtubule networks were quantified and plotted by a high content system. Data are the mean ± S.E. from three independent experiments, and at least 100 HA-positive cells were analyzed for each transfection. No obvious change to the microtubule network could be observed compared with the control cells.

To explore which site on MARK2 was phosphorylated by PKA, we analyzed the MARK2 sequence (Fig. 3*B*) and found that Ser-409 in the spacer domain of MARK2 matched the optimal PKA substrate motif RR*X*(S/T) (Fig. 3*B*). Furthermore, a search of protein phosphorylations in eukaryotic databases showed that tandem mass spectrometry studies (17, 18) previously detected the phosphorylated MARK2 Ser-409 moiety in cells, although the upstream kinase responsible for this phosphorylation is unknown. Therefore, we supposed that PKA may act as the upstream kinase of MARK2 by directly phosphorylating Ser-409.

The nano HPLC-MS/MS analysis using a high resolution Q Exactive mass spectrometer was carried out to confirm the phosphorylating site MARK2 targeted by PKA. The Ser-409 phosphorylated peptide (RFpSDQAAGPAIPTSNSYSK, pS is phosphorylated Ser) of MARK2 incubated with PKA was detected, whereas no phosphorylation at Ser-409 was identified in the protein from the group of MARK-WT alone (Fig. 3*G*). Besides, the autoradiography showed that PKAc phosphorylated the wild-type MARK2, and mutation of the Ser residue at 409 to Ala significantly reduced phosphorylation. The immunoblotting analysis of the SDS gel against MARK2 antibody also showed that the M_r of MARK2 shifted upward in the presence of PKAc, which was obviously reversed by mutation of Ser-409 to Ala (Fig. 3*C*). Both autoradiography and MS/MS analysis obviously indicated that PKAc could phosphorylate MARK2 at Ser-409, although the possibility that PKA phosphorylates other sites on MARK2 cannot be excluded.

PKA Inhibits MARK2 Kinase Activity in Vitro, Depending on Ser-409 Phosphorylation—To investigate whether PKA inhibits the kinase activity of MARK2 after its phosphorylation, we used

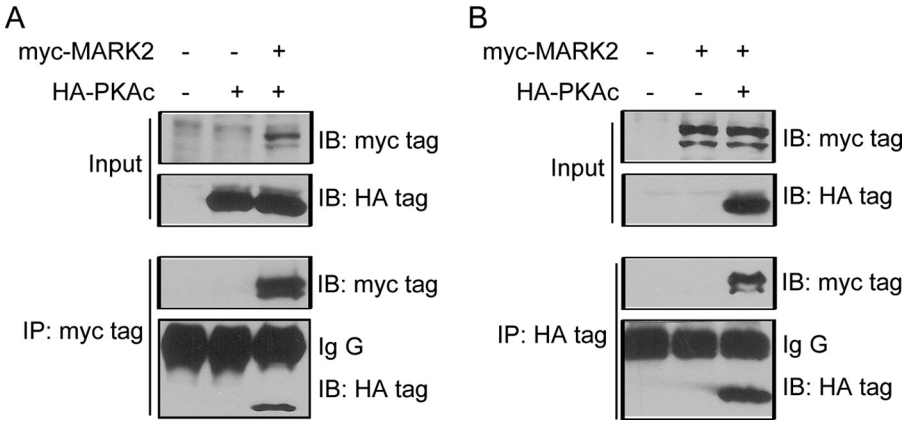

FIGURE 2. **MARK2 interacts with PKA in HEK293 cells.** *A*, Myc-tagged MARK2 WT or control plasmid was coexpressed with HA-tagged PKAc in HEK293 cells. The proteins immunoprecipitated (*IP*) with anti-myc antibody were immunoblotted (*IB*) with antibody against myc or HA. *B*, lysates of HEK293 cells transfected with myc-tagged MARK2 WT alone or together with HA-tagged PKAc were immunoprecipitated using anti-HA antibody and immunoblotted with antibody against myc or HA.

a bacterially expressed PKAc and MARK2 in an *in vitro* kinase activity assay. In the analysis of MARK2 proteins, including MARK2 WT, MARK2 S409A with or without PKA, or MARK2 S409E kinase activity against a well known substrate, the AMARA peptide was performed by a radioactivity incorporation assay. LKB1 was used to pre-phosphorylate and activate the *E. coli* expressed MARK2 proteins (MARK2 WT, MARK2 S409A, MARK2 S409E), with no activity against AMARA peptide (Fig. 3*D*). Fig. 3*F* showed that both the wild-type MARK2 and the mutant MARK2 S409A had similarly high kinase activities, whereas the activity of the mutant MARK2 S409E was reduced to 60.67% that of the control (***, $p < 0.001$).

The activity of wild-type MARK2 was significant inhibited by the PKAc, with 53.60% activity of the wild type (**m $p = 0.0016$) (Fig. 3*D*). The kinase-dead mutant of PKAc (PKA-K_D), without autophosphorylation at Thr197 (Fig. 3*Ei*), had no significant inhibitory activity on MARK2 ($p = 0.25$) (Fig. 3*Eii*). However, the activity of the MARK2 S409A mutant was hardly affected by the PKAc ($p = 0.99$) (Fig. 3*F*). PKAc alone showed no kinase activity against AMARA peptide (Fig. 3*D*).

