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Cyclic AMP-dependent protein kinase regulates 9G8-mediated alternative splicing of tau exon 10

Jianlan Gu [a,c,1], Jianhua Shi [b,d,1], Shiliang Wu [a], Nana Jin [b,d], Wei Qian [b,c], Jianhua Zhou [b], Inge-Grundke Iqbal [d], Khalid Iqbal [d], Cheng-Xin Gong [b,d], Fei Liu [b,d,*]

[a] Department of Biochemistry and Molecular Biology, Medical School, Soochow University, 199 Renai Road, Soochow, Jiangsu 215123, China
[b] Jiangsu Key Laboratory of Neuroregeneration, Nantong University, 19 Qixiu Road, Nantong, Jiangsu 226001, China
[c] Department of Biochemistry and Molecular Biology, School of Medicine, Nantong University, 19 Qixiu Road, Nantong, Jiangsu 226001, China
[d] Department of Neurochemistry, New York State Institute for Basic Research in Developmental Disabilities, 1050 Forest Hill Road, Staten Island, NY 10314, USA

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ABSTRACT

Alternative splicing of tau exon 10 generates tau isoforms with three or four microtubule-binding repeats, named 3R- or 4R-tau. Normal adult human brain expresses equal levels of them. Imbalance of 3R-tau and 4R-tau associates with several tauopathies. Splicing factor 9G8 suppresses tau exon 10 inclusion and its function is regulated by phosphorylation. Here, we found that cyclic AMP-dependent protein kinase (PKA) phosphorylated 9G8. The catalytic subunits α and β of PKA interacted with 9G8, and activation of PKA enhanced the interaction. Up-regulation of PKA activity prevented 9G8 from inhibition of tau exon 10 inclusion. These findings provide novel insights into the regulation of tau exon 10 splicing and further our understanding of neurodegeneration associated with dysregulation of tau exon 10 splicing.

Structured summary of protein interactions:
9G8 physically interacts with **PKA catalytic subunit** by coimmunoprecipitation (View interaction)

1. Introduction

Tau is a major neuronal microtubule-associated protein and plays an important role in the assembly and stabilization of microtubules. Hyperphosphorylation of tau leads to its aggregation into neurofibrillary tangles, a hallmark of Alzheimer disease (AD) and related neurodegenerative diseases called tauopathies [1–4]. Adult human brain expresses six tau isoforms from a single gene by alternative splicing of exons 2, 3, and 10 of its pre-mRNA [5]. The exon 10 encodes the second microtubule binding repeat [6], and its alternative splicing generates tau isoforms with three or four microtubule-binding repeats (3R-tau or 4R-tau), which are under developmental and cell type-specific regulation. In normal adult human brain, approximately equal levels of 3R- and 4R-tau are expressed [6,7]. Alteration in 3R-tau/4R-tau ratio is sufficient to trigger neurodegeneration in frontotemporal dementia and might also play a role in other neurodegenerative disorders, such as Pick's disease, corticobasal degeneration, or progressive nuclear palsy [8–11].

Alternative splicing of tau exon 10 is regulated by several trans-acting factors, including serine/arginine-rich (SR) proteins [12–16]. 9G8, one of the SR proteins, is believed to be involved in both constitutive splicing and alternative splicing of many pre-mRNAs. It can help mRNA export to the cytoplasm and enhance the expression of unspliced mRNA [17]. Recent studies have shown that 9G8 inhibit tau exon 10 inclusion [18,19]. The localization and functions of 9G8 are tightly regulated by phosphorylation, and overexpression of dual-specificity tyrosine-phosphorylated and regulated kinase 1A (Dyrk1A) drives 9G8 to cytosolic compartment and suppresses its function in tau splicing [19].

Cyclic AMP (cAMP)-dependent protein kinase (PKA) is a key kinase that interacts with many proteins involved in the etiology of AD as well as other tauopathies. It has been shown that PKA phosphorylates tau at several sites and primes phosphorylation of tau by glycogen synthase kinase-3β [20]. In the absence of cAMP, PKA is an inactive tetrameric holoenzyme consisting of two catalytic (PKA-C) subunits and two regulatory (PKA-R) subunits. Stimulation by cAMP dissociates PKA-R from PKA-C and causes a fraction of the C subunit translocated into the nucleus. Apart from regulation of transcription, little is known about the function of the

* Corresponding author at: Department of Neurochemistry, New York State Institute for Basic Research in Developmental Disabilities, 1050 Forest Hill Road, Staten Island, NY 10314, USA. Fax: +1 718 494 1080.
E-mail address: feiliu63@hotmail.com (F. Liu).
[1] These authors contribute equally to this work.

