Regulation of the alternative splicing of tau exon 10 by SC35 and Dyrk1A

Wei Qian1,2, Hongwei Liang1, Jianhua Shi1,2, Nana Jin1, Inge Grundke-Iqbal3, Khalid Iqbal3, Cheng-Xin Gong3 and Fei Liu1,3,*

1Jiangsu Key Laboratory of Neuroregeneration, 2Department of Biochemistry, Medical School, Nantong University, Nantong, Jiangsu, P. R. China and 3Department of Neurochemistry, New York State Institute for Basic Research in Developmental Disabilities, Staten Island, NY, USA

Received December 21, 2010; Revised March 16, 2011; Accepted March 17, 2011

ABSTRACT

Abnormal alternative splicing of tau exon 10 results in imbalance of 3R-tau and 4R-tau expression, which is sufficient to cause neurofibrillary degeneration. Splicing factor SC35, a member of the superfamily of the serine/arginine-rich (SR) proteins, promotes tau exon 10 inclusion. The molecular mechanism by which SC35 participates in tau exon 10 splicing remains elusive. In the present study, we found that tau pre-mRNA was coprecipitated by SC35 tagged with HA. Mutation of the SC35-like exonic splicing enhancer located at exon 10 of tau affected both the binding of SC35 to tau pre-mRNA and promotion of tau exon 10 inclusion, suggesting that SC35 acts on the SC35-like exonic splicing enhancer to promote tau exon 10 inclusion. Dyrk1A (dual-specificity tyrosine-phosphorylated and regulated kinase 1A) phosphorylated SC35 in vitro and interacted with it in cultured cells. Overexpression of Dyrk1A suppressed SC35’s ability to promote tau exon 10 inclusion. Downregulation of Dyrk1A promoted 4R-tau expression. Therefore, upregulation of Dyrk1A in Down syndrome brain or Alzheimer’s brain may cause dysregulation of tau exon 10 splicing through SC35, and probably together with other splicing factors, leading to the imbalance in 3R-tau and 4R-tau expression, which may initiate or accelerate tau pathology and cause neurofibrillary degeneration in the diseases.

INTRODUCTION

Tau is a neuronal microtubule-associated protein that promotes microtubule (MT) assembly and stabilizes MT network. Therefore, tau plays important roles in neuronal morphogenesis, axon polarity and axonal transport (1,2). Aggregation of hyperphosphorylated tau in the brain causes diverse set of sporadic and familial neurodegenerative diseases called tauopathies (3,4).

The human tau gene lies on the long arm of chromosome 17 and contains 16 exons from which 6 different tau isoforms are generated in the adult central nervous system by alternative splicing of exons 2, 3 and 10 (5). The alternative splicing of exon 10 of the tau gene results in the presence or absence of the second MT-binding repeats, leading to the expression of tau containing either four (4R-tau) or three MT-binding repeats (3R-tau) (6,7). Approximately equal amount of 3R-tau and 4R-tau are expressed in normal adult human brain (8,9).

At least 39 different mutations in the tau gene have been identified from patients with frontotemporal dementia with Parkinsonism linked to chromosome 17 (FTDP-17) (10–12). More than a half of these mutations only alters splicing of exon 10 and consequently disrupts 3R-tau/4R-tau balance but does not disrupt tau’s primary sequence (13). Thus, alteration in the 3R-tau/4R-tau ratio is sufficient to trigger neurodegeneration and dementia. Dysregulation of alternative splicing of human tau exon 10 is one of the important etiologic mechanisms in the pathogenesis of tauopathies.

Alternative splicing is regulated by multiple exonic and intronic cis-element and trans-acting splicing factors. Serine/arginine-rich (SR) proteins are one group of the splicing factors involved in alternative splicing (14,15). All SR proteins are highly conserved in eukaryotes and have a modular organization. They contain an N-terminal RNA-recognition motif (RRM) that interacts with the pre-mRNA and a C-terminal arginine–serine-rich (RS) domain that promotes protein–protein interactions within the splicing complex (16,17).

*To whom correspondence should be addressed. Tel: +1 718 494 4820; Fax: +1 718 494 1080; Email: feiliu63@hotmail.com

The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors.

© The Author(s) 2011. Published by Oxford University Press. This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (http://creativecommons.org/licenses/by-nc/2.5), which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.
SC35 is one of the SR proteins, which was identified by monoclonal antibodies directed against purified spliceosomes (18). Previous studies reported that the extent of SC35 recruitment to alternatively spliced transcripts of tau exon 10 is related to exon 10 inclusion (19). Tau exon 10 contains a SC35-like enhancer at 5′-end (20,21). However, there is no direct evidence that SC35 acts on SC35-like enhancer to promote tau exon 10 inclusion.

SR protein is a phosphoprotein. Its function is highly regulated by the phosphorylation. Several kinases could phosphorylate SR proteins and regulate their function (22–25). We recently reported that dual-specificity tyrosine-phosphorylated and regulated kinases 1A (Dyrk1A) phosphorylates SF2/ASF, a SR protein and inhibits its promotion of tau exon 10 inclusion (26). Dyrk1A is a proline- and arginine-directed Ser/Thr kinase. It lies at the Down syndrome (DS) critical region of chromosome 21 and contributes to several phenotypes of DS in transgenic mice (27,28). Individuals with DS develop Alzheimer-type neurofibrillar degeneration as early as the fourth decade of life (29). Overexpression of Dyrk1A due to an extra-copy of chromosome in DS brain leads to the dysregulation of tau exon 10, resulting in an increase in 3R-tau expression and causing early onset of tau pathology in DS brain (26). The early onset of tau pathology in DS could also be caused or promoted by over-production of amyloid-β peptide as a result of an extra copy of the gene for amyloid-β precursor protein, which is also located on chromosome 21. Overexpression of Dyrk1A changes the distribution of SC35 from speckles to more diffuse in nucleus (30), suggesting that Dyrk1A may also modulate SC35’s function.

