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Regulation of Alternative Splicing of Human Tau Exon 10 by Phosphorylation of Splicing Factors

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Tau is a microtubule-associated protein whose transcript undergoes regulated splicing in the mammalian nervous system. Exon 10 of the gene is an alternatively spliced cassette that is adult-specific and encodes a microtubule-binding domain. Mutations increasing the inclusion of exon 10 result in the production of tau protein which predominantly contains four microtubule-binding repeats and were shown to cause frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17). Here we show that exon 10 usage is regulated by CDC2-like kinases CLK1, 2, 3, and 4 that phosphorylate serine–arginine-rich proteins, which in turn regulate pre-mRNA splicing. Cotransfection experiments suggest that CLKs achieve this effect by releasing specific proteins from nuclear storage sites. Our results show that changing pre-mRNA-processing pathways through phosphorylation could be a new therapeutic concept for tauopathies.

INTRODUCTION

Tau is a microtubule-associated protein expressed in the nervous system, where it plays a role in the polymerization and stabilization of microtubules (Delacourte and Buee, 1997). The human tau gene consists of 16 exons from which six tau protein isoforms are generated in the adult human brain by alternative splicing (Andreadis *et al.*, 1992; Goedert *et al.*, 1989a). The interaction of tau with microtubules occurs at the C-terminus of the tau protein, where it contains imperfect repeats that act as microtubule-binding domains (Himmler *et al.*, 1989). One of these repeats is encoded by exon 10 that is alternatively spliced in humans, giving rise to tau with three (3R, exon 10−) or four (4R, exon 10+) microtubule-binding domains. Alternative splicing of exon 10 is under developmental control in both rodents and humans, with exon 10 inclusion being adult specific. However, in adult human and primate brains, exon 10 remains regulated, whereas in rodents, it is used constitutively (Goedert *et al.*, 1989b; Grover *et al.*, 1999; Kosik *et al.*, 1989). Although knock-out experiments have revealed that tau gene loss can be compensated for during brain formation (Harada *et al.*, 1994; Ikegami *et al.*, 2000; Takei *et al.*, 2000), tau protein is extensively studied because it is involved in the pathology of a number of neurodegenerative diseases (Garcia and Cleveland, 2001; Spillantini and Goedert, 1998). Recently, mutations in the tau gene have been associated with frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17)[3] (Hutton *et al.*, 1998; Poorkaj *et al.*, 1998; Spillantini *et al.*, 1998, 2000a). These mutations were missense, deletion, silent, or in-

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[3] Abbreviations used: FTDP-17, frontotemporal dementia and parkinsonism linked to chromosome 17; DARPP-32, dopamine and cyclic AMP-regulated phosphoprotein, relative molecular mass 32,000; SR protein, serine–arginine-rich protein.

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tronic. Most missense mutations act at the protein level and reduce the ability of tau to interact with microtubules (Hasegawa *et al.,* 1998). Intronic mutations and the exonic mutations N279K, L284L, N296N, S305N, and S305S affect pre-mRNA processing and lead to an increase of exon 10 usage by changing either the 5′ splice site composition or exonic elements in exon 10 (Clark *et al.,* 1998; D'Souza *et al.,* 1999; Hasegawa *et al.,* 1999; Hutton *et al.,* 1998; Spillantini *et al.,* 1998, 2000b; Stanford *et al.,* 2000; Varani *et al.,* 1999). As a result, the approximately equal ratio of 4R and 3R isoforms is shifted in favor of 4R tau. This change in isoform ratio ultimately leads to the filamentous tau pathology composed of 4R tau. Overexpression of a human 4R isoform in mice leads to a hind-limb clasping phenotype and abnormal tau and neurofilament axonal swellings in the spinal cord (Duff *et al.,* 2000). In healthy individuals, the normal balance between 3R and 4R tau is achieved by regulated alternative splicing, an important mechanism for creating different protein isoforms from a single gene, which is used in about 60% of all human genes (Mironov *et al.,* 1999; Stamm *et al.,* 2000; International human genome sequencing consortium, 2001). It has been estimated that up to 15% of genetic defects caused by point mutations in humans manifest themselves as pre-mRNA splicing defects through changes in splice site sequences (Krawczak *et al.,* 1992; Nakai and Sakamoto, 1994). Splice site choices are regulated by degenerate sequence elements in the RNA, such as the branch point and the 5′ and 3′ splice sites, as well as by enhancer or silencer elements (Elliot, 2000; Green, 1991; Krämer, 1996; Moore, 2000). The high fidelity of splice site selection is the result of cooperative binding of transacting factors to these sequences (Hertel *et al.,* 1997). The loose consensus sequences, found in exonic elements that can work as either enhancers or silencers, are probably necessary to allow the proper protein coding (Elliot, 2000). Their importance is underlined by several diseases caused by mutations in exonic enhancers (Cooper and Mattox, 1997; Daoud *et al.,* 2000; Philips and Cooper, 2000; Stoss *et al.,* 2000).