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C subunit in the nucleus. It is known that PKA phosphorylates several splicing factors and is involved in the pre-mRNA splicing, including tau pre-mRNA [21–23]. The role of PKA in the 9G8 mediated alternative splicing of tau exon 10 was unclear. In the present study, we found that PKA interacted with and phosphorylated 9G8. Activation of PKA or overexpression of PKA-C inhibited 9G8-promoted tau exon 10 exclusion. These results suggest that PKA may be involved in the regulation of tau alternative splicing via 9G8 and in tau pathogenesis in AD and related tauopathies.

2. Materials and methods

2.1. Plasmids, antibodies, and other reagents

pCEP4/9G8 tagged with HA (hemagglutinin tag) at C-terminal was a gift from Dr. Tarn of the Institute of Biomedical Sciences, Academia Sinica, Taiwan. pCI/SI9-LI10 containing a tau minigene, SI9/LI10, comprising tau exons 9, 10, and 11 and part of intron 9 and the full-length of intron 10, pCI/PKA-Cα, pCI/PKA-Cβ and pGEX-4T/9G8 have been described previously [19,24,25]. The catalytic subunit of PKA, monoclonal anti-HA, forskolin and bd-cAMP were bought from Sigma (Sigma, St. Louis, MO, USA). Polyclonal anti-PKA-Cα and anti-PKA-Cβ, siRNAs of mouse 9G8, human PKA-Cα and PKA-Cβ were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Polyclonal anti-CREB and anti-p-CREB were from Cell Signaling Technology. Peroxidase-conjugated anti-mouse and anti-rabbit IgG were obtained from Jackson Immuno Research Laboratories (West Grove, PA, USA); Alexa 488-conjugated goat anti-mouse IgG, and Alexa 555-conjugated goat anti-rabbit IgG and TO-PRO-3 iodide (642/661) were from Invitrogen (Invitrogen, Carlsbad, CA, USA). The ECL kit was from Thermo Fisher Scientific (Rockford, IL, USA), and [γ-^{32}P]ATP and [^{32}P] orthophosphate were from MP Biomedicals (Irvine, CA, USA).

2.2. Cell culture and transfection

HEK-293FT, N2a and HeLa cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA, USA) at 37 °C (5% CO_2). All transfections were performed in triplicates with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) or FuGENE HD (Roche Diagnotics, Indianapolis, IN, USA) according to the manufacturer's instructions. For N2a cells, after transfection, the cells were differentiated with 20 μM retinoic acid in DMEM supplemented with 2% FBS.

2.3. In vitro phosphorylation of 9G8 by PKA

GST-9G8 or GST (0.2 mg/ml) was incubated with various concentrations of PKA-C in a reaction buffer consisting of 50 mM HEPES, pH 6.8, 10 mM $MgCl_2$, 10 mM β-mercaptoethanol, 1.0 mM EGTA, and 0.2 mM [γ-^{32}P] ATP (500 cpm/pmol). After incubation at 30 °C for 30 min, the reaction was stopped by boiling with an equal volume of 2× Laemmli sample buffer. The reaction products were separated by SDS–PAGE. Incorporation of ^{32}P was detected by exposure of the dried gel to phosphor-image system (BAS-1500, Fuji film).

2.4. Mass spectrometry

GST-9G8 fusion protein was phosphorylated by PKA-C as described above. In order to maximize the yield of the phosphorylated protein, the reaction was carried out for 1 h with high amounts of the kinase (6:1 molar ratio of GST-9G8 and PKA-C). The phosphorylated products were separated in SDS–PAGE and stained by Coomassie blue. The GST-9G8-containing gel piece was in-gel trypsin digested. Proteolytic peptides were extracted from the gel, followed by TiO2 immobilized metal affinity chelate (IMAC) enrichment for the phosphopeptides. The resulting fraction was concentrated and reconstituted in 10 μl of 5% formic acid for LC–MS/MS (liquid chromatography–mass spectrometry/mass spectrometry) analysis.