The *in vitro* kinase activity assay showed that PKAc could significantly inhibit the kinase activity of MARK2 WT. The mutation of MARK2 Ser-409 to Ala did not affect MARK2 enzyme activity but blocked the inhibitory effects of PKAc on MARK2 kinase activity. The mutation of MARK2 Ser-409 to Glu significant decreased the kinase activity, mimicking the inhibitory effects of PKAc. These results clearly indicated that PKA significantly inhibits MARK2 kinase activity by phosphorylating Ser-409.

PKA Counteracts the Effects of MARK2 on Microtubule Stability, Which Depends on Ser-409 Phosphorylation, in HEK293 Cells—To investigate the role of the predicted PKA phosphorylation site Ser-409 of MARK2 in the regulation of microtubule stability, EGFP-MARK2 S409A or MARK2 S409E was cotransfected with or without HA-PKAc into HEK293 cells. As shown in Fig. 4*A*, MARK2 S409A overexpression caused microtubule disruption in 66% of the transfected HEK293 cells, similar to the microtubule disrupted cells percentage (60%) caused by MARK2 WT overexpression ($p = 0.35$). However, PKA did not reverse the microtubule disruption induced by MARK2 S409A. Microtubule disruption was caused in 61% of the MARK2 S409A and HA-PKAc co-expressed cells, not significantly different from that of the MARK2 S409A expressed alone ($p = 0.68$) (Fig. 4, *A* and *C*), whereas PKAc did not change the MARK2 S409A protein homeostasis (Fig. 4*B*). This suggested that the mutation of Ser to Ala at 409 blocked the phosphorylation of this site by PKA, leading to the inability of PKA to counteract MARK2.

As shown in Fig. 4, *D* and *E*, only 27% of the MARK2 S409E-overexpressing cells had disrupted microtubules, similar to the control cells ($p = 0.079$) and significantly less than that of the MARK WT ($p = 0.013$) or MARK2 S409A-overexpressing cells ($p = 0.003$). In agreement with the kinase activity data *in vitro*, the overexpression of the MARK2 mutant S409E alone, mimicking a continuously phosphorylated state at Ser-409, did not disrupt the microtubule stability, similar to the rescue effect of active PKA on MARK2 WT. Together, our data support that PKA rescues the disruption of microtubule stability induced by MARK2 by phosphorylating MARK2 on Ser-409.

PKA Interacts with MARK2 in the Embryonic Brain and Primary Neurons—Because MARK2 elicits profound effects on neuronal differentiation (10, 19, 20), we further assessed the biological relevance of the PKA-MARK2 interaction in the nervous system. The complex containing PKA and MARK2 was immunoprecipitated from the E18 mouse brain (Fig. 5*A*), demonstrating that PKA and MARK2 endogenously interact. MAP2 could also be detected in the PKA-MARK2 immune complex (Fig. 5*A*). It suggested that MAP2 (or other proteins) as the substrate of MARK2 and the anchoring protein of PKA (48) may facilitate the interaction between PKA and MARK2 in brain.

To monitor the state of endogenous MARK2 and PKA in primary neurons, we probed primary hippocampal neurons with antibodies against MARK2 and PKAc. As shown in Fig. 5*B*, PKAc and MARK2 were mostly distributed in the cytoplasm and neurite protrusions. The colocalization of PKAc and MARK2 was partial and mainly occurred in certain zones near neurites. The partial localization of PKAc and MARK2 was observed in the tips of neurites in growth cones and some segments along neurites. All of these loci of colocalization are