Two major classes of proteins have been shown to interact with exonic sequence elements: the hnRNPs (Weighardt *et al.,* 1996) and the SR proteins (Fu, 1995; Tacke and Manley, 1999). These proteins can interact with spliceosomal components (Wu and Maniatis, 1993) and mark or hide splice sites located nearby. As a result, their relative concentrations can dictate splice site usage, both *in vivo* and *in vitro* (Cáceres *et al.,* 1994; Mayeda and Krainer, 1992; Screaton *et al.,* 1995; Wang and Manley, 1995). The expression levels of various SR proteins (Ayane *et al.,* 1991; Mayeda and Krainer, 1992; Screaton *et al.,* 1995; Zahler *et al.,* 1993) and hnRNPs (Kamma *et al.,* 1995) are variable among tissues and could therefore account for differences in splice site selection. Several examples of antagonistic splicing factors have been described (Cáceres *et al.,* 1994; Gallego *et al.,* 1997; Jumaa and Nielsen, 1997; Mayeda *et al.,* 1993; Polydorides *et al.,* 2000), where one factor promotes inclusion of an exon and the other factor promotes its skipping. In the nucleus, factors involved in pre-mRNA processing are concentrated in speckles—dynamic structures (Misteli *et al.,* 1997) that are sensitive to phosphorylation (Nayler *et al.,* 1998b). A number of kinases, phosphorylating RS domains, have been described, among them a U1 70K-associated kinase (Woppmann *et al.,* 1993), SRPK1 (Gui *et al.,* 1993), and SRPK2 (Wang *et al.,* 1998). In addition, four kinases termed CLK (cdc2-like kinases) have been shown to phosphorylate SR proteins and to regulate their subnuclear localization (Colwill *et al.,* 1996a,b; Nayler *et al.,* 1997, 1998b). Phosphorylation of SR proteins leads to their release from speckles and probably increases their concentration in the nucleoplasm. As alternative splice site selection is regulated by the relative concentrations of transacting factors, CLK kinases can indirectly govern splice site selection.

Here we show that CLK kinases cause exon 10 skipping of tau, even when regulatory elements of this exon are changed by mutation. These data suggest that a change in phosphorylation of splicing factors could be of therapeutic usage in FTDP-17 and that a misregulation of these factors could be a cause for tauopathies.

RESULTS

mCLK1–4 Promote Skipping of Tau Exon 10

In order to analyze transacting factor regulation of exon 10 splicing, we constructed the minigene SV9/10L/11 consisting of exon 10 and its flanking exon and intron regions (Fig. 1A) (Gao *et al.,* 2000). The mutations N279K, L284L, S305N, M16, and C16 involved in FTDP-17 were introduced into this wild-type background (Fig. 1B).

Previously, we showed that the SR proteins SF2/ASF, SRp55, and SRp75, splicing regulatory proteins containing an arginine–serine-rich domain (Manley and Tacke, 1996), promote skipping of exon 10 (Gao *et al.,* 2000). This shows that alternative splicing patterns of exon 10 can be modified *in vivo.* We reasoned that a phospho-

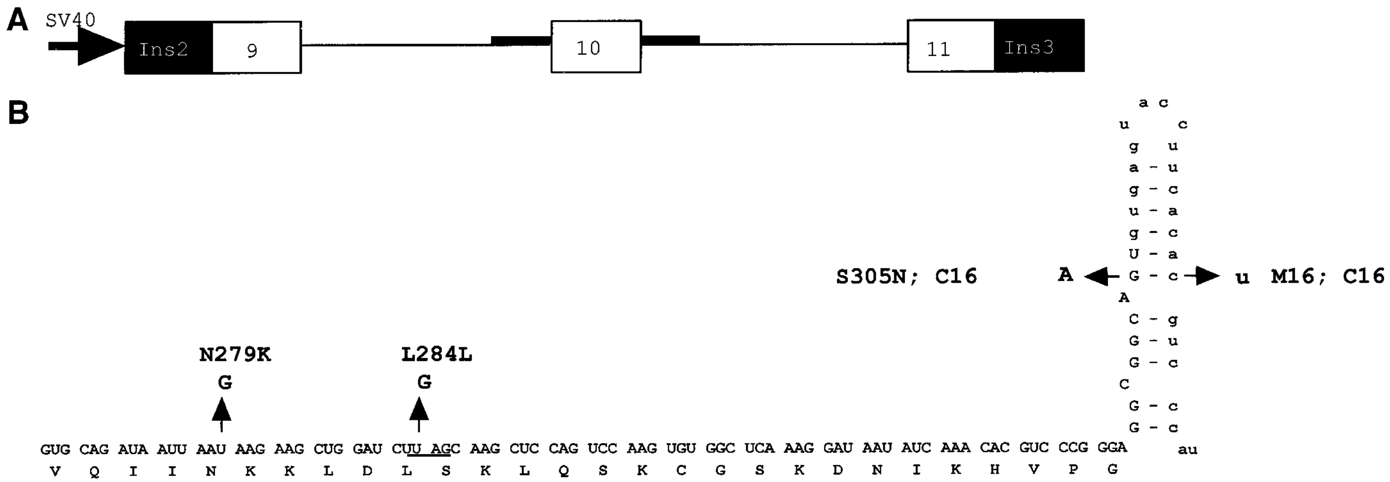

FIG. 1. Exon 10 sequence and mutations. (A) Schematic representation of the expression construct SV9/10L/11. Exon 10 is flanked by tau exons 9 and 11. Exons 9 and 11 are fused with insulin exons 2 and 3, respectively. Primers located in these insulin exons are used for amplification. Thick lines around exon 10 indicate the flanking intronic regions (471 nt downstream, 408 nt upstream). The SV40 promotor is indicated with an arrow. (B) Sequence of the human tau pre-mRNA encoding exon 10 and its flanking intronic regions. Mutations found in FTDP-17 are indicated. C16 is a double compensatory mutant.

