

Glycogen Synthase Kinase-3 Plays a Crucial Role in Tau Exon 10 Splicing and Intranuclear Distribution of SC35

IMPLICATIONS FOR ALZHEIMER'S DISEASE*

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Tauopathies, including Alzheimer's disease, are neurodegenerative disorders in which tau protein accumulates as a consequence of alterations in its metabolism. At least three different types of alterations have been described; in some cases, an aberrant mRNA splicing of tau exon 10 occurs; in other cases, the disorder is a consequence of missense mutations and, in most cases, aberrant tau hyperphosphorylation takes place. Glycogen synthase kinase-3 (GSK-3) has emerged as a key kinase that is able to interact with several proteins involved in the etiology of Alzheimer's disease and other tauopathies. Here, we have evaluated whether GSK-3 is also able to modulate tau-mRNA splicing. Our data demonstrate that GSK-3 inhibition in cultured neurons affects tau splicing resulting in an increase in tau mRNA containing exon 10. Pre-mRNA splicing is catalyzed by a multimolecular complex including members of the serine/arginine-rich (SR) family of splicing factors. Immunofluorescence studies showed that after GSK-3 inhibition, SC35, a member of the SR family, is redistributed and enriched in nuclear speckles and colocalizes with the kinase. Furthermore, immunoprecipitated SC35 is phosphorylated by recombinant GSK-3 β . Phosphorylation of a peptide from the SR domain by GSK-3 revealed that the peptide needs to be prephosphorylated, suggesting the involvement of a priming kinase. Our results demonstrate that GSK-3 plays a crucial role in tau exon 10 splicing, raising the possibility that GSK3 could contribute to tauopathies via aberrant tau splicing.

Tau is a brain microtubule-associated protein that promotes microtubule stability (1, 2). Tau plays a key role in the morphogenesis of neurons, and to a lesser extent, the morphogenesis of glial cells, where it is also present at low levels (3). The human *tau* gene contains 16 exons from which different tau isoforms are generated by alternative splicing (4, 5). Some of these isoforms are selectively expressed during embryonic and early postnatal development (5–7), whereas in the adult central nervous system, six different tau isoforms are expressed that

differ in the presence or absence of exons 2, 3, and 10 (8). Exon 10 encodes one of the four repeat sequences (8, 9) that form the microtubule-binding domain (8, 10). The presence of exon 10 results in tau with four repeat microtubule-binding sequences (4R), whereas the alternatively spliced isoforms without exon 10 have only three of these sequences (3R).

A group of neurodegenerative disorders is referred to as tauopathies because tau protein is assembled into aberrant aggregates in the brains of affected individuals (11). One of these tauopathies, frontotemporal dementia with parkinsonism associated to chromosome 17 (FTDP-17),¹ is an inherited dementia that occurs as a consequence of tau mutations in both coding and non-coding regions (12–14). Most exonic mutations are single amino acid substitutions that result in the reduced ability of tau to interact with microtubules (8, 9). On the other hand, some exonic and virtually all the intronic FTDP-17 tau mutations affect RNA processing, resulting in an increase in tau 4R isoforms (8, 12–20) with the only exception being the exonic A280K mutation that results in a decrease in tau 4R isoforms (15–21). Thus, alteration in the tau 4R/tau 3R proportion of isoforms is sufficient to trigger neurodegeneration in frontotemporal dementia, and probably, this might also play a role in other neurodegenerative disorders such as progressive nuclear palsy or corticobasal degeneration, which are characterized by an increase in tau 4R isoforms (11). Thus, gaining insight to the regulation of alternative splicing of human tau exon 10 has been of critical interest (15, 22).

RNA splicing is a complex mechanism wherein five different small nuclear ribonucleoproteins, components of the spliceosome, play a role together with other factors (23, 24). Among these other factors are a family of proteins called SR proteins because they contain an arginine- and serine-rich domain in their C-terminal portion that influences the selection of alternative splicing sites (25, 26).

Glycogen synthase kinase (GSK-3) has emerged as a key kinase that is able to interact with many of the proteins involved in the etiology of Alzheimer's disease (AD), the most common of tauopathies, as well as other tauopathies (27). GSK-3 is a serine/threonine kinase originally identified due to its role in glycogen metabolism regulation and which is most abundant in the central nervous system (28). GSK-3 β accumulates in the cytoplasm of AD pretangle neurons and in tau deposits in most tauopathies (29), and its distribution in brains staged for AD neurofibrillary changes is coincident with the sequence of development of these

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¹ The abbreviations used are: FTDP-17, frontotemporal dementia with parkinsonism associated to chromosome 17; GSK-3, glycogen synthase kinase; AD, Alzheimer's disease; SR, serine/arginine-rich; RT, reverse transcription; PBS, phosphate-buffered saline; DIV, days *in vitro*.