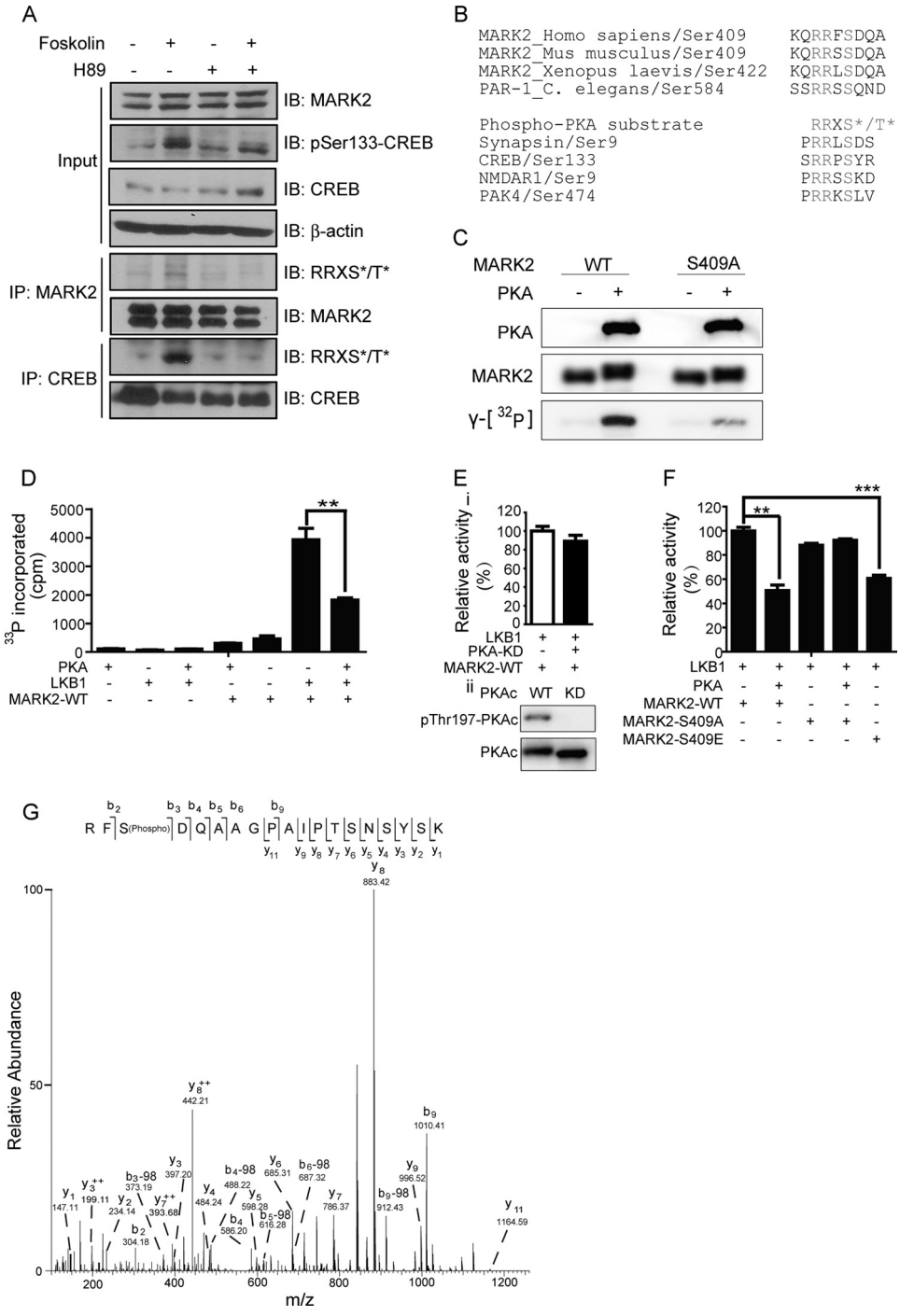

FIGURE 3. **PKA phosphorylates MARK2 at a novel site, Ser-409.** *A*, Western blot (*IB*) analysis directed against phospho-PKA substrates (RR*X*(S*/T*)) in endogenous MARK2 or CREB immunoprecipitated (*IP*) from HEK293 cells pretreated with 10 μM H89 (a PKA inhibitor) for 0.5 h before treatment with 10 μM forskolin for 30 min. *B*, sequence alignment of MARK orthologues illustrates conservation of the RR*X*(S/T) motif in MARK2 between species. The kinases and transcription factors that contain the RR*X*(S/T) motif were confirmed as phosphorylation substrates of PKA. *NMDAR*, NMDA receptor. *C*, recombinant MARK2 WT or MARK2 S409A (400 nM) was incubated with or without PKAc in the presence of 100 μM ATP and 5 μCi of [γ-^{32}P]ATP per reaction at 30 °C for 4 h. Reaction products were analyzed by autoradiography. *D*, recombinant MARK2 WT were incubated with or without recombinant PKAc (400 nM) at 30 °C for 4 h in 50 mM HEPES, pH 7.5, 10 mM $MgCl_2$, 1 mM EGTA, 0.01% Brij-35, and 100 μM ATP. Because the *E. coli*-derived recombinant MARK2 has no phosphorylation modification and low kinase activity, 20 nM LKB1 was added to pre-phosphorylate and activate MARK2 proteins *in vitro*. The analysis of the indicated protein kinase activity was performed using a ^{33}P incorporation assay against a well known substrate of MARK2, the AMARA peptide. Radioactivity that had been incorporated in AMARA was determined by liquid scintillation counting in a Wallac MicroBeta plate counter. Neither PKAc nor LKB1 showed any activity against AMARA peptide. *E*, analysis of the activity of MARK2 WT (400 nM) with or without a kinase-dead mutant of PKAc (PKAc K72I, PKA-PD) (400 nM) were performed using a ^{33}P incorporation assay against AMARA. *F*, analyses of the activities of MARK2 proteins (MARK2 WT with or without PKAc, MARK2 S409A with or without PKAc, MARK2 S409E) were performed using a ^{33}P incorporation assay against AMARA. All reactions were repeated in three independent experiments. **, $p < 0.005$; ***, $p < 0.001$. *G*, detection of phosphorylation at Ser-409 in MARK2 by MS/MS analysis. Recombinant MARK2 WT were incubated with or without recombinant PKAc (400 nM) at 30 °C for 4 h in 50 mM HEPES, pH 7.5, 10 mM $MgCl_2$, 1 mM EGTA, 0.01% Brij-35, and 100 μM ATP. Shown is a high resolution MS/MS spectrum of a tryptic peptide (RFpSDQAAGPAIPTSNSYSK) from MARK2 by higher energy collision dissociation (*HCD*) in a Q Exactive mass spectrometer. The fragmented y and b ions indicated that Ser-409 of the protein was phosphorylated. The neutral loss of 98 Da corresponding to the mass of a phosphoryl group is marked.