2.5. Co-immunoprecipitation

HEK-293FT cells were transfected with pCEP4/9G8-HA for 40 h as described above and treated with 5 or 10 μm forskolin for 8 h, then the cells were washed twice with phosphate-buffered saline (PBS), and lysed by sonication in lysate buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM Na_3VO_4, 50 mM NaF, 2 mM EDTA, 1 mM PMSF, and 10 μg/ml of aprotinin, leupeptin and pepstatin). The cell lysate was centrifuged at 16000×g for 10 min. The extract was incubated with anti-HA overnight at 4 °C, and then protein-G-conjugated agarose beads were added. After 4 h of incubation at 4 °C, the beads were washed with lysate buffer twice and with tris-buffered saline (TBS) twice, and bound proteins were eluted by boiling in 2× Laemmli sample buffer. The samples were subjected to Western blot analysis with the indicated primary antibodies.

2.6. Co-localization of PKA with 9G8

HeLa cells were plated in 24-well plates onto coverslips 1 day before transfection at 30–40% confluence. The cells were transfected with pCEP4/9G8-HA for 40 h and treated with 10 μm forskolin for 30 min to activate PKA. Then the cells were fixed with 4% paraformaldehyde in PBS for 30 min at room temperature. After washing with PBS, the cells were blocked with 10% goat serum in 0.2% Triton X-100/PBS for 2 h at 37 °C, and then incubated with mouse anti-HA (1:1000) and rabbit anti-PKA-C (1:50) overnight at 4 °C. The cells were incubated for 1 h with secondary antibody (Alexa 488-conjugated goat anti-mouse IgG, 1:1000, Alexa 555-conjugated goat anti-rabbit IgG, 1:1000) plus TO-PRO-3 iodide at room temperature. After washing with PBS, the cells were mounted with Fluoromount-G, and observed with a Nikon TCS-SP2 laser-scanning confocal microscope.

2.7. Knockdown of 9G8 or PKA catalytic subunits with RNA interference

N2a or HEK-293FT cells were transfected with various amounts of short interfering RNAs, si9G8, siPKA-Cα and siPKA-Cβ (Santa Cruz Biotechnology) using Lipofectamine 2000 according to the manufacturer's instructions. After 48 h transfection, protein or RNA was extracted as described above or below. The same amount of scramble siRNA was used for control.

2.8. Quantitation of tau exon 10 splicing by reverse transcription–PCR (RT–PCR)

Total cellular RNA was extracted from cultured cells by using the RNeasy mini kit (Qiagen, GmbH, Germany) according to the manufacturer's instruction. One microgram of total RNA was used for first-strand cDNA synthesis with oligo-$(dT)_{15-18}$ by using the Omniscript reverse transcription kit (Qiagen). PCR was performed by using PrimeSTAR™ HS DNA Polymerase (Takara Bio Inc., Otsu, Shiga, Japan) with forward primer 5′-GGTGTCCACTCCCAGTTCAA-3′ and reverse primer 5′-CCCTGGTTTATGATGGATGTTGCCTAAT-GAG-3′ to measure alternative splicing of tau exon 10. The PCR conditions were: 98 °C for 5 min, 98 °C for 10 s, 68 °C for 40 s for 30 cycles and then 68 °C 10 min for extension. The PCR products

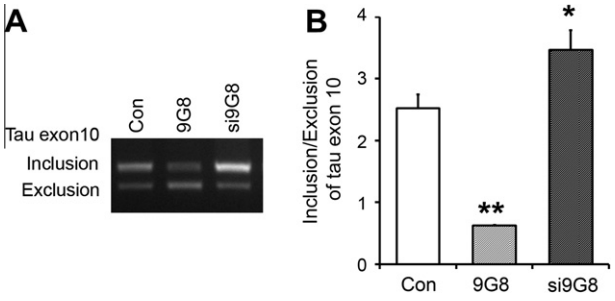


Fig. 1. 9G8 inhibits tau exon 10 inclusion. (A) N2a cells were co-transfected with pCI/SI9-LI10 mini-tau gene and pCEP4/9G8 or si9G8 using Lipofectamine 2000 for 48 h. The total RNA was then extracted and amplified by RT–PCR, followed by gel electrophoresis to measure tau exon 10 splicing. (B) The ratio of inclusion/exclusion of tau exon 10 was calculated after densitometry. The data are represented as mean ± S.D. *, $p < 0.05$; **, $p < 0.01$ vs. controls.

were resolved on 1.5% agarose gels, visualized by ethidium bromide staining and quantitated using the Molecular Imager system (Bio-Rad).