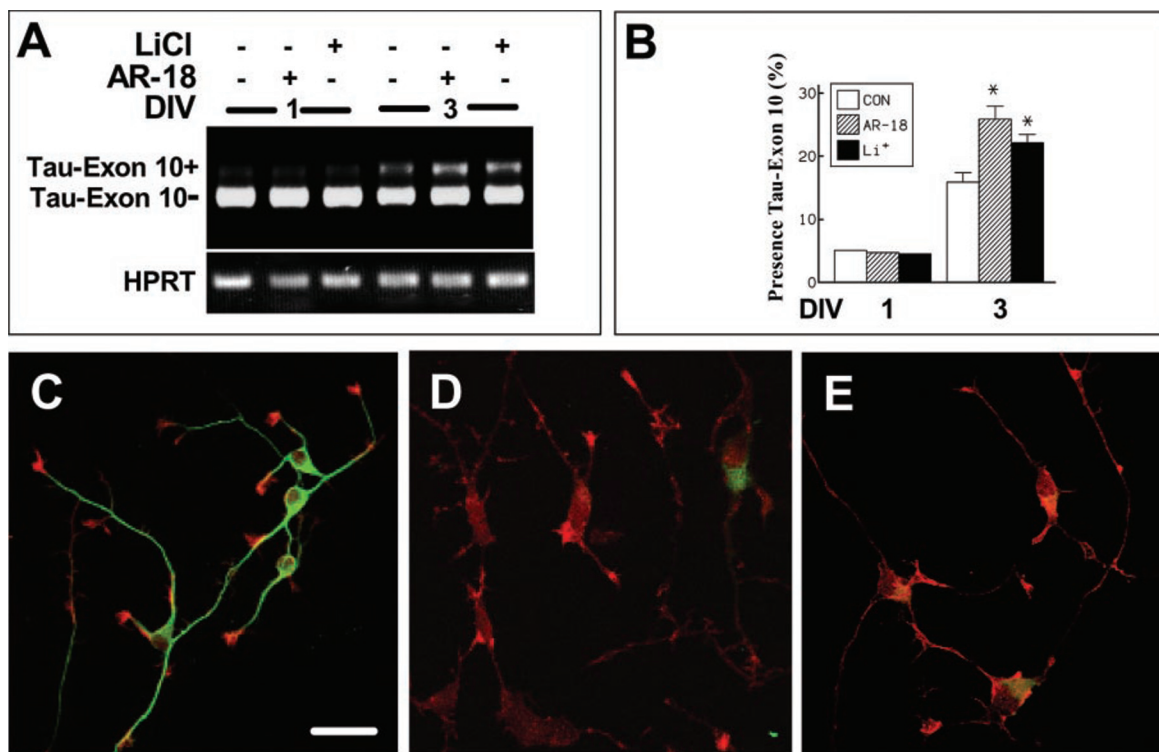


FIG. 1. Analysis of tau exon 10 alternative splicing by RT-PCR. As shown in A, total RNA from cultured cortical neurons at DIV1 and -3 was prepared. Vehicle, lithium chloride (20 mM), or AR-18 (33 μ M) was added 12 h before RNA extraction. RT-PCR was between tau exon 9 and exon 11 (exon 10+ products are 401 bp, and exon 10- products are 301 bp). A representative gel is shown. RT-PCR for murine hypoxanthine-guanine phosphoribosyltransferase (*HPRT*) amplification is shown as a loading control (bottom panel). B, quantization of tau exon 10 inclusion. Each bar represents the mean of at least three separate experiments being 100% the sum of both bands (tau exon 10- and tau exon 10+). Errors bars indicate standard deviations. *, value significantly different ($p < 0.05$) from those non-treated. CON, control. C, confocal micrograph showing cortical neurons maintained in culture for 3 days that were double-labeled with a monoclonal antibody against phospho-tau (PHF-1, green) and TexasRed-phalloidin (red). D, effect of lithium (20 mM) on PHF-1 IR. E, effect of AR-18 (33 μ M). Neurons were incubated for 12 h with the inhibitor. Cortical neurons were fixed and processed for immunofluorescence as described under "Experimental Procedures." Bar, 50 μ m.