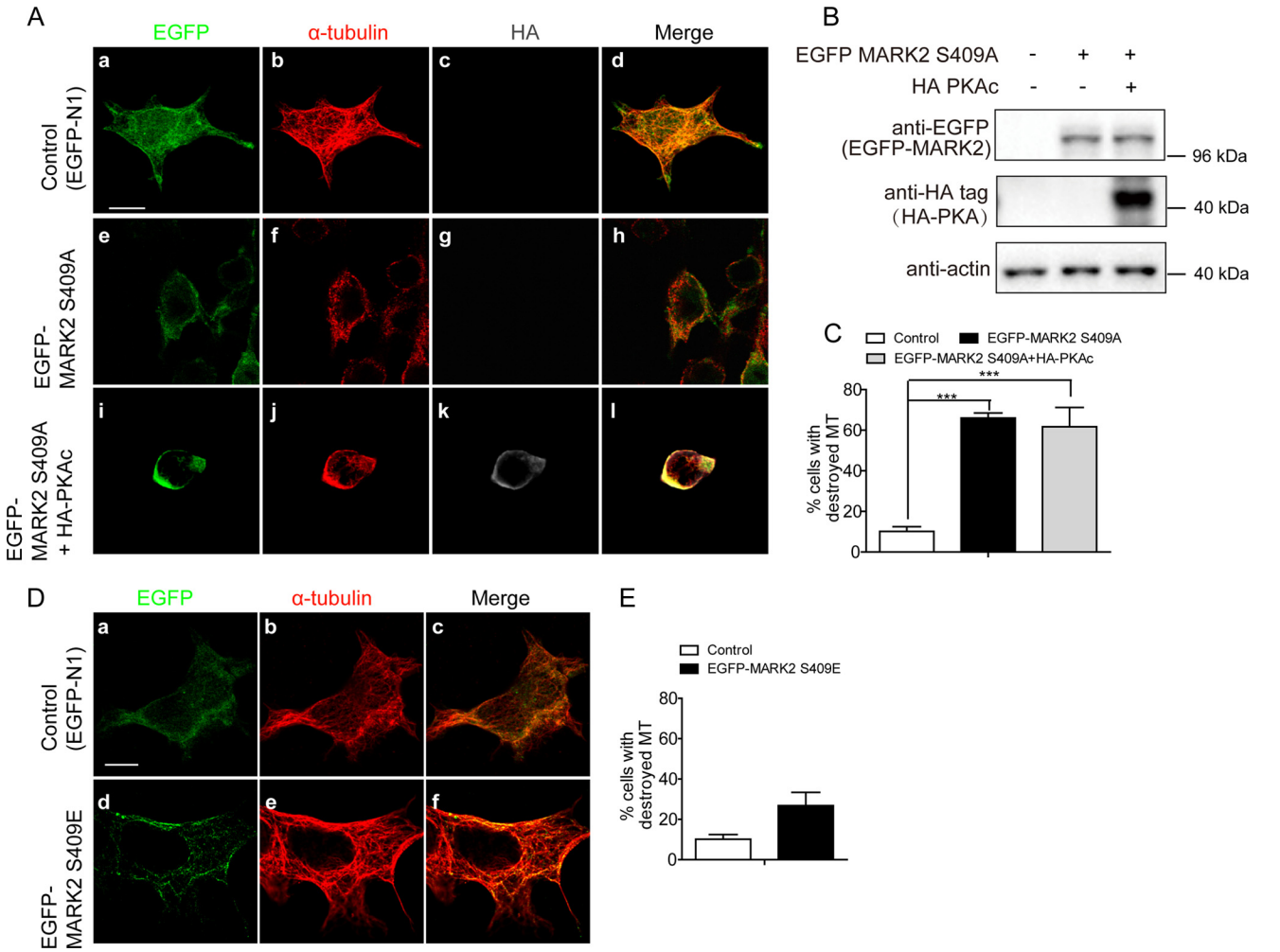

FIGURE 4. **PKA counteracts the effects of MARK2 on microtubule stability by phosphorylating MARK2 Ser-409 in HEK293 cells.** Immunochemistry of HEK293 cells transfected with EGFP-MARK2 S409A with or without HA-PKAc (*A*, *B*, and *C*) or EGFP-MARK2 S409E (*D* and *E*). After 24 h, the cells were fixed and immunostained for HA (*gray*) and α-tubulin (*red*). *A* and *D*, images were captured by a confocal microscopy. *Scale bars* in all images are 10 μm. *B*, Western blot analysis of the cell lysates directed against EGFP, HA, and endogenous protein actin. *C* and *E*, EGFP-positive cells (control, EGFP-MARK2 S409A, EGFP-MARK2 S409E) or EGFP and HA-double positive cells (EGFP-MARK2 S409A+HA-PKAc) with disrupted microtubule networks were quantified and plotted by a high content system. Data are the mean ± S.E. from three independent experiments and at least 100 EGFP-positive cells or EGFP, and HA double-positive cells were analyzed for each transfection. ***, $p < 0.001$. *MT*, microtubule.

involved in the formation and maintenance of neurite outgrowth, indicating that endogenous MARK2 and PKA may interact to regulate neurite outgrowth.