rylation-mediated release of SR proteins could have a similar effect and therefore tested the murine kinases CLK1, 2, 3, and 4 in similar transfection experiments in HEK293 cells. Without addition of CLKs, exon 10 was skipped in about 30% of the minigene-derived RNA (Fig. 2A). This percentage increases in the presence of CLK1 and CLK4 to about 45% ($P = 0.056$ and $P = 0.062$, respectively) and is highest with CLK2 ($P < 0.0001$) followed by CLK3 ($P < 0.01$), where between 60 and 70% of exon 10 is skipped (Fig. 2C). As this effect could have been the result of different expression levels, we analyzed protein lysates from the same transfection experiments and found similar protein amounts for all four kinases (Fig. 2B), indicating comparable expression of CLK1–4. Without addition of CLKs, exon 10 was skipped in about 30% of the minigene-derived RNA. This percentage increases in the presence of CLK1 and CLK4 to about 45% and is highest with CLK2 and CLK3, where it is between 60 and 70% (Fig. 2C). It should be noted that overexpression of CLKs does not change the splicing patterns of other minigenes (data not shown), e.g., GABA$_A\gamma 2$ alternative 24-nt exon (Ashiya and Grabowski, 1997), clathrin light-chain B exon EN (Stamm *et al.,* 1992, 1999), insulin receptor exon 11 (Kosaki and Webster, 1993), and SWAP exon 2A (Sarkissian *et al.,* 1996). Only with the SRp20 exon 4 was a similar effect on exon skipping found (Stoss *et al.,* 1999). We conclude that CLKs can stimulate skipping of tau exon 10 *in vivo,* with CLK2 having the strongest effect.

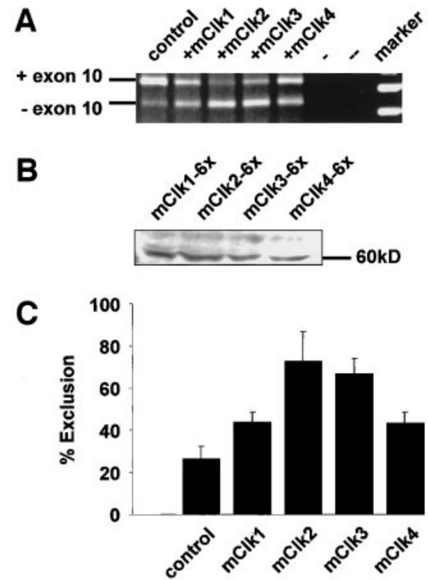

FIG. 2. Influence of CLK kinases on exon 10. (A) One microgram of the tau 9–10–11 minigene was cotransfected with 0.5 μg of the mouse CLK kinases indicated (mCLK1–4). Control is transfection with pEGFP-C2. The splice products have sizes of 336 and 429 nt, respectively. – and –– are control reactions without cDNA template and reverse transcription, respectively. (B) Western blot demonstrating equal expression of the mCLK1–4 protein. Fifty microliters of protein lysate transfected with his-tagged CLKs 1–4 was analyzed using Ni-HPRT (Qiagen). (C) Statistical evaluation of at least four independent experiments. The percentage exclusion of exon 10 is indicated. Error bars indicate standard deviations of at least four independent experiments.

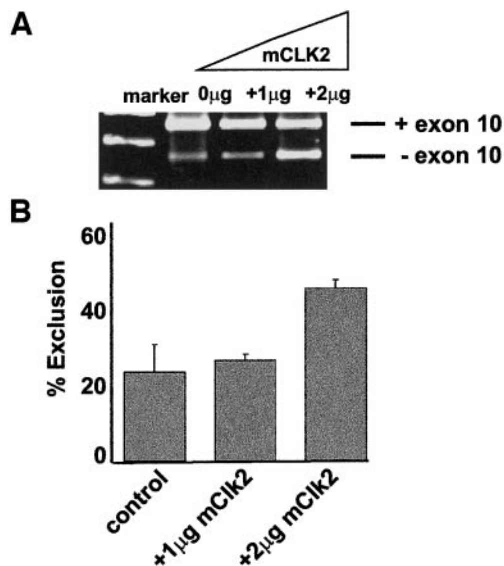

FIG. 3. Influence of mCLK2 on exon 10 in heterologous background. (A) Two micrograms of the pSPL3wt minigene containing exon 10 flanked by tat exons was cotransfected with increasing amounts of mCLK2 expression constructs into HEK293 cells. Lane 1, no CLK2 was added; lane 2