changes (30, 31). GSK-3 β has been shown to phosphorylate tau in most sites hyperphosphorylated in AD-tau in transfected cells (32). Overexpression of GSK-3 β in the brain of conditional transgenic mice resulted in tau hyperphosphorylation and somatodendritic localization of tau (33). Furthermore, β -amyloid toxicity has been shown to be mediated by an increase in GSK-3 activity (34, 35). All these data point toward GSK-3 β as a key enzyme in regulating tau metabolism. Here, we have studied whether GSK-3 activity, apart from affecting tau phosphorylation, also affects the regulation of alternative splicing of tau exon 10, the other main alteration found in tau metabolism in neurodegenerative disorders.

EXPERIMENTAL PROCEDURES

Antibodies—The following antibodies were used: according to the residue numbering of the longest human tau isoform of 441 amino acids, antibody PHF-1 (a gift from Dr. Peter Davies) recognizes tau protein when serines 396 and 404 are phosphorylated. Sources of commercial antibodies were: anti-GSK-3 β (Transduction Laboratories), anti-GSK-3 α/β (pY^{279/216}) (BioSource), anti-SC35 (Sigma), and anti- β -tubulin (Sigma).

Primary Culture—Primary cultures of cortical neurons were prepared according to modifications of established procedures (36). Briefly, pups were sacrificed at postnatal day 1. Cortical tissue was then dissected and dissociated individually from each pup with the papain dissociation system (Worthington Biochemical Corp.). Cortical neurons were maintained in neurobasal medium (Invitrogen) supplemented with 1% B-27, 5% fetal calf serum, 0.5 mM glutamine, 100 units/ml penicillin, and 100 mg/ml streptomycin and grown on 3 μ g/ml laminin (Sigma) and 10 μ g/ml poly-L-lysine-coated cover plates. The cells were incubated in 95% air, 5% CO₂ in a humidified incubator at 37 $^{\circ}$ C.

Immunofluorescence—After treatments, cell cultures were fixed with phosphate-buffered saline (PBS) containing paraformaldehyde (4%) for 30 min. After several washes with PBS, the cells were preincubated in

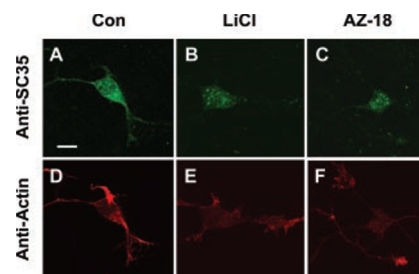


FIG. 2. SC35 nuclear localization. Cortical neurons were incubated for 12 h in the absence (A and D) or presence of 10 mM LiCl (B and E) or 33 μ M AR-18 (C and F). Cultures were fixed at DIV3. The localization of SC35 was determined by indirect immunofluorescence with monoclonal antibody SC35 and fluorescein isothiocyanate-conjugated secondary antibody (green, A-C). Actin was labeled with TexasRed-phalloidin (red, D-F). Neurons were fixed and processed for immunofluorescence as described under "Experimental Procedures." Bar, 20 μ m.

PBS containing 0.1% Triton X-100 and 1% fetal calf serum for 30 min. After a brief wash with PBS, they were incubated for 1 h with primary antibodies at the following dilutions: anti-SC35 (1/100), anti- β -galactosidase (1/1000), anti-GSK-3 β (1/50), anti-PHF-1 (1/100), anti-GSK-3 α/β (pY^{279/216}) (1/200). Cultures were extensively washed and then incubated with the appropriate secondary antibody, conjugated either with fluorescein isothiocyanate or with TexasRed (Jackson Laboratories, West Grove, PA) for 45 min. For some experiments, TexasRed-phalloidin (Molecular Probes) was included with the secondary antibody to visualize F-actin. After washing, they were immediately mounted with Fluoromount. Immunofluorescence was performed using confocal scanning microscopy (Bio-Rad) coupled to a vertical microscopy Axioskop2 (Zeiss).