In the present study, we investigated the molecular mechanism by which SC35 regulates tau exon 10 splicing and Dyrk1A regulates SC35-mediated tau exon 10 splicing. The findings of this study suggest that SC35 promotes exon 10 inclusion by acting on the SC35-like enhancer and that Dyrk1A phosphorylates SC35 and suppresses its function in promotion of tau exon 10 inclusion.

MATERIALS AND METHODS

Plasmids and antibodies

pCEP4/SC35-HA was a gift from Dr Tarn of the Institute of Biomedical Sciences, Academia Sinica, Taiwan. Mammalian expression vector pcDNA3.1 containing either rat Dyrk1A or kinase-dead Dyrk1A K188R were described previously (26). pCl/S19–S110 containing a tau minigene, S19/S110, comprising tau exons 9, 10 and 11, part of introns 9 and 10 was as described (31). Mouse monoclonal antibody 8D9 was raised against a histidine-tagged protein containing the first 160 residues of rat Dyrk1A (32). Rabbit polyclonal anti-HA, mouse monoclonal anti-HA and mouse monoclonal anti-β-actin were from Sigma (St Louis, MO, USA). Rabbit polyclonal anti-tau (R134d) was described previously (33). Peroxidase-conjugated anti-mouse and anti-rabbit IgG were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA, USA); tetramethyl rhodamine isothiocyanate (TRITC)-conjugated goat anti-rabbit IgG, and fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG, siRNAs of human or mouse Dyrk1A, and siRNA of mouse SC35 were from Santa Cruz Biotechnology (Santa Cruz, CA). The ECL kit was from Thermo Scientific (Rockford, IL, USA), and [γ-32P] ATP and [35S] orthophosphate were from MP Biomedicals (Irvine, CA, USA). Alkaline phosphatase from bovine calf intestine was from Sigma (St Louis, MO, USA)

Plasmid construction and DNA mutagenesis

pGEX-2T/SC35 was constructed by PCR amplification from pCEP4/SC35 and subcloned into pGEX-2T to express GST-SC35 protein. The deletion mutations of SC35 were generated by amplifying an individual fragment, which contains part of the SC35 coding region into the HindIII–XhoI sites of pCEP4. Mutants of S19/S110 were created by site-directed mutagenesis using KOD-Plus-Mutagenesis kit (TOYOBO) with primers (forward, 5′-ctcaacagggatggtatttaatgatctg3′ and reverse, 5′-aaagacatgctattatattcggacccgctgac3′) for S19/S110 E10A5G, primers (forward, 5′-ctcaacagggatgtattcattaaaaaagaagttattctag3′ and reverse, 5′-aaagacatgctattatattcggacccgctgac3′) for S19/S110 E10G6A and primers (forward, 5′-tctcaacagggatggtatttaatgatctg3′, and reverse, 5′-aaagacatgctattatattcggacccgctgac3′) for S19/S110 E10Δ3.

Cell culture and transfection

COS-7, HEK-293T, N2a, 3T3, SH-SY5Y and HeLa cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Invitrogen, Carsbad, CA, USA) at 37°C (5% CO2). Transfections were performed with Lipofectamine 2000 (Invitrogen, Carsbad, CA, USA) or FuGene 6 (Roche, Indianapolis, IN), according to the manufacturer’s instructions.

Expression and purification of recombinant SC35

For purification of GST-SC35, pGEX-2T/SC35 plasmid was transformed into BL21 (DE3) host strain for expression. A 1 l culture of Escherichia coli harboring pGEX-2T/SC35 plasmid was grown at 37°C to OD600 ~0.6, and induced with 0.5 mM IPTG at 16°C overnight before harvesting by centrifugation. Cells were re-suspended in 30 ml of lysis buffer (50 mM Tris–HCl, pH 7.8, 150 mM NaCl, 1 mM DTT, 1 mM EDTA and protease inhibitor cocktail) and lysed with sonication. All purification procedures were carried out at 4°C. The total soluble fraction was recovered by centrifugation at 36000 g for 60 min, and loaded onto 10 ml of glutathione sepharose beads. The resin was washed with 2 M NaCl high salt buffer (50 mM Tris–HCl, pH 7.8, 1 mM DTT, 1 mM EDTA and protease inhibitor cocktail) and lysed with sonication. All purification procedures were carried out at 4°C. The total soluble fraction was recovered by centrifugation at 36000 g for 60 min, and loaded onto 10 ml of glutathione sepharose beads. The resin was washed with 2 M NaCl high salt buffer (50 mM Tris–HCl, pH 7.8, 1 mM DTT, 1 mM EDTA, 10% glycerol) and subsequently with 50 mM NaCl low salt buffer. The GST-fused SC35 bound to GST beads was eluted with 10 mM glutathione in 50 mM Tris–HCl, pH 8.0, 50 mM NaCl and dialyzed against 50 mM Tris–HCl, pH 7.4). The purified GST-SC35 was aliquoted and stored at –80°C.

Downloaded from https://academic.oup.com/nar/article-abstract/39/14/6162/1380716 on 28 July 2018 by guest
For purification of HA-SC35 from cultured mammalian cells, pCEP4-SC35 was transfected into HEK-293T Cells with FuGENE 6 for 48 h, and then cells were lysed in 0.5 ml of IP lysis/wash buffer (Pierce Crosslink Immunoprecipitation Kit). One milligram of cell lysate protein was added to protein G-Sepharose beads cross-linked to anti-HA and incubated overnight at 4°C. After extensively washing, SC35 was eluted in three successive 100 μl fractions with elusion buffer provided in the kit and neutralized with 1 M Tris.