2.9. Statistical analysis

Where appropriate, the data are presented as the means ± S.D. Data points were compared by the unpaired two-tailed Student's *t* test, and the calculated *p* values are indicated in the figures.

3. Results

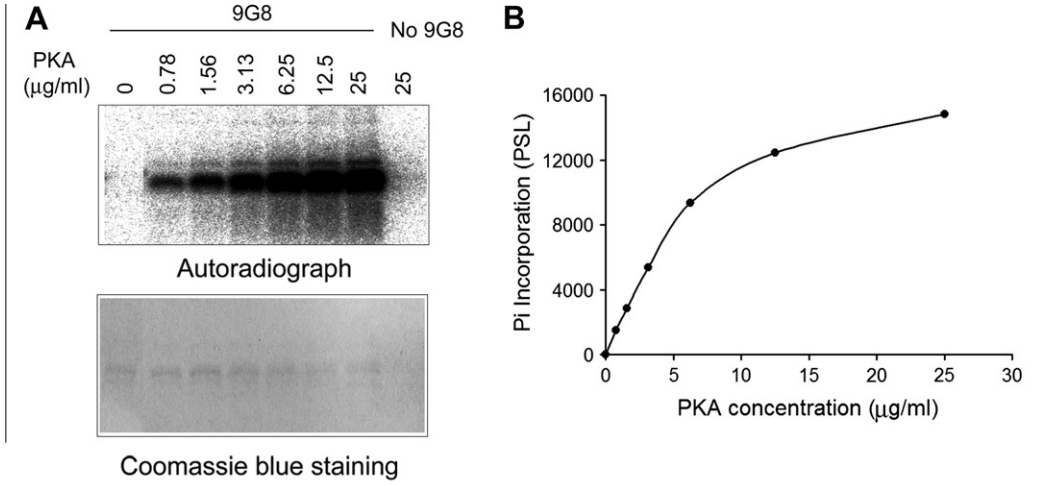
3.1. 9G8 inhibits tau exon 10 inclusion

To examine the role of 9G8 in tau exon 10 splicing, we co-transfected mini-tau gene pCI/SI9-LI10 (comprising tau exons 9, 10, and 11 and part of intron 9 and the full-length of intron 10) together with pCEP4/9G8 or si9G8 into N2a cells, and measured the alternative splicing products of tau exon 10 by RT–PCR. We found that over-expression of 9G8 inhibits tau exon 10 inclusion markedly, while knock-down of 9G8 by siRNA increased tau exon 10 inclusion significantly (Fig. 1), confirming that 9G8 suppresses tau exon 10 inclusion. These results are consistent with our previous observations using HEK-293T cells [19].

3.2. PKA phosphorylates 9G8 in vitro

The biological activity of 9G8 is tightly regulated by its phosphorylation [19]. To study whether PKA phosphorylates and regulates 9G8, we incubated GST-9G8 or GST with various concentration of PKA catalytic subunit in vitro and found that GST-9G8 was phosphorylated by PKA in an enzyme concentration-dependent manner (Fig. 2A and B). Under the same condition, incubation of GST with PKA-C did not lead to any detectable phosphorylation [24].

To map the phosphorylation sites of 9G8 by PKA, we phosphorylated GST-9G8 with 25 μg/ml of PKA for 60 min. The reaction



C GST-9G8 fusion protein

MSPILGYWKI KGLVQPTRLL LEYLEEKYEE HLYERDEGDK WRNKKFELGL
EFPNLPYYID GDVKLTQSMA IIRYIADKHN MLGGCPKERA EISMLEGAVL
DIRYGVSRIA YSKDFETLKV DFLSKLPEML KMFEDRLCHK TYLNGDHVTH
PDFMLYDALD VVLYMDPMCL DAFPKLVCFK KRIEAIPQID KYLKSSKYIA
WPLQGWQATF GGGDHPPKSD LVPRGSPEFP MSRYGRYGGE TKVYVGNLGT
GAGKGELERA FSYYGPLRTV WIARNPPGFA FVEFEDPRDA EDAVRGLDGK
VICGSRVRVE LSTGMPRRSR FDRPPARRPF DPNDRCYECG EKGHYAYDCH
RYSRRRRSRS RSRSHSRSRG RRYSRSRSRS RGRRSRSASP RRSRSISLRR
SRSASLRRSR SGSIKGSRYF QSPSRSRSRS RSISRPRSSR SKSRSPSPKR
SRSPSGSPRR SASPERMD