PKA Counteracts MARK2 in Primary Neuron Neurite Outgrowths in a Ser-409 Phosphorylation-dependent Manner—The overexpression of MARK2, but not its kinase-defective mutant, in undifferentiated primary hippocampal cultures impairs neuronal microtubule stability, thereby blocking neurite outgrowth (20). To investigate the influence of PKAc on this function of MARK2, primary hippocampal neurons at day *in vitro* 0 were cotransfected with EGFP-MARK2 WT and HA-PKAc. These neurons were examined at day *in vitro* 2 for neurite outgrowth by assaying the expression of axon marker Tau. Remarkably, the overexpression of MARK2 in hippocampal neurons induced a defective neurite outgrowth and decreased the total length of neurite branches (TLNB) (Fig. 6, *A*, *e–h*) compared with the neurons transfected with pEGFP-N1 (Fig. 6, *A*, *a–d*). The TLNB of neurons with MARK2 overexpression was reduced to 0.1958 that of the TLNB of the control neurons ($p <$ 0.001) (Fig. 6*C*). However, most of the cells cotransfected with EGFP-MARK2 WT and HA-PKAc exhibited normal morphology (Fig. 6, *A*, *i–l*) and a normal TLNB (1.18-fold the TLNB of the control neurons) ($p = 0.069$) (Fig. 6*C*). Ectopic expression of PKAc alone in hippocampal neurons exhibited normal neurite outgrowth (Fig. 6*B*) and TLNB ($p = 0.053$, Fig. 6*D*).

The MARK2 S409A mutant induced significantly defective neurite outgrowth (*white arrow* indicated cells in Fig. 6, *E*, *e–h*) and decreased the TLNB to 0.17 that of the control neurons ($p < 0.001$) (Fig. 6*G*). The TLNB of the neurons cotransfected with PKAc and MARK2 S409A (*white triangles* indicated cells in Fig. 6, *E*, *i–l*) was 0.21-fold that of the TLNB of the empty vector-transfected neurons (Fig. 6*G*). Both morphological (Fig. 6E, *i–l*) and statistical analysis ($p = 0.31$) (Fig. 6*G*) showed that PKA had no rescue effect on the defective neurite outgrowth induced by MARK2 S409A compared with neurons transfected with MARK2 S409A alone.

As noted above for HEK293 cells, the primary neurons transfected with EGFP-MARK2 S409E alone exhibited normal

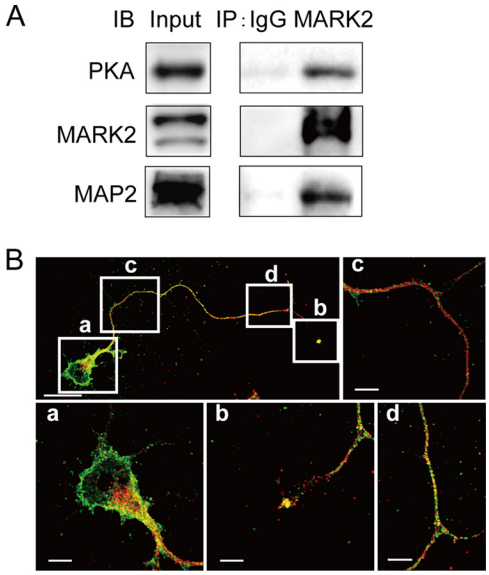

FIGURE 5. **PKA interacts with MARK2 in the embryonic brain and primary neurons.** *A*, coimmunoprecipitation (*IP*) of endogenous proteins from E18 mouse brain. Anti-MARK2 antibody or control IgG was used for immunoprecipitation. MARK2, PKAc, and MAP2 were detected by immunoblotting (*IB*) with specific antibodies. *B*, immunofluorescence of endogenous MARK2 (*green*) and PKAc (*red*) in hippocampal neurons at day *in vitro* 2. Images were captured by confocal microscopy. The *scale bars* in the overview images represent 20 μm; those in *panels a*, *b*, *c*, and *d* represent 5 μm.

TLNB (Fig. 6, *F* and *H*), similar to the neurons transfected with the control vector ($p = 0.085$), suggesting that mimicking continuous phosphorylation of MARK2 at Ser-409 is able to restore the defects of neurite growth caused by MARK2 overexpression. These results indicate that PKA prevents defects of neurite outgrowth induced by MARK2 by phosphorylating MARK2 at Ser-409.

PKA Inhibits Tau Ser-262 Phosphorylation Caused by MARK2—Tau is a well known MAP that plays an important role in neurodegenerative disorders. One of the Tau protein main functions is to modulate the stability of axonal microtubule. As a downstream substrate of MARK2, Tau is phosphorylated by MARK2 at Ser-262, and ectopic expression of MARK2 increases the phosphorylation of Tau Ser-262 and loss of axons. We examined Tau Ser-262 phosphorylation in primary hippocampal neurons to evaluate the kinase activity of MARK2 and the biological significance of the PKA-MARK2 interaction.

Immunoblotting analysis indicated that the overexpression of PKAc alone did not influence Tau Ser-262 phosphorylation (Fig. 7*A*), but it inhibited the Tau Ser-262 phosphorylation caused by MARK2 WT (Fig. 7*B*). MARK2 S409A impaired this inhibition because of its unregulated S409A mutation (Fig. 7*C*). Cells expressing MARK2 S409E exhibited decreased phosphorylation at Tau Ser-262 compared with cells expressing MARK2 S409A (Fig. 7*C*). This result suggests that PKA inhibits MARK2-induced Tau Ser-262 phosphorylation by phosphorylating MARK2 at Ser-409.