In Vitro Kinase Assays—To immunoprecipitate SC35, 500 μ g of cerebral cortex was incubated with 5 μ g of mouse monoclonal anti-SC35 in

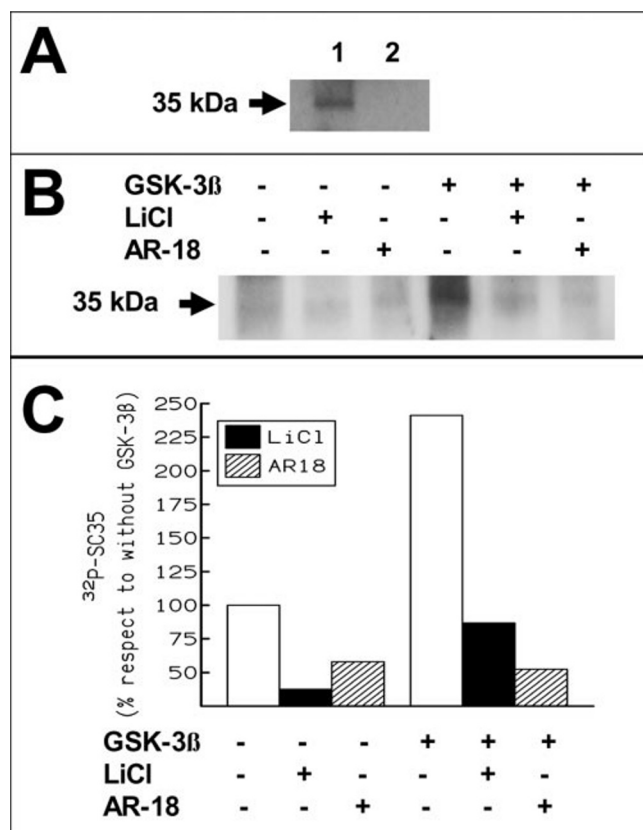


FIG. 3. GSK-3 β phosphorylation of SC35 in an *in vitro* kinase assay. As shown in *A*, protein from total forebrain was incubated with (lane 1) or without (lane 2) mouse monoclonal anti-SC35 (dilution, $1/100$). ECL detection reagents (Amersham Biosciences) were used for immunodetection. Immunoprecipitated samples were then resolved on a 10% SDS-polyacrylamide gel, transferred to a nitrocellulose membrane, and tested with monoclonal anti-SC35 ($1/100$) antibody. As shown in *B*, immunoprecipitated SC35 protein was incubated in the absence or presence of purified GSK-3 β plus [γ - 32 P]ATP as described under "Experimental Procedures." The assay was done in the absence or presence of GSK-3 β inhibitors (20 mM LiCl or 33 μ M AR-18). The samples were resolved on a 10% SDS-polyacrylamide gel and exposed to autoradiography. *C*, quantitative data showing the increase in 32 P incorporation in immunoprecipitated SC35.

immunoprecipitation lysis buffer (1% Triton X-100, 150 mM NaCl, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM EGTA, 0.4 mM sodium orthovanadate, 0.4 mM phenylmethylsulfonyl fluoride, 1% Nonidet P-40) overnight at 4 °C with gentle agitation. Extracts were incubated with 5 μ g of rabbit anti-mouse (Dako) for 2 h and then with 30 μ l of protein A-Sepharose (Sigma) for another additional 1 h at 4 °C. The immobilized immune complex was washed twice with immunoprecipitation lysis buffer and then resuspended in kinase buffer (20 mM Tris-HCl, pH 7.5, 5 mM Mg $_2$ Cl $_2$, 1 mM dithiothreitol, 250 μ M ATP, 20 mM LiCl, and 1 μ M okadaic acid) to prephosphorylate with "cold" phosphate the sites phosphorylated by coimmunoprecipitated kinases different from GSK-3. The sample was incubated at 30 °C for 30 min with shaking and then passed over a G-25 sizing column to remove ATP and LiCl. Afterward, the sample was phosphorylated in the same kinase buffer but with 10 μ M ATP (1000 cpm/pmol of [γ - 32 P]ATP; Amersham Biosciences), and 0.1 μ g/ μ l recombinant GSK-3 β (Sigma). The samples were incubated for 15 min at 30 °C, and Laemmli sample buffer was added to stop the reaction. Samples were boiled for 5 min, and proteins were separated in 10% SDS-polyacrylamide gels. The gels were vacuum-dried and autoradiographed. The phosphorylated bands were quantitated with a densitometer.