**Phosphorylation of SC35 by Dyrk1A in vitro**

For phosphorylation of SC35 by Dyrk1A in vitro, GST-SC35 or GST (0.2 mg/ml) was incubated with various concentrations of Dyrk1A in a reaction buffer consisting of 50 mM Tris–HCl, pH 7.4, 10 mM β-mercaptoethanol, 0.1 mM EGTA, 10 mM MgCl2 and 0.2 mM [γ-32P] ATP (500 cpm/pmol). After incubation at 30°C for 30 min, the reaction was stopped by adding an equal volume of 2× Laemmli sample buffer and boiling. The reaction products were separated by SDS–PAGE. Incorporation of 32P was detected by exposure of the dried gel to phosphorimaging system (BAS-1500, Fuji film).

**Dephosphorylation of SC35 by alkaline phosphatase in vitro**

SC35 was overexpressed in HEK-293T cells and immunoprecipitated with anti-HA crosslinked onto protein G beads as described above. The immunocomplex on the beads was dephosphorylated with alkaline phosphatase in reaction buffer (50 mM Tris–HCl, pH 8.5, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin and 1 mM AEBSF) for 30 min at 37°C. The dephosphorylated product was eluted with 2× Laemmli buffer and boiling and subjected to western blot analysis.

**GST pull down**

GST or GST-SC35 was purified by affinity purification with glutathione Sepharose without elution from the beads. Beads coupled with GST or GST-SC35 were incubated with crude extract from rat brain homogenate in buffer (50 mM Tris–HCl, pH 7.4, 8.5% sucrose, 50 mM NaF, 1 mM Na3VO4, 0.1% Triton X-100, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin and 10 μg/ml pepstatin). After 4 h incubation at 4°C, the beads were washed with washing buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl and 1 mM dithiothreitol) six times and the bound proteins were eluted by boiling in Laemmli sample buffer, and the samples were subjected to western blot analysis.

**CO-IMMUNOPRECIPITATION**

HEK-293T cells were co-transfected with pCEP4-SC35-HA and pcDNA3.1-Dyrk1A for 48 h. The cells were washed twice with phosphate-buffered saline (PBS) and lysed by sonication in lysate buffer containing phosphatase and protease inhibitors (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 50 mM NaF, 1 mM Na3VO4, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 2 μg/ml leupeptin and 2 μg/ml pepstatin). Insoluble materials were removed by centrifugation; Protein G beads were incubated with anti-HA overnight at 4°C, and then the antibody bound beads were incubated with the cell lysate. After a 4 h incubation at 4°C, the beads were washed with lysate buffer twice and with Tris-buffered saline twice, and bound proteins were eluted by boiling in Laemmli sample buffer. The samples were subjected to western blot analysis with the indicated primary antibodies.

**Co-localization study**

HeLa cells were plated in 24-well plates onto coverslips 1 day prior to transfection at 30–40% confluence. These cells were transfected with HA-tagged SC35 constructs or co-transfected with Dyrk1A as described above. Two days after transfection, the cells were washed with PBS and 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 incubated with rabbit anti-HA (1:200) and mouse anti-Dyrk1A (8D9, 1:5000) overnight at 4°C. After washing and incubation with secondary antibodies (TRITC-conjugated goat anti-rabbit IgG and FITC-conjugated goat anti-mouse IgG, 1:200), the cells were washed extensively with PBS and incubated with 5 μg/ml Hoechst 33342 for 5 min at room temperature. The cells were washed with PBS, mounted with Fluoromount-G and visualized with a Leica TCSP2 laser-scanning confocal microscope.

**Quantitation of tau exon 10 splicing by reverse transcription–PCR**

Total cellular RNA was isolated from cultured cells by using an RNAsol mini kit (Qiagen GmbH). Six hundred nanograms of total RNA was used for first-strand cDNA synthesis with oligo (dT)18 by using an Omniscript reverse transcription kit (Qiagen GmbH). PCR was performed by using Prime-STARTM HS DNA Polymerase (Takara Bio Inc., Otsu, Shiga, Japan) with primers (forward 5’-GGTG TCCACCTCCAGTCA-3’ and reverse 5’-CCCTGTT TTATGATGGATGTTGCTCTAATGAG-3’) for transfected pCI/S19–S110, and with primers (forward 5’-AAC ACCGCCCACCCGGGAG-3’ and reverse 5’-GTCTGTCT TTGGCTTTGGCATTTCA-3’) for endogenous mouse tau to measure alternative splicing of tau exon 10 under conditions: denaturation for 5 min at 98°C was followed by 30 cycles with denaturation for 10 s at 98°C, annealing for 15 s at 55°C, polymerization for 30 s at 72°C and a final extension for 10 min at 72°C. The PCR products were resolved on 1.5% agarose gels and quantified using the Molecular Imager system (Bio-Rad).