LC-MS/MS
Red: Matched peptides Underline: 9G8

D

^{215}SPSPKRSR^{222}
^{223}SPSGSPRR^{230}

Fig. 2. PKA phosphorylates 9G8 in vitro. (A) GST-9G8 (0.2 mg/ml) was incubated with various concentrations of PKA-C at 30 °C for 30 min, and the reaction products were separated by SDS–PAGE and visualized with Coomassie blue staining (lower panel). The ^{32}P incorporated into 9G8 in the dried gel was measured by using a phosphorimaging device (BAS-1500, Fuji) (upper panel). (B) Incorporation of ^{32}P into GST-9G8 was plotted against PKA concentration. (C) The amino acid sequence derived by mass spectrometry analysis of GST-9G8 phosphorylated by PKA. The red-colored sequence indicates the peptide fragments detected by LC–MS/MS, and the underlined sequence indicates the 9G8 sequence. (D) Two phosphopeptides from the PKA-phosphorylated 9G8 samples detected by LC–MS/MS.

products were then resolved by SDS–PAGE, followed by in-gel trypsin digestion and LC–MS/MS. We identified two phosphopeptides from the phosphorylated GST-9G8 digest, which were both located at the C-terminal end of 9G8 molecule (Fig. 2C and D). Because the known PKA-phosphorylated sites are not followed by proline [26], we speculate that PKA phosphorylates 9G8 at serine 221 and serine 225.

3.3. PKA modulates tau exon 10 splicing and interacts with 9G8

To study whether activation of PKA affects 9G8-regulated tau exon 10 splicing, we co-transfected pCI/SI9-LI10 with pCEP4/9G8 into HEK-293FT cells for 40 h, and then treated the cells with 10 μM forskolin or 50 μM bd-cAMP for 8 h to activate PKA. The splicing products of tau exon 10 were then quantitated by RT–PCR. We found that in the pCI/SI9-LI10-transfected HEK-293FT cells, 9G8 overexpression inhibited and forskolin and bd-cAMP promoted tau exon 10 inclusion (Fig. 3A). However, forskolin or bd-cAMP treatment did not promote tau exon 10 inclusion in 9G8-overexpressed cells (Fig. 3A), suggesting that 9G8 counteracts tau exon 10 inclusion induced by activation of PKA.

9G8 mainly locates in the nucleus. Activation of PKA by cAMP might lead to a fraction of the catalytic subunit translocating into the nucleus. To study the subcellular localization of 9G8 and PKA-C, we transfected pCEP4/9G8-HA into HeLa cells and then examined their localizations by confocal microscopy after immunofluorescent staining with anti-HA and anti-PKA-C. We found that 9G8 was localized extensively in the nucleus and forskolin treatment did change its subcellular localization (Fig. 3B). PKA catalytic subunit (PKA-C) was located in both cytoplasm and nucleus. A fraction of PKA-C in nucleus was co-localized with 9G8 (Fig. 3B). Forskolin treatment increased PKA-C nuclear localization and enhanced its co-localization with 9G8 (Fig. 3B).

There are three isoforms, α, β, and γ, of PKA catalytic subunits. PKA-Cα and PKA-Cβ express ubiquitously, but PKA-Cγ only expresses in testis [27–29]. To investigate the interaction of 9G8 with PKA-Cα and β, we overexpressed HA-tagged 9G8 in HEK-293FT cells for 48 h, immunoprecipitated 9G8 with anti-HA from the cell