A peptide corresponding to residues 180–197 of SC35 protein, SC35 $^{180-197}$ (RSRSRSRSRSRSPPPVSK), as well as a prephosphorylated form P-SC35 $^{180-197}$ (RSRSRSRSRSR(P)RSPPPV(S)(P)K), were chemically synthesized by Neosystem S.A. (Strasbourg, France). The phosphorylation assay was carried out in a final volume of 50 μ l at 37 °C in 50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM dithiothreitol, and 50 mM NaCl. Phosphorylation was initiated by 0.1 μ g/ μ l recombinant

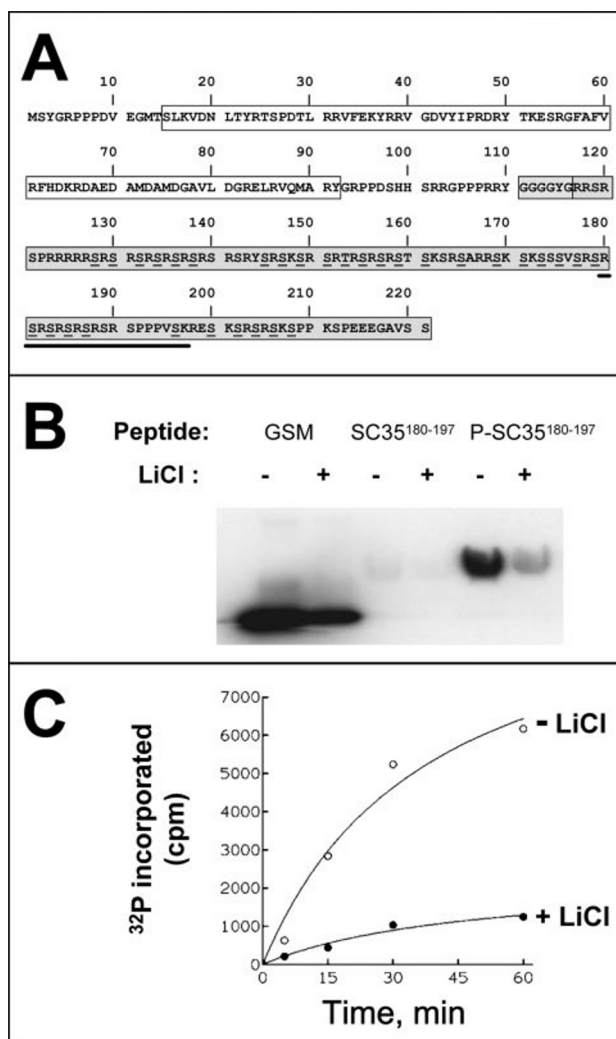


FIG. 4. Domains and putative GSK-3 phosphorylation sites on SC35 protein. Phosphorylation by GSK-3 β is shown. *A*, gray box, Arg/Ser-rich (RS domain); shaded box, Gly-rich (Hinge region); white box, RNA recognition motif. Underlined amino acids are putative GSK-3 phosphorylation sites. The SC35 $^{180-197}$ peptide is shown underlined. Data from Swiss Protein Database accession number for SC35 from *Mus musculus* Q62093. As shown in *B*, GSK-3 β phosphorylates a primed peptide (P-SC35 $^{180-197}$) to a significantly higher extent than unprimed peptide (SC35 $^{180-197}$). As a control, GSK-3 β was incubated with a substrate as well recognized as GSM peptide (43). The effect of 20 mM LiCl in the assay is shown. As shown in *C*, radioactivity incorporated in the P-SC35 $^{180-197}$ peptide was measured in the presence (filled symbol) and in the absence (open symbol) of lithium chloride as a function of incubation time.

GSK-3 β and [γ - 32 P]ATP plus MgCl $_2$ (50 μ M (1 μ Ci of assay medium) and 10 mM, respectively, final concentrations). SDS-PAGE sample buffer was added to stop the reaction, and samples were electrophoresed on a 15% SDS gel. The gels were vacuum-dried and autoradiographed. The phosphorylated bands were quantitated with a densitometer. The difference between the kinase activity in the presence or absence of 20 mM LiCl was considered to be a measure of GSK-3 protein kinase activity.