**Electrophoretic mobility shift assay**

Tau RNA primer of the wild-type SC35-like element 5’-G UGCAGAUUUUUAAAGAGCUGGAACUU-3’ (Tau-RNA) or RNA primer of SC35-like element deleted 5’-GUAUUUUUAAGAGCUGGAACUU-3’ (Tau-RNAaSC35like) was labeled with [γ-32P]ATP (4500 Ci/mM) using T4 polynucleotide kinase (New England Biolabs)
was eluted with elution buffer (1% SDS, 0.1 M NaHCO$_3$ (10 mM Tris pH 8.0, 1 mM EDTA). Immune-complex deoxycholate and 1 mM EDTA), and with TE buffer (X-100 and 2 mM EDTA), with LiCl buffer (10 mM and 2 mM EDTA), with high-salt buffer (20 mM Tris–HCl, pH 8.1, 150 mM NaCl, 0.1% SDS, 1% Triton X-100 was washed sequentially with low-salt buffer (20 mM Tris–HCl, pH 8.1, 0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 167 mM NaCl, 1× Roche protease inhibitors cocktail and 50 U/ml RNasin* Plus RNase Inhibitor) on ice for 10 min, and centrifuged at 2000 g for 5 min to pellet nuclei. The nuclear fraction was sonicated in buffer B (50 mM Tris–HCl, pH 8.1, 1% SDS, 10 mM EDTA, 1× protease inhibitors cocktail and 50 U/ml RNasin* Plus RNase Inhibitor). After centrifugation at 16000 g for 10 min, the supernatant was subject to immunoprecipitation with anti-HA in IP buffer (16.7 mM Tris–HCl, pH 8.1, 167 mM NaCl, 0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 1× protease inhibitors cocktail and 50 U/ml RNasin* Plus RNase Inhibitor) for 2 h. Immune-complex was washed sequentially with low-salt buffer (20 mM Tris–HCl, pH 8.1, 150 mM NaCl, 0.1% SDS, 1% Triton X-100 and 2 mM EDTA), with high-salt buffer (20 mM Tris–HCl, pH 8.1, 500 mM NaCl, 0.1% SDS, 1% Triton X-100 and 2 mM EDTA), with LiCl buffer (10 mM Tris–HCl, pH 8.1, 250 mM LiCl, 1% NP-40, 1% deoxycholate and 1 mM EDTA), and with TE buffer (10 mM Tris pH 8.0, 1 mM EDTA). Immune-complex was eluted with elution buffer (1% SDS, 0.1 M NaHCO$_3$ and 50 U/ml RNasin* Plus RNase Inhibitor). The crosslinking was reversed by incubation with 200 mM NaCl at 65°C for at least 2 h. After digestion with 0.4 mg/ml Proteinase K (Invitrogen) at 42°C for 45 min and 1 mg/ml of RQ1 RNase-free Dnase (Promega) at 37°C for 15 min, respectively, RNA was extracted by RNeasy Mini Kit (Qiagen) and subjected to first-strand cDNA synthesis with random primer or Oligo-(dT)$_{15}$–18 by using the Omniscript Reverse Transcription Kit (Qiagen). cDNA was amplified by PrimeSTAR TM HS DNA Polymerase (Takara Bio Inc.) with two sets of primers against tau introns 9 and 10: one set primers: Forward 5’-AGGCGGGTCCAGGGTGGCTGTGTCAC TCATCC-3’, Reverse 5’-CTAATAATTCAGCCACAGCACGCGCATGGGACG-3’; another set of primers: Forward 5’-AGGGTGCGGGCAATGTCACTCATCGAAA GTGGAGGCG-3’, Reverse 5’-GGATTATTTCTATG CAGTTGTCAGAAAGTGTACGC-3’. An initial denaturation for 5 min at 98°C was followed by 30 cycles with denaturation for 10 s at 98°C, annealing for 15 s at 55°C, polymerization for 30 s at 72°C and a final extension for 10 min at 72°C. PCR products were separated on a 1.5% agarose gel and visualized by ethidium bromide staining.

**RESULTS**

SC35 promotes tau exon 10 inclusion

To elucidate the role of SC35 on tau exon 10 splicing, we co-transfected mini-tau gene pCI/SI9–SI10, consisting of tau exons 9, 10 and 11, part of introns 9 (SI9) and 10 (SI10) (31) together with various amount of pCEP4/SC35 into HEK-293T cells. The transfected cells were harvested at 48 h and analyzed for the splicing product of tau exon 10 by reverse transcription (RT)–PCR. We found that overexpression of SC35 promotes the tau exon 10 inclusion concentration dependently. The ratio of inclusion/exclusion of tau exon 10 increased along with transfection concentration and the peak appeared at 0.8 μg/well pCEP4/SC35 transfection (Figure 1A). To examine whether the effect of SC35 on tau exon 10 splicing was cell type specific, we also co-transfected pCI/SI9–SI10 and

![Figure 1. SC35 promotes tau exon 10 inclusion.](https://example.com/figure1.png)
SC35 binds to the pre-mRNA of tau via SC35-like element of exon 10

To determine the molecular mechanism by which SC35 promotes the inclusion of tau exon 10, we co-transfected pCI/SI9–SI10 with pCEP4/SC35-HA into HEK-293T cells, and immunoprecipitated SC35 with anti-HA antibody from the cell lysates. The co-immunoprecipitated pre-mRNA of tau with SC35 by anti-HA was amplified with RT–PCR by using two kinds of primers, random primer and oligo dT primer, for reverse transcription and two sets of primers against introns 9 and 10 of tau to get 194 and 294 bp of PCR products, respectively (Figure 2A). We observed that pre-mRNA of the mini-tau-gene was co-immunoprecipitated with SC35 (Figure 2A and B), suggesting that SC35 could act on the pre-mRNA of tau.

Next, we determined which domain of SC35 was responsible for recruitment to exon 10 of pre-mRNA of the mini-tau-gene. SC35 contains one RNA-recognition motif (RRM) at its N-terminal half and a characteristic SR-rich protein–protein interaction domain at its C-terminus. We transfected a set of previously characterized domains of SC35 (Figure 2C) together with the pCI/SI9/SI10 mini-tau-gene into HEK-293T cells. After 48 h transfection, RNA-IP was also used to detect the binding ability between the fragments of SC35 and pre-mRNA of the mini-tau-gene. We found that HA-SC35FL and SC35FL–191 promoted tau exon 10 inclusion (Figure 2E). These results indicate that SC35 binds with pre-mRNA of mini-tau-gene through its RRM and that both RRM and RS domains are required to promote tau exon 10 inclusion.