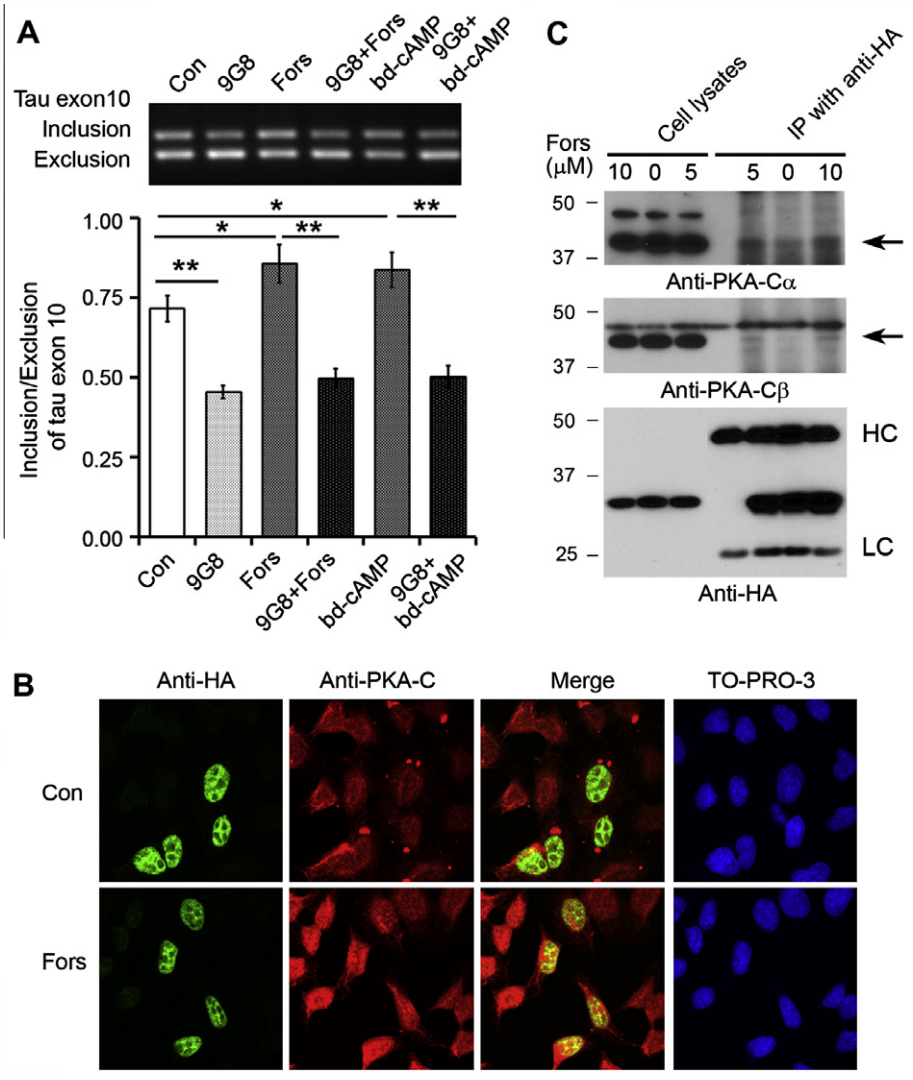


Fig. 3. PKA modulates tau exon 10 splicing and interacts with 9G8. (A) The pCI/SI9-LI10-transfected HEK-293FT cells were transfected with pCEP4/9G8-HA for 40 h or treated with either 10 μM forskolin or 50 μM bd-cAMP for 8 h, or both. The total RNA was then extracted and subjected to RT–PCR and gel electrophoresis to measure the tau exon 10 splicing. The lower graph represents the mean ± S.D after densitometry. *, $p < 0.05$; **, $p < 0.01$. (B) HeLa cells were transfected with pCEP4/9G8-HA for 40 h, and some cells were then treated with 10 μM of forskolin for 8 h. The cells were fixed and immune-stained by monoclonal anti-HA and polyclonal anti-PKA-C, followed by Alexa 555-conjugated goat anti-rabbit IgG (red) and Alexa 488-conjugated goat anti-mouse IgG (green), respectively. TO-PRO-3 (blue) was used for nuclear staining. (C) HEK-293FT cells were transfected with pCEP4/9G8-HA for 40 h and then treated with two concentrations of forskolin for 8 h. The cell extracts were incubated with anti-HA to immune-precipitate 9G8, and the immune-complex was detected by using antibodies against PKA-Cα and Cβ. The arrows indicate PKA-C. HC, IgG heavy chain; LC, IgG light chain.

lysates, and then measured whether PKA-Cα and β were co-immunoprecipitated by anti-HA with Western blots. We found that PKA-Cα, but not PKA-Cβ, was co-immunoprecipitated by anti-HA (Fig. 3C), suggesting that 9G8 interacts with PKA-Cα, but not PKA-Cβ, at the basal condition.