Reverse Transcription of RNA and Polymerase Chain Reaction (RT-PCR)—Exon 10 splicing was assayed by RT-PCR as reported previously (37). Total RNA from different primary cultures was prepared using the reagent TRIzol (Invitrogen) and following the supplier's protocol. Reverse transcription was performed using the First cDNA synthesis kit (Roche Applied Science) on 5 μ g of RNA with oligo(dT) primers. PCR was performed with the oligonucleotides R1 (5'-GGCGAATTCGGATCATGCCAGACCTGAAGAATG-3') and R2 (5'-GGCCTGCAGTTACTCGCGAAGTTCAGCTTGTTG-3'). The amplifications were performed basically with the following protocol: 30 cycles of 94 °C for 45 s, 55 °C for 1 min, and 72 °C for 1 min. The PCR products were resolved on a 1.8% agarose gel and stained with ethidium bromide. Two populations of RNA (coding for tau proteins containing three or four tubulin-binding

motifs) are detected. The exon 10⁻ (tau 3R) and exon 10⁺ (tau 4R) products as amplified by RT-PCR are 301 and 401 bp, respectively. As a loading control, RT-PCR for hypoxanthine-guanine phosphoribosyltransferase was performed for each sample. The used primers for murine hypoxanthine-guanine phosphoribosyltransferase were 5'-CCTG-GTTAAGCAGTACAGCC-3' and 5'-TCAAGGGCATATCCAACAACA-3'. The amplification protocol was: 20 cycles with denaturation for 15 s at 95 °C, annealing for 30 s at 60 °C, and elongation for 1 min at 72 °C. Differences among groups were analyzed by Student's unpaired *t* test to determine significant differences between means.

RESULTS

GSK-3 Inhibition Results in Decreased Tau Exon 10 Alternative Splicing in Cortical Neurons—Alternative splicing of exon 10 was investigated in cultured cortical neurons. Similar to early postnatal brain development, there is a progression in the expression from 3R tau expression to both 4R tau and 3R tau in cortical cultured neurons (Fig. 1A). At the first day *in vitro* (DIV1), almost all the tau mRNA detected by RT-PCR ($96 \pm 3\%$) is exclusively the isoform 3R-tau (Fig. 1, A and B). At DIV3, a change in tau mRNA splicing takes place, and 4R isoforms can be detected by RT-PCR. More precisely, at DIV3, exon 10 is present in $15.9 \pm 1.5\%$ of total tau-RNA (Fig. 1, A and B). These studies demonstrate that the mechanism for the developmental regulation of the splicing of tau exon 10 is also present in cultured cortical neurons. We next studied the effect of incubation of neurons with the GSK-3 inhibitors lithium (35, 38) and AR-A014418 (AR-18) (39) on the 3R and 4R proportion of tau isoforms. We first verified that 20 mM LiCl in the culture medium efficiently inhibited GSK-3 in the primary cultures. As shown in Fig. 1, C and D, tau phosphorylation at the GSK-3-dependent epitope recognized by antibody PHF-1 (40, 41) was reduced to background levels. Lithium treatment was done in the presence of 10 mM *myo*-inositol to avoid any effect of the inositol depletion caused by inositol monophosphatase inhibition (35). The same analysis was carried out with 33 μ M AR-18, a concentration that inhibits tau phosphorylation in tau transfected cells (39). We then analyzed whether the inhibition of GSK-3 by lithium and AR-18 treatments affects the expression of tau exon 10 at different culture times. As shown in Fig. 1, A and B, RT-PCR analysis on RNA isolated from control-, lithium-, and AR-18-treated cultures showed that GSK-3 inhibition resulted in increased proportion of 4R isoforms. These data demonstrate that alternative splicing of exon 10 is regulated by GSK-3.

The Nuclear Distribution of SC35 Is Sensitive to GSK-3 Activity—Pre-mRNA splicing is catalyzed by a multimolecular complex including members of the SR family of splicing factors. These splicing factors usually concentrate in nuclear speckles. Taking into account that one of the two exon splicing enhancers present within tau exon 10 consists of a potential SC35-binding element (22) and that GSK-3 activity affects tau pre-mRNA splicing, we analyzed the effects of GSK-3 inhibition on the subcellular localization of SC35. Cortical neurons in culture (DIV3) displayed a diffuse predominantly nucleoplasmic staining pattern for SC35 (Fig. 2A) as evidenced by confocal microscopy analysis. However, after GSK-3 inhibition with lithium, SC35 accumulates in nuclear speckles (Fig. 2B). A similar effect was observed with the more selective GSK-3 inhibitor AR-18 (Fig. 2C).