SC35-dependent splicing enhancers are known to contain UGCNGYY sequence (37). A SC35-like enhancer located at the 5’-end of tau exon 10 contains the sequence TGCAGAT (38). Whether the binding between SC35 and pre-mRNA of tau exon 10 depends on SC35-like enhancer remains elusive. To answer this question, HA-tagged SC35 was overexpressed in HEK-293T cells and purified using Protein G beads crosslinked with anti-HA monoclonal antibody. Two major forms of SC35 were eluted in the first fraction, and only one form was found in fractions 2 and 3 (Figure 3A, left panel). It was well-known that phosphorylation affects the gel mobility.
of proteins. To define whether phosphorylation slows down the mobility of SC35, we immunopurified SC35 with anti-HA crosslinked protein G beads, and then dephosphorylated it with alkaline phosphatase and determined by anti-HA and 1H4, an antibody to phosphorylated SR proteins. E1, elution fraction 1; E2, elution fraction 2; E3, elution fraction 3; ALP, alkaline phosphatase. (B) SC35 bound to RNA of tau exon 10. Immunopurified SC35 (E1 and E2) was incubated with tau pre-mRNA (tau-RNA) or SC35-like enhancer deleted tau pre-mRNA (tau-RNA/C1SC35 like) pre-labeled with γ-32P ATP. The incubation products were subjected to native-gel electrophoresis. After drying, the gel was analyzed with phosphoimaging device (BAS-1800, Fujifilm). (C) Schematic of mutations of mini-tau-gene on SC35-like enhancer of tau exon 10. (D) Mutations of SC35-like enhancer affected SC35 promoted tau exon 10 inclusion. Different mutations of mini-tau-gene, pCI/SI9–SI10, at SC35 like enhancer were transfected alone or together with pCEP4/SC35. RT–PCR was carried out to measure tau exon 10 splicing after 36 h transfection.
SC35-like enhancer, GCAGATA, also suppressed the exon 10 inclusion (Figure 3D, lane 7). Overexpression of SC35 increased tau exon 10 inclusion markedly in SI9–SI10 and in SI9–SI10E10A4G, but much less in SI9–SI10E10G5A and SI9–SI10E10A2–8 (Figure 3D). These results verify that SC35-like enhancer acts as a splicing enhancer and that the promotion of tau exon 10 inclusion by SC35 depends on the SC35-like enhancer located on tau exon 10.

SC35 interacts with Dyrk1A

To address whether Dyrk1A regulates SC35 activity in splicing, we first studied the interaction between SC35 and Dyrk1A. GST-pull down was used to detect the protein–protein interaction in vitro. We found that Dyrk1A from rat brain extract was pulled down with GST-SC35, but not with GST itself (Figure 4A). To further validate the interaction, co-immunoprecipitation and confocal microscopy were employed in cultured cells. Dyrk1A could be co-immunoprecipitated with HA-SC35 by anti-HA antibody (Figure 4B), confirming the interaction between SC35 and Dyrk1A.

To study the interaction of SC35 with Dyrk1A in intact cells, we coexpressed HA-SC35 and Dyrk1A in HeLa cells and then immunostained the cells with anti-HA and anti-Dyrk1A. By employing confocal microscopy, we observed that both Dyrk1A and SC35 were mainly located in the nucleus, colocalized and enriched in the nuclear speckles, giving further evidence to their possible interaction in cultured cells (Figure 4C).

Dyrk1A inhibits SC35’s activity to promote tau exon 10 inclusion

Our observations of the physical interaction between SC35 and Dyrk1A led us to further investigate the functional relationship between them. We incubated GST-SC35 with Dyrk1A in vitro and found that GST-SC35, but not GST, was phosphorylated by Dyrk1A in an enzyme concentration-dependent manner (Figure 6A and B).

Then we studied the impact of Dyrk1A on the biological activity of SC35. pcDNA3/Dyrk1A or pcDNA3/Dyrk1AK188R alone, or in combination with pCEP4/SC35, and the amounts of exon 10 inclusion and exclusion were measured by RT–PCR. We found that Dyrk1A, but not Dyrk1AK188R, a dead enzyme, suppressed SC35’s activity to promote tau exon 10 inclusion (Figure 6C). These results suggest that phosphorylation of SC35 by Dyrk1A inhibits its activity to promote tau exon 10 inclusion.

To confirm that Dyrk1A suppresses tau exon 10 inclusion, we knocked down the expression of Dyrk1A by its siRNA (26) in pCI/SI9–SI10 transfected HEK-293FT cells and then measured splicing products by RT–PCR. We observed that transfection of Dyrk1A siRNA enhanced the SC35 promoted tau exon 10 inclusion dose dependently (Figure 6D). To determine the role of Dyrk1A in endogenous tau exon 10 splicing, we transfected siRNA of Dyrk1A into N2a or SH-SY5Y cells, both expressing tau, for 48 h, and then measured the products of the splicing by RT–PCR. We found that transfection with
Dyrk1A siRNA significantly increased 4R-tau expression in both cells (Figure 6E), suggesting that Dyrk1A also suppresses exon 10 inclusion of endogenous tau.

**DISCUSSION**

The present study provides the first directly experimental evidence that SC35 acts on the SC35-like enhancer located at the 5′-end of tau exon 10 and promotes tau exon 10 inclusion. Dyrk1A interacts with and phosphorylates SC35 and inhibits its activity to promote tau exon 10 inclusion. Taken together with our recent findings that Dyrk1A phosphorylates splicing factor SF2/ASF and suppresses SF2/ASF-promoted tau exon 10 inclusion (26,39), we conclude that up-regulation of Dyrk1A in DS individuals due to trisomy 21 may suppress the function of SC35 and SF2/ASF in promoting tau exon 10 inclusion and lead to increased 3R-tau expression, which may initiate or accelerate tau pathology in DS brain. In addition, overproduction of amyloid-β peptide, as a result of an extra copy of the gene for amyloid-β precursor protein located on chromosome 21, in DS brain may also initiate or accelerate tau pathology. Therefore, the early onset of tau pathology in DS could result from the overexpression of both Dyrk1A and amyloid-β precursor protein.