DISCUSSION

MARK2 may have different roles in various cell types and organisms, but it generally plays a central role in regulating microtubule stability and neurite outgrowth by phosphorylating MAPs (MAP2, MAP4, and Tau) (5–8, 21). MARK2 contributes to the detachment of MAPs from microtubules, thereby causing microtubule destabilization (7, 22–25).

Many signaling molecules regulate microtubule dynamics and neuronal outgrowth by affecting MARK2 activity through the phosphorylation of MARK2 at various sites and binding with different domains of MARK2 (26–28). MARK2 is activated by phosphorylation at a conserved threonine moiety (threonine 208) and inactivated by phosphorylation at a serine moiety (serine 212), both of which are situated in the activation loop of the catalytic domain. Activation of MARK2 can be achieved by MARK kinase (MARKK)/1001 amino acids (TAO1) (9), a member of the Ste20 kinase family, or by LKB1 at threonine 208 (29). Glycogen synthase kinase 3 serves as an inhibitory kinase by phosphorylating MARK2 at serine 212 (30).

Some regulatory sites other than Thr-208 and Ser-212 have been discovered in MARK2. Calmodulin-dependent kinase I activates MARK2 by phosphorylating MARK2 at serine 92 and threonine 294, thus promoting neurite outgrowth (31). Similarly, atypical protein kinase C (aPKC) inhibits MARK2 through phosphorylation at threonine 595. This induces binding of MARK2 to the scaffold protein 14-3-3/PAR5, which results in relocation of the kinase from the cell membrane to the cytosol (10, 32, 33). Protein kinase D directly phosphorylates MARK2 at serine 400 to positively regulate 14-3-3 binding and negatively regulate membrane association (34).

MARK2 is also regulated by mechanisms other than phosphorylation. Death-associated protein kinase (DAPK) inhibits microtubule assembly by activating MARK2. The death-associated protein kinase death domain but not catalytic activity is responsible for this activation by binding to the MARK2 spacer region, thereby disrupting an intramolecular interaction that inhibits MARK2 (35). PAK5, a neuronal member of the p21-activated kinase family, suppresses the activity of MARK2 (36). This inhibition requires binding between the PAK5 and MARK2 catalytic domains but does not require phosphorylation. These molecules perturb MARK2 activity, thereby affecting microtubule stability and neuronal outgrowth.

Our study reveals for the first time that PKA acts as an inhibitor of MARK2. PKA negatively regulated MARK2 via phosphorylation at a novel site, Ser-409. Forskolin induced PKA to phosphorylate MARK2 at its RR*X*(S/T) motif (Fig. 3*A*). Sequence analysis, autoradiography, and MS/MS analysis helped us predict that Ser-409 was the most likely PKA target (Fig. 3, *B*, *C*, and *G*). PKA inhibited MARK2 kinase activity *in vitro* and significantly rescued the microtubule disruption induced by MARK2. The mutation of MARK2 Ser-409 to Ala or Glu blocked or mimicked, respectively, the inhibitory effects of PKA on MARK2. We also found mutation at Ser-409 of MARK2 does not affect its formation of an immune complex with PKA (data not shown). Although the possibility that PKA phosphorylates other sites on MARK2 cannot be excluded, phosphorylation at Ser-409 by PKA was shown to be necessary for its inhibition of MARK2 function.

Phosphorylation at other sites inside and outside of the kinase domain of MARK2 either affects the activity of the kinase domain (calmodulin-dependent kinase I at Ser-92 and Thr-294, activator) or mediates the interaction with regulatory