SC35 Is Phosphorylated by GSK-3 β in Vitro—The effect of GSK-3 activity on SC35 localization raised the possibility that this SR protein might be a substrate for phosphorylation by GSK-3. We therefore tested the ability of GSK-3 to phosphorylate SC35. SC35 was immunoprecipitated from brain homogenates (Fig. 3A), and the immunoprecipitate was incubated with recombinant GSK-3 β in the presence or absence of lithium as well as AR-18. After resolving the samples on SDS-PAGE

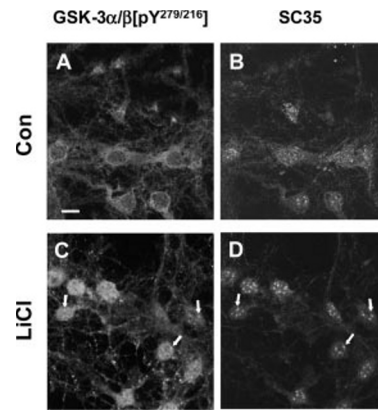


FIG. 5. Active form of GSK-3 colocalizes with SC35 in speckled regions of the nucleus after lithium treatment. A confocal micrograph showing cortical neurons maintained in culture for 3 days and that were double labeled with a monoclonal antibody against GSK-3 α/β (pY^{279/216}) (A) and SC35 (B) is shown. The effect of lithium (20 mM) on GSK-3 α/β (pY^{279/216}) (C) and SC35 (D) is also shown. Neurons were incubated for 12 h with the inhibitor. Cortical neurons were fixed and processed for immunofluorescence as described under "Experimental Procedures." Bar, 20 μ m. Arrows show speckled immunolabeling for GSK-3 α/β (pY^{279/216}) (C) and SC35 (D) after lithium treatment. Con, control.

and exposing them to autoradiography, we observed a significant increase in ³²P incorporation in SC35 (about 2.5-fold over control; Fig. 3, B and C). The radioactive incorporation was mediated by GSK-3 β as it was inhibited by lithium chloride and the more selective inhibitor AR-18.

The motif (S/T)XXX(S/T) is a consensus site recognized by GSK-3, especially when it is phosphorylated in +4 S/T (42). Such a motif is present in tandem in the N-terminal site of SC35 (Fig. 4), suggesting that it is the region phosphorylated by GSK-3 β . A peptide corresponding to predicted phosphorylation sequences was synthesized (Fig. 4A, residues 180–197). Fig. 4, B and C, shows that unphosphorylated peptide is a poor substrate for the kinase. However, when the same experimental protocol was carried out with primed peptide, a strong incorporation of radioactivity that was inhibited by lithium was observed (Fig. 4, B and C). In fact, the P-SC35^{180–197} peptide is a substrate for the kinase as good as GSM peptide, a peptide from glycogen synthase that is widely used to study GSK-3 activity (43). The radioactivity incorporated in SR-18P peptide, in our experimental conditions, is 270 ± 50 cpm nmol⁻¹ min⁻¹, whereas the radioactivity incorporated in GSM peptide is 340 ± 35 cpm nmol⁻¹ min⁻¹.

SC35 Colocalizes with GSK-3 to Speckled Regions in the Nucleus—To determine whether a cross-talk occurs between SC35 and GSK-3 in cortical cultures, double immunolabeling experiments were performed with antibody SC35 and polyclonal GSK-3 antibody, which recognize active forms of GSK-3 α/β (GSK-3 α/β (pY^{279/216})), in a confocal laser microscopy analysis. In the absence of any treatment, fluorescence staining for GSK-3 α/β (pY^{279/216}) (Fig. 5A) was mainly cytoplasmic, and within the nucleus, it was diffusely distributed. However, after lithium treatment, it was then redistributed to nuclear speckles (Fig. 5C) in colocalization with SC35 (Fig. 5D). These results indicate that GSK-3 inhibition enhances GSK-3 and SC35 colocalization in speckles.

DISCUSSION

The present results provide the first direct experimental evidence of the involvement of GSK-3 in tau mRNA splicing, thereby pointing to a novel role for GSK-3. Regarding the involved mechanism, we provide experimental data showing that GSK-3 phosphorylates SC35 and that GSK-3 activity af-

fects SC35 localization in nuclear speckles. More precisely, we show that inhibition of GSK-3 results in increased exon 10+ tau transcription and increased nuclear localization of SC35 in speckles.