Tau exon 10 is flanked by large introns 9 (13.6 kb) and 10 (3.8 kb). It has two weak splice sites, a weak 5′ splice site and a weak 3′ splice site (10,12,38). Several cis-elements in exon 10 and intron 10, which modulate the use of the weak 5′- and 3′-splice sites, have been identified and extensively characterized (20,21). The 5′ end of exon 10 contains three ESEs (exonic splicing enhancer), a SC35-like enhancer, a polypurine enhancer (PPE) and an A/C-rich enhancer (ACE). Splicing factors act on these elements and regulate the alternative splicing of tau exon 10. Mutations in these elements, including C1280, N279K and L284L, cause FTDP17. SC35-dependent splicing enhancers have the sequence of UGCNGYY (where Y = C or U, and N is any base) (37). The 5′-end of exon 10 of tau pre-mRNA contains the sequence, UGCAGAU, that matches with the above consensus sequence and was named as SC35-like enhancer (38). The present study showed that deletion of this element resulted in increased tau exon 10 exclusion, supporting that the SC35-like element acts as an enhancer. Mutation of guanosine to adenosine (UGCA GU to UGCA AU) led tau exon 10 exclusion, confirming that the G at base 5 is required for the enhancer. However, mutation of adenosine to guanosine at base 4 (UGC A GU to UGCGAU) increased tau exon 10 inclusion, suggesting that the fourth base at SC35-dependent splicing enhancer, UGCGNYY, could modulate its splicing activity.

As implied by the name, SC35 acts on SC35-dependent splicing enhancer and promotes the exon inclusion. In the present study, by employing EMSA and RNA-IP, we
demonstrated that SC35 mainly acts on SC35-like enhancer to promote the exon 10 inclusion and that the regulatory efficiency is dependent on SC35-like enhancer sequence. It is well known that in SR proteins, RRM interacts with the pre-mRNA and RS domain promotes protein–protein interactions within the splicing complex. SC35 has a RRM at its N-terminus and a RS domain at C-terminus. In this study, we observed that RRM bound to the pre-mRNA most strongly, and RS domain weakly interacts with the pre-mRNA. However, both RRM and RS domains are required for promoting tau exon 10 inclusion.

It has been reported that the serine residues within the RS domains of SR proteins are extensively phosphorylated. First, this phosphorylation appears to influence the subcellular localization of SR proteins (22,24). Second, phosphorylation affects protein interactions involving SR proteins (23,40). Both of them may change the ability of SR proteins in splicing function. To date, several kinases, including SR-protein kinases 1 and 2 (SRPK1 and SRPK2), the cell cycle-dependent dual specificity kinase (Clk/Sty), Akt/protein kinase B and DNA topoisomerase I (Topo I), have been reported to phosphorylate and regulate localization and function of SR proteins (22,24,41,42). SC35 is also phosphorylated by GSK-3β at N-terminal of SC35 primed by other kinases. Inhibition of GSK-3 increases 4R-tau expression (43). Amyloid-β peptide treatment reduces 4R-tau expression via the GSK-3β-SC35 pathway (44). We previously found Dyrk1A phosphorylates SR proteins SF2/ASF and regulates their function in tau splicing (26). SF2/ASF plays a very important role in tau exon 10 inclusion (45). Dyrk1A phosphorylates and drives SF2/ASF into nuclear speckles, and prevents it from facilitating tau exon 10 inclusion (26). Overexpression of Dyrk1A due to trisomy 21 in DS brain leads to increased 3R-tau/4R-tau ratio, an imbalance that is known to associate with neurofibrillar...
degeneration in Pick disease and some cases of FTDP-17 (26). Mutations of L266V, G272Y, D280, E9+33, E10+19 and E10+29 result in an increase in tau exon 10 exclusion. The molecular mechanism of 3R-tau associated neurofibrillary degeneration in Pick’s disease is unclear. It was reported that as compared with controls, the mRNA level of Dyrk1A in the hippocampus of patients with AD is increased (46). Our unpublished data also demonstrated that Dyrk1A was truncated and activated by calpain 1 in AD brain. Moreover, calpain I also proteolyzes GSK-3β and PKA-Cy, which promotes tau exon 10 exclusion and 3R-tau expression (47–49). Dyrk1A is a proline directed Ser/Thr kinase and phosphorylates Thr or Ser at the consensus motif of RX(X)T/SP of numerous proteins. SC35 harbors four Dyrk1A-consensus motifs. They are located at both RRM and RS domain. However, the exact phosphorylation sites of SC35 by Dyrk1A remain to be determined.

In the present study, we demonstrated that Dyrk1A interacted with SC35 through the RRM of SC35, phosphorylated SC35 effectively and suppressed tau exon 10 inclusion, suggesting that an upregulation of Dyrk1A may cause an imbalance in the alternative splicing of tau exon 10, leading to or accelerating tau pathology in DS, AD and related disorders via ASF and SC35, as well as other splicing factors.

In summary, SC35 specifically binds to pre-mRNA product of the SC35-like enhancer on tau exon 10 both in vitro and in vivo. SC35 promotes tau exon 10 inclusion that is dependent on the SC35-like enhancer of SC35. Dyrk1A interacts and phosphorylates SC35, resulting in an inhibition of tau exon 10 inclusion promoted by SC35. These findings provide a new insight into mechanisms of the regulation of tau exon 10 splicing and shed new light into the dysregulation of tau exon 10 splicing in DS where there is an extra copy of Dyrk1A. These findings can help in development of novel therapeutic strategies to prevent or inhibit neurofibrillary degeneration in tauopathies.