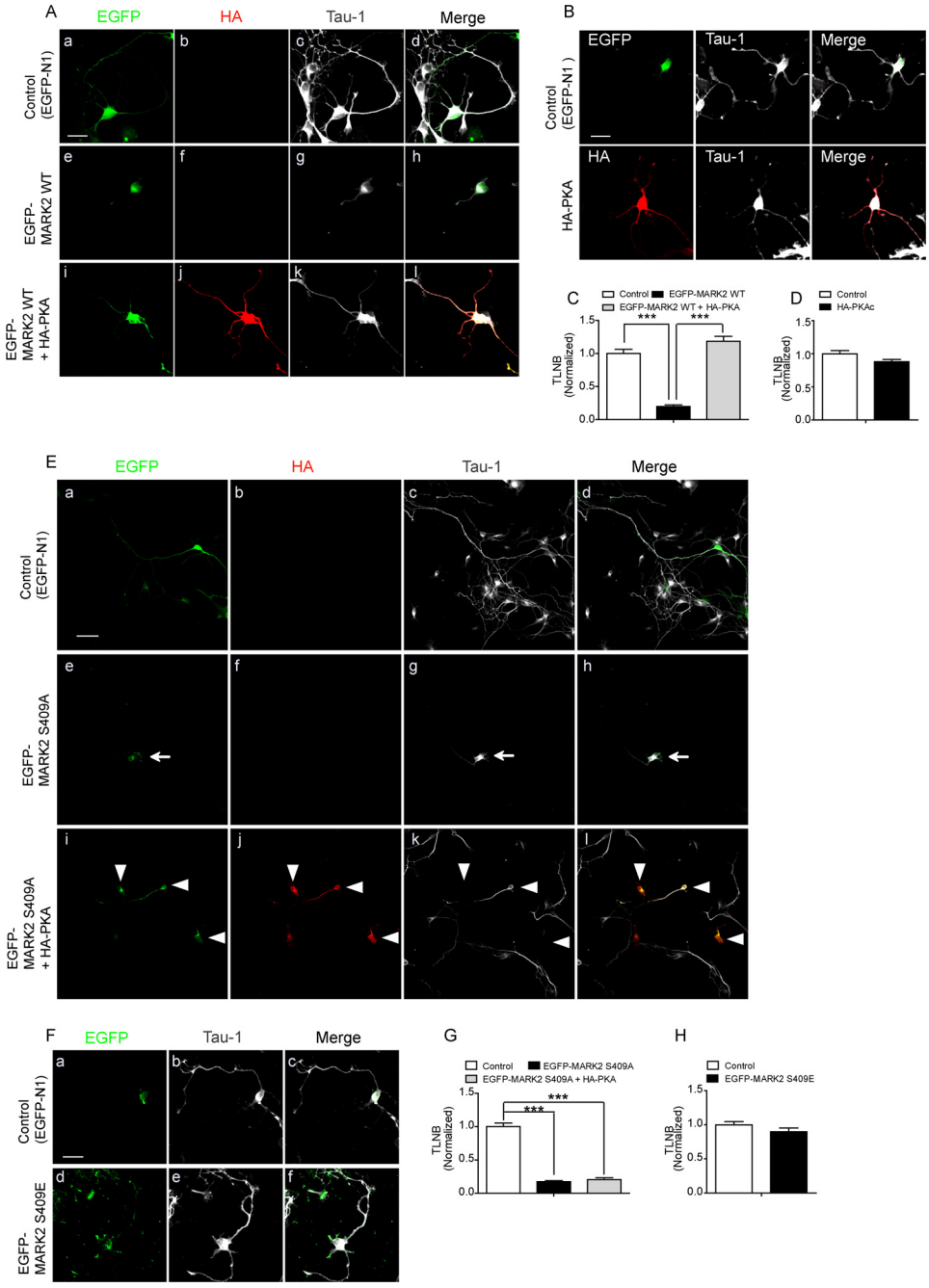

FIGURE 6. **PKA counteracts MARK2 inhibition of neurite formation by phosphorylating Ser-409.** Primary hippocampal neurons were transfected with EGFP-MARK2 with or without HA-PKAc (*A* and *C*), HA-PKAc (*B* and *D*), EGFP-MARK2 S409A with or without HA-PKAc (*E* and *G*), or EGFP-MARK2 S409E alone (*F* and *H*). At day *in vitro* 2, transfected cells were stained with antibodies against HA and Tau1. *A*, *B*, *E*, and *F*, all images were captured by confocal microscopy. The *white arrows* indicate EGFP-positive neurons (MARK2 S409A-transfected cells), and the *solid white triangles* indicated the EGFP and HA-double positive neurons (MARK2 S409A and PKA double-transfected cells). The *scale bars* in all images represent 20 μm. *C*, *D*, *G*, and *H*, quantitative analysis of the TLNBs. Data are the mean ± S.E. ***, $p < 0.001$. All experiments were repeated three times. At least 30 cells for each transfection were analyzed per experiment.

proteins (atypical PKC at Thr-595, protein kinase D at Ser-400, both inhibitors). Serine 409 is outside the kinase domain, between the reported two inhibitory sites, Thr-595 and Ser-400. It is interesting that PKA shows a stronger inhibitory effect on MARK2 in a cellular functional assay than that in kinase activity assay *in vitro*. We speculate that phosphorylation at Ser-409 by PKA may also mediate the interaction with regulatory proteins such as 14-3-3. The details of how phosphorylation at Ser-409 affects MARK2 activity are still unclear and will be further investigated.

PKA plays an important role in the regulation of microtubule stability and neuronal polarity. Forskolin attenuates the microtubule disassembly induced by the anti-microtubule agent nocodazole (11). Furthermore, Aglah *et al.* (12) reported that cAMP promotes neurite outgrowth and extension through PKA but independently of Erk activation in cultured rat motor