To know whether activation of PKA enhances the interaction of 9G8 with PKA-C, we treated 9G8-overexpressed cells with 5 or 10 μM forskolin for 8 h to activate PKA and measured the levels of PKA-Cα and PKA-Cβ in anti-HA-9G8-immunoprecipitated complexes. We found that both PKA-Cα and PKA-Cβ were co-immunoprecipitated by anti-HA from the cells treated with forskolin (Fig. 3C), suggesting that activation of PKA enhances the interaction of 9G8 with PKA-Cα and PKA-Cβ.

3.4. Overexpression of PKA catalytic subunits modulates 9G8-regulated tau exon 10 splicing

To study the effects of PKA and 9G8 on tau exon 10 splicing, we overexpressed PKA-Cα, PKA-Cβ and 9G8 individually and in combination in HEK-293FT cells transfected with pCI/SI9-LI10, and then measured the splicing products of tau exon 10 by RT–PCR. We found that overexpression of either PKA-Cα or PKA-Cβ increased the PKA kinase activity, as evidenced by marked increase in phosphorylation of its endogenous substrate CREB at Ser133 (pS133-CREB) (Fig. 4A) and promoted tau exon 10 inclusion dramatically (Fig. 4B) [24]. Co-overexpression of PKA-Cα or PKA-Cβ with 9G8 reversed 9G8's inhibitory action on tau exon 10 inclusion (Fig. 4B). These results indicate a negative regulation of PKA on 9G8's function in tau exon 10 splicing.

4. Discussion

Alternative splicing of tau exon 10 results from action of trans-acting factors, also named splicing factors, on cis-elements located mainly on exon 10 and intron 10. 9G8 is a splicing factor, and its overexpression suppresses tau exon 10 inclusion [12,19]. Down-regulation of 9G8 by its siRNA significantly promotes tau exon 10 inclusion, further supporting the involvement of 9G8 in the alternative splicing of tau exon 10. Like other SR proteins, the function of 9G8 is tightly regulated by its phosphorylation [19]. In the present study, we demonstrated for first time that 9G8 is phosphorylated by PKA, which modulates 9G8-mediated tau exon 10 splicing.

PKA phosphorylates numerous proteins at Thr or Ser commonly in the consensus sequence of R/KR/KX(X)T/S [26]. 9G8 contains 8 of this motifs, $_{123}$RRRR**S**$_{128}$, $_{125}$RRSR**S**$_{130}$, $_{140}$RRY**S**$_{144}$, $_{152}$RRSR**S**$_{157}$,$_{161}$RRSR**S**$_{165}$, $_{168}$RRSR**S**$_{173}$, $_{176}$RRSR**S**$_{181}$, $_{228}$KRSR**S**$_{233}$, and $_{237}$RRSA**S**$_{242}$, suggesting that 9G8 is putative good substrate of PKA. These putative PKA phosphorylation sites are located at its RRM2 (RNA recognition motif 2) and RS domain. In the present study, we demonstrated that PKA phosphorylated 9G8 effectively in vitro. We further identified two phospho peptides, $_{215}$SPSPKRSR$_{222}$ and $_{223}$SPSGSPRR$_{230}$, by mass-spectrum analysis. Because the known PKA-phosphorylated sites are not followed by proline [26] and two of the three Ser residues in each of the two identified peptides are followed by proline, we speculate that PKA phosphorylates 9G8 at Ser221 and Ser225. However, these two sites do not belong to any of the above-mentioned putative PKA-phosphorylated sites. It is likely that there are other PKA phosphorylation sites of 9G8 that we failed to identify due to poor recovery of the trypsin-digested peptides. These phosphorylation sites that we failed to identify are probably located in the above consensus sequence. These consensus motifs are rich of arginine/lysine residues. It is not surprising that trypsin may had cleaved these sequences and made the identification of phospho-Ser and phospho-Thr within these sequences difficult.