ACKNOWLEDGEMENTS

The authors thank Ms. M. Marlow for editorial suggestions, Ms. J. Murphy for secretarial assistance, Jianhua Zhou and Yu-Wen Hwang for the gifts of pCI/SI9–SI10 and pcDNA/Dyrk1A and Tianyi Zhang for technical assistance.

FUNDING

The National Natural Science Foundation of China (30770468 and 81030059 to F.L.); A project funded by the Priority Academic Program Development of Jiangsu Higher Education Institution (PAPD); Alzheimer’s Association (IIRG-10-173154 to F.L.); Basic Research Program of Jiangsu Education Department (10KJA310040 to F.L.). Funding for open access charge: The National Natural Science Foundation of China.

Conflict of interest statement. None declared.

REFERENCES

1. Schoenfeld, T.A. and Obar, R.A. (1994) Diverse distribution and function of fibrous microtubule-associated proteins in the nervous system. Int. Rev. Cytol., 151, 67–137.

2. Mandelkow, E. and Mandelkow, E.M. (1995) Microtubules and microtubule-associated proteins. Curr. Opin. Cell Biol., 7, 72–81.

3. Ballatore, C., Lee, V.M. and Trojanowski, J.Q. (2007) Tau-mediated neurodegeneration in Alzheimer’s disease and related disorders. Nat. Rev. Neurosci., 8, 665–672.

4. Hernandez, F. and Avila, J. (2007) Tauopathies. Cell Mol. Life Sci., 64, 2219–2233.

5. Goedert, M. and Spillantini, M.G. (2001) Tau gene mutations and neurodegeneration. Biochem. Soc. Symp., 69, 59–71.

6. Goedert, M., Spillantini, M.G., Potter, M.C., Ulrich, J. and Crowther, R.A. (1989) Cloning and sequencing of the cDNA encoding an isoform of microtubule-associated protein tau containing four tandem repeats: differential expression of tau protein mRNAs in human brain. EMBO J., 8, 393–399.

7. Andreadis, A., Brown, W.M. and Kosik, K.S. (1992) Structure and novel exons of the human tau gene. Biochemistry, 31, 10626–10633.

8. Kosik, K.S., Orecchio, L.D., Bakalis, S. and Neve, R.L. (1989) Developmentally regulated expression of specific tau sequences. Neuron, 2, 1389–1397.

9. Goedert, M. and Jakes, R. (1990) Expression of separate isoforms of human tau protein: correlation with the tau pattern in brain and effects on tubulin polymerization. EMBO J., 9, 4225–4230.

10. Hutton, M., Lendon, C.L., Rizzu, P., Baker, M., Frolich, S., Houlden, H., Pickering-Brown, S., Chakrvartty, S., Isaacs, A., Grover, A. et al. (1998) Association of missense and 5′-splice-site mutations in tau with the inherited dementia FTDP-17. Nature, 393, 702–705.

11. Poorkaj, P., Bird, T.D., Wisniewski, E., Garruto, R.M., Anderson, L., Andreadis, A., Wiederholt, W.C., Raskind, M. and Schellenberg, G.D. (1996) Tau is a candidate gene for chromosome 17 frontotemporal dementia. Ann. Neurol., 43, 815–825.

12. Spillantini, M.G., Murrell, J.R., Goedert, M., Farlow, M.R., Klug, A. and Ghetti, B. (1998) Mutation in the tau gene in familial and sporadic cases of frontotemporal dementia. Proc. Natl Acad. Sci. USA, 95, 7737–7741.

13. Liu, F. and Gong, C.X. (2008) Tau exon 10 alternative splicing and tauopathies. Mol. Neurodegener., 3, 8.

14. Dreyfuss, G., Kim, V.N. and Katozka, N. (2002) Messenger-RNA-binding proteins and the messages they carry. Nat. Rev. Mol. Cell Biol., 3, 195–205.

15. Graveley, B.R. (2000) Sorting out the complexity of SR protein functions. RNA, 6, 1197–1211.

16. Caceres, J.F., Misteli, T., Smerdon, G.R., Spector, D.L. and Krainer, A.R. (1997) Role of the modular domains of SR proteins in subnuclear localization and alternative splicing specificity. J. Cell Biol., 138, 225–238.

17. Zeller, A.M., Lan, W.S., Stolc, J.A. and Roth, M.B. (1992) SR proteins: a conserved family of pre-mRNA splicing factors. Genes Dev., 6, 837–847.

18. Fu, X.D. and Maniatis, T. (1999) Factor required for mammalian spliceosome assembly is localized to discrete regions in the nucleus. Nature, 343, 437–441.

19. Mabon, S.A. and Misteli, T. (2005) Differential recruitment of pre-mRNA splicing factors to alternatively spliced transcripts in vivo. PLoS Biol., 3, e374.

20. D’Souza, I. and Schellenberg, G.D. (2005) Regulation of tau isoform expression and dementia. Biochim. Biophys. Acta, 1739, 104–115.

21. Andreadis, A. (2005) Tau gene alternative splicing: expression patterns, regulation and modulation of function in normal brain and neurodegenerative diseases. Biochim. Biophys. Acta, 1739, 91–103.

22. Gui, J.F., Lane, W.S. and Fu, X.D. (1994) A serine kinase regulates intracellular localization of splicing factors in the cell cycle. Nature, 369, 678–682.

23. Wang, H.Y., Lin, W., Dyck, J.A., Yeakley, J.M., Songyang, Z., Cantley, L.C. and Fu, X.D. (1998) SRPK2: a differentially
expressed SR protein-specific kinase involved in mediating the interaction and localization of pre-mRNA splicing factors in mammalian cells. J. Cell Biol., 140, 737–750.