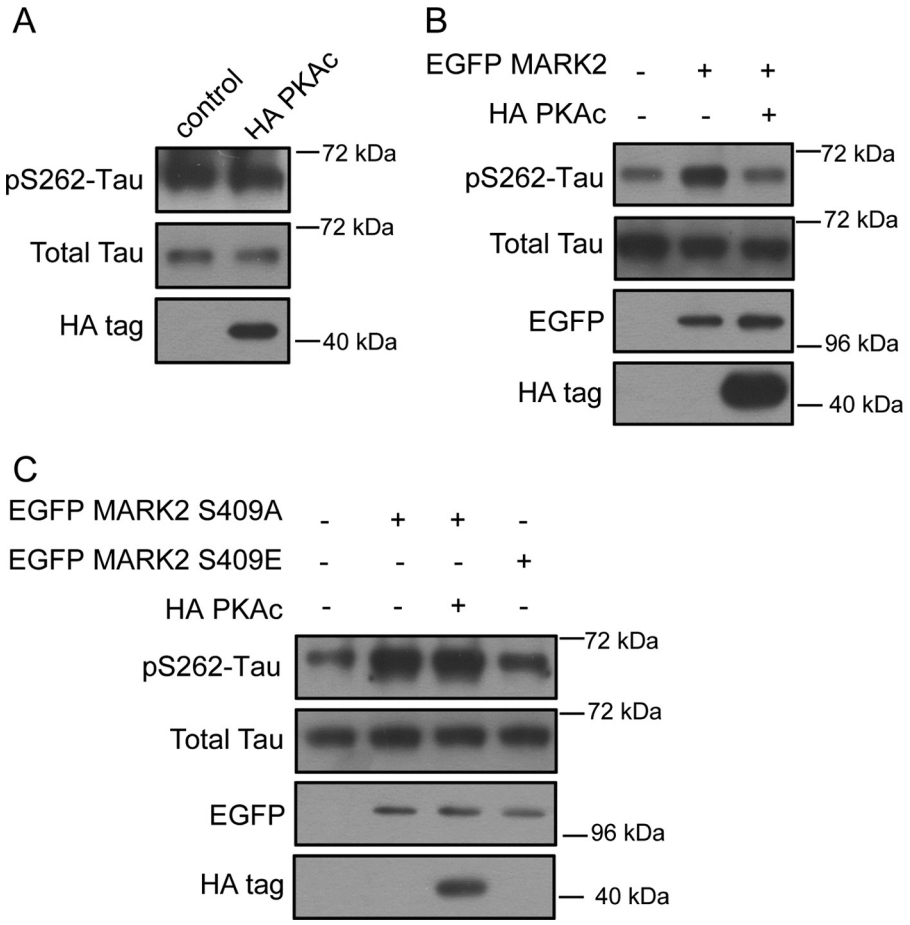

FIGURE 7. **PKAc inhibits Tau Ser-262 phosphorylation caused by MARK2.** Primary hippocampal neurons were transfected with HA-PKAc alone (*A*), EGFP-MARK2 with or without HA-PKAc (*B*), EGFP-MARK2 S409A with or without HA-PKAc, or EGFP-MARK2 S409E alone (*C*). Cell lysates were processed for the detection of phospho-Tau Ser-262 by immunoblot.

neurons. However, the above studies provided little evidence of the mechanisms that link activated PKA to microtubule stabilization or neurite outgrowth promotion.

PKA likely regulates microtubule stability and neuronal outgrowth by directly phosphorylating microtubule-associated proteins. Alexa *et al.* (37) reported that MAP2c can be phosphorylated by PKA at threonine 220 and that this phosphorylation may be a primary determinant of microtubule function because MAP2c specifically dephosphorylated at this site binds and stabilizes microtubules stronger than either fully phosphorylated or nonphosphorylated MAP2c. In neurons, PKA can also phosphorylate Tau at Ser-214 (*e.g.* during mitosis). This phosphorylation strongly decreases the Tau-microtubule interaction (38–40). The role of PKA in neuronal outgrowth remains controversial because many of the permissive substrates of PKA can also phosphorylate Tau. Glycogen synthase kinase-3, which is inhibited by PKA, is one example (38, 41). We suggest that PKA, as an upstream activator and deactivator of many substrates, plays an essential role in regulating microtubule dynamics, exerting complex effects rather than a single, unidirectional effect according to the specific cell type and specific physiological condition. Here PKA negatively regulated MARK2 to restore microtubule stability and neurite outgrowth by phosphorylating Ser-409 of MARK2. This finding will further expand our understanding of the PKA downstream network that is important for regulating microtubule stability and neuronal outgrowth.

Recent studies on human pre-implantation embryonic development have shown a large increase of MARK2 and PKA expression at certain stages (42). The rise in the MARK2 protein level might influence microtubule stability. Hence, it is likely beneficial to limit the activity of MARK2 to a degree that would not disrupt embryonic development, and this could be accomplished by an elaborate regulatory mechanism, such as inhibition by PKA. PKA likely inhibits MARK2 by phosphorylating Ser-409 under the specific temporal and spatial conditions in development, which would keep the activity of MARK2 in dynamic balance. MARK2 might have many upstream regulators, and PKA has many downstream effectors; thus, the significance of the PKA inhibition of MARK2 during biological processes, especially neuronal development, should be further investigated.

The hyperphosphorylation of Tau is an early step in the degeneration of neurons in Alzheimer disease (AD) and other tauopathies (39). Nishimura *et al.* (43) showed that *Drosophila* PAR-1 kinase initiated Tau toxicity by triggering a temporally ordered phosphorylation process. PAR-1 directly phosphorylates Tau at Ser-262 and Ser356. This phosphorylation event is a prerequisite for the action of downstream kinases, including glycogen synthase kinase-3 and cyclin-dependent kinase 5

(Cdk5) to phosphorylate several other sites and generate disease-associated phospho-epitopes (43). The augmented interactions between MARK2 and Tau in AD brain sections suggest that MARK2 may play an important role in the early phosphorylation of Tau in AD, possibly qualifying as a therapeutic target for intervention to prevent disease progression (44, 45). Impairment of the cAMP/PKA/CREB pathway may contribute to the learning/memory deficits in AD (46, 47). Here, we demonstrated the inhibition of MARK2 by PKA as a new mechanism of microtubule regulation, and the roles of PKA and MARK2 in AD pathogenesis should be further investigated.

In summary, PKA works as a negative upstream regulator of MARK2 in regulating microtubule stability and neurite outgrowth by phosphorylating Ser-409 of MARK2. Evidence of direct cross-talk between PKA and MARK2 might reveal a novel molecular pathway that affects microtubule stability and neuronal differentiation. The identification of a PKA-MARK2 interaction provides new insight into both the PKA and MARK2 signal networks.

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