PKA is activated by cAMP-induced dissociation of its regulatory subunits from its catalytic subunits. A fraction of the catalytic subunit can translocate into the nucleus and phosphorylates nucleic proteins, such as 9G8. We also observed that activation of PKA

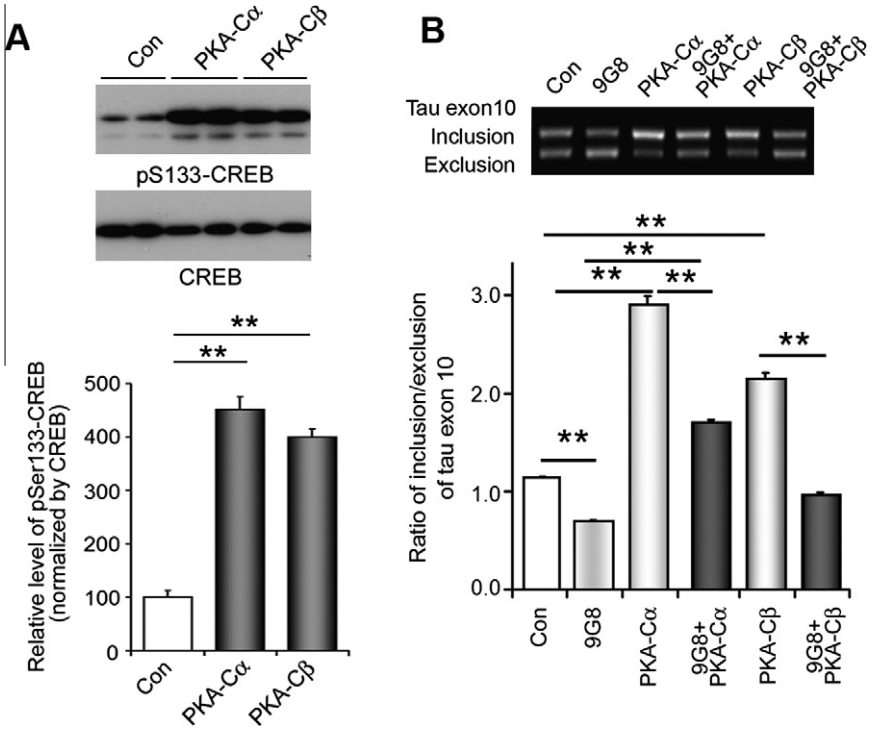


Fig. 4. PKA and 9G8 regulate tau exon 10 splicing. (A) HEK-293FT cells were transfected with pCI/PKA-Cα, or pCI/PKA-Cβ. After 48 h transfection, the phosphorylated CREB and CREB were analyzed by Western blots and quantification is shown in the lower panel. (B) pCI/SI9-LI10-transfected HEK-293FT cells were transfected with 9G8 or with either PKA-Cα or PKA-Cβ individually or in combination. The total RNA was extracted 48 h later and the splicing products of tau exon 10 were detected by RT–PCR. Quantification of the gel electrophoresis of the PCR products is shown in the lower panel. The data are represented as mean ± S.D. *, $p < 0.05$; **, $p < 0.01$.

by forskolin treatment significantly promoted its translocation to the nucleus from the cytoplasm and enhanced its interaction with 9G8. 9G8 is also a shuttle protein and participates in the transportation of mRNA to cytoplasm [30]. The shuttle of 9G8 is regulated by phosphorylation. Overexpression of both Dyrk1A and 9G8 led to their translocation from the nucleus to the cytoplasm and thus diminished their effects on tau exon 10 splicing [19]. In the present study, we found that PKA had no effect on the subcellular localization of 9G8 or its subnuclear localization.

Tau exon 10 alternative splicing is regulated by several splicing factors, including 9G8, alternative splicing factor (ASF), and SC35. ASF promotes tau exon 10 inclusion and its activity in tau exon 10 splicing is enhanced by PKA [31]. In contrast with ASF, 9G8 suppresses tau exon 10 inclusion [19] and PKA inhibits 9G8 suppressed tau exon 10 inclusion. Thus, activation of PKA would increase tau exon 10 inclusion through at least two splicing factors, 9G8 and ASF. We previously found that PKA is down-regulated in AD brain [27]. Taken together, the down-regulated PKA may lead to an imbalance of tau exon 10 splicing and increased 3R-tau via 9G8 and ASF in AD brain. Such an altered 4R-tau/3R-tau ratio has been reported in AD [3,24,32].

Acknowledgments

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