The exact molecular mechanism by which GSK-3 affects pre-mRNA splicing remains to be determined. However, taking into account that (i) SR proteins are trans-acting factors that play an important role in splicing process through binding to splicing enhancers and mediating protein-protein interaction (44); (ii) SR proteins are targeted to stores with the appearance of speckles through their RS domain (45, 46); and (iii) that they are released from them upon phosphorylation (47), the more likely hypothesis is that SC35 is released from speckles upon phosphorylation by GSK-3. However, SC35 phosphorylation by GSK-3 seems to require prephosphorylation by priming kinases. Several protein kinases that regulate SR proteins could be possible candidates as priming kinases. The SR protein kinase family and Clk kinase family are kinases that phosphorylate the RS domains of SR proteins (48). Clk kinases are implicated in alternative splicing of human tau exon 10 (49). We demonstrate the involvement of GSK-3 β in SC35 distribution and alternative splicing of human tau exon 10. Taking into account that GSK-3 β phosphorylates immunoprecipitated SC35, the most likely explanation suggests a relationship among protein kinases that has been implicated in the phosphorylation of SR proteins and GSK-3. SR proteins released into the nucleoplasm are able to regulate alternative splicing by forming part of a multimolecular complex called the spliceosome, whose main components are small nuclear ribonucleoproteins, heteronuclear RNA-associated proteins, and SR proteins. If GSK-3 inhibition is occurring, SC35 and likely other SR proteins are concentrated in speckles, resulting in an inhibition of tau mRNA splicing.

Our data raise the question regarding the physiological relevance of nuclear GSK-3. It has been described that nuclear GSK-3 is involved in phosphorylation of cyclin D1 (50), heat shock factor-1 (51–53), nuclear factor of activated T-cells (54), and cAMP-response element-binding protein (55). In addition, proapoptotic stimuli induce nuclear accumulation of GSK-3 β in SH-SY5Y (56, 57) and PC12 cells (57) as well as *in vivo* in neurons after ischemic insult (57). In this report, we demonstrate that GSK-3 colocalizes with SC35 in nuclear speckles after inhibition and is able to phosphorylate SC35 *in vitro*, demonstrating a novel role for the kinase in alternative splicing.

The motif (S/T)XXX(S/T) is a consensus site recognized by GSK-3 (42). That motif is present in tandem in the N-terminal site of SC35 (Fig. 4), and we have here demonstrated that a peptide from that region is efficiently phosphorylated by the kinase but only when the peptide is phosphorylated, suggesting the requirement of a priming kinase. Thus, this consensus motif must first be phosphorylated by a priming kinase at the Ser that is four amino acids C-terminal to the target site, which primes the substrate for phosphorylation by GSK-3 β . The RS domain functions as a protein interaction domain. The serines within the domain are phosphorylated, being as the subcellular localization of SR proteins is dependent of its phosphorylation status. The interaction with other proteins is also altered by phosphorylation. The DNA topoisomerase I and two families of kinases, the SR protein kinase (SRPK1 and -2), and the Clk/Sty family are involved in SR domain phosphorylation (for a review of SR proteins, see Ref. 44). Our results implicate a new kinase, GSK-3 β , in SC35 phosphorylation and likely phosphorylation of other SR proteins.

The relevance of our data in relation to neurodegenerative diseases such as tauopathies can be a matter of debate, and the contribution of the tau isoforms for the development of neuro-

degeneration remains to be determined. This is important because data from FTDP-17 patients have demonstrated that the only tau mutations found in some of them have been those which affect the splicing of exon 10 and which increase the ratio of four-repeat with respect to three-repeat isoforms (58). This is in contrast with Pick's disease in which tau isoforms are predominantly found with three repeats. Therefore, an imbalance among tau isoforms and probably the type of neuron involved are relevant for the development of the illness. In any case, taking into account that the binding of tau to microtubules, as well as to cell membranes, is regulated by phosphorylation (59, 60) and that hypophosphorylated tau binds with high affinity to microtubules (whereas hyperphosphorylated tau, like that present in AD, shows a very low capacity to bind to microtubules (61, 62)), it is tempting to speculate that GSK-3 inhibition would favor the expression of tau 4R isoforms that are able to stabilize microtubules better than tau 3R isoforms (63).

In summary, GSK-3 is an enzyme that has been found at the convergence of pathways that are altered in AD and other tauopathies. GSK-3 has several roles in AD and tauopathies including tau hyperphosphorylation (32, 41, 64), modulation of presenilin (34, 65), and amyloid toxicity (34, 35). We have demonstrated here that GSK-3 modulates tau splicing, which is altered in some tauopathies, likely through SC35 protein, a member of the SR family of splicing factors.

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