24. Colwill,K., Pawson,T., Andrews,B., Prasad,J., Manley,J.L., Bell,J.C. and Duncan,P.I. (1996) The Clk/Sty protein kinase phosphorylates SR splicing factors and regulates their intranuclear distribution. EMBO J., 15, 265–275.

25. Rossi,F., Labourier,E., Forne,T., Divita,G., Derancourt,J., Riou,J.F., Antoine,E., Cathala,G., Brunel,C. and Tazi,J. (1996) Specific phosphorylation of SR proteins by mammalian DNA topoisomerase I. Nature, 381, 80–82.

26. Shi,J., Zhang,T., Zhou,C., Chohan,M.O., Gu,X., Wegiel,J., Zhou,J., Hwang,Y.W., Iqbal,K., Grundke-Iqbal,I. et al. (2008) Increased dosage of Dyrk1A alters alternative splicing factor (ASF)-regulated alternative splicing of tau in Down syndrome. J. Biol. Chem., 283, 28660–28669.

27. Arron,J.R., Winslow,M.M., Polleri,A., Chang,C.P., Wu,H., Yu,Q., Guo,J. and Zhou,J. (2004) A minimal length between tau exon 10 and 11 is required for correct splicing of exon 10. Brain Res., 1010, 69–80.

28. Liu,F., Iqbal,K., Grundke-Iqbal,I., Hart,G.W. and Gong,C.X. (2004) O-GlcNAcylates regulate phosphorylation of tau: a mechanism involved in Alzheimer’s disease. Proc. Natl Acad. Sci. USA, 101, 10804–10809.

29. Niranjanakumari,S., Lasda,E., Brazas,R. and Garcia-Blanco,M.A. (2002) Reversible cross-linking combined with immunoprecipitation to study RNA-protein interactions in vivo. Methods, 26, 182–190.

30. Gilbert,S.L., Pebrun,S.R. and Sharp,P.A. (2000) XIST RNA associates with specific regions of the inactive X chromosome. J. Biol. Chem., 275, 36491–36494.

31. Sun,B.K., Deaton,A.M. and Lee,J.T. (2006) A transient heterochromatin state in X preempts X inactivation choice without RNA stabilization. Mol. Cell, 21, 617–628.

32. Schaal,T.D. and Maniatis,T. (1999) Selection and characterization of pre-mRNA splicing enhancers: identification of novel SR protein-specific enhancer sequences. Mol. Cell. Biol., 19, 1705–1719.

33. D’Souza,I. and Schellenberg,G.D. (2000) Determinants of 4-repeat tau expression. Coordination between enhancing and inhibitory splicing factors for exon 10 inclusion. J. Biol. Chem., 275, 17700–17709.

34. Zhong,X.Y., Wang,P., Han,J., Rosenfeld,M.G. and Fu,X.D. (2009) SR proteins in vertical integration of gene expression from transcription to RNA processing to translation. Mol. Cell, 35, 1–10.

35. Xiao,S.H. and Manley,J.L. (1998) Phosphorylation-dephosphorylation differentially affects activities of splicing factor ASF/SF2. EMBO J., 17, 6359–6367.

36. Sun,B.K., Deaton,A.M. and Lee,J.T. (2006) A transient mechanism involved in Alzheimer's disease. J. Biol. Chem., 281, 24569–24575.

37. Blaustein,M., Pelisch,F., Tano,T., Munoz,M.J., Wengier,D., Quadrana,L., Sanford,J.R., Muschietti,J.P., Kornblith,A.R., Caceres,J.F. et al. (2005) Concerted regulation of nuclear and cytoplasmic activities of SR proteins by AKT. Nat. Struct. Mol. Biol., 12, 1037–1044.

38. Hernandez,F., Perez,M., Lucas,J.J., Mata,A.M., Bhat,R. and Avila,J. (2004) Glycogen synthase kinase-3 plays a crucial role in tau exon 10 splicing and intranuclear distribution of SC35. Implications for Alzheimer’s disease. J. Biol. Chem., 279, 3801–3806.

39. Chen,K.L., Yuan,R.Y., Hu,C.J. and Hsu,C.Y. (2010) Amyloid-beta peptide alteration of tau exon-10 splicing via the GSK3beta-SC35 pathway. Neurobiol. Dis., 40, 378–385.

40. D’Souza,I. and Schellenberg,G.D. (2006) Arginine/serine-rich protein interaction domain-dependent modulation of a tau exon 10 splicing enhancer: altered interactions and mechanisms for functionally antagonistic FTDP-17 mutations Delta280K and N279K. J. Biol. Chem., 281, 2460–2469.

41. Kimura,R., Kamino,K., Yamamoto,M., Nuriya,A., Kida,T., Kazui,H., Hashimoto,R., Tanaka,T., Kudo,T., Yamagata,H. et al. (2007) The Dyrk1A gene, encoded in chromosome 21 Down syndrome critical region, bridges between beta-amyloid production and tau phosphorylation in Alzheimer disease. Hum. Mol. Genet., 16, 15–23.

42. Goni-Oliver,P., Lucas,J.J., Avila,J. and Hernandez,F. (2007) N-terminal cleavage of GSK-3 by calpain: a new form of GSK-3 regulation. J. Biol. Chem., 282, 22406–22413.

43. Liang,Z., Liu,F., Grundke-Iqbal,K., Iqbal,K. and Gong,C.X. (2007) Down-regulation of cAMP-dependent protein kinase by over-activated calpain in Alzheimer disease brain. J. Neurochem., 103, 2462–2470.

44. Shi,J., Qian,W., Yin,X., Iqbal,K., Grundke-Iqbal,K., Gu,X., Ding,F., Gong,C.X. and Liu,F. (2011) Cyclic AMP-dependent protein kinase regulates the alternative splicing of tau exon 10: a mechanism involved in tau pathology of Alzheimer's disease. J. Biol. Chem., doi:10.1074/jbc.M110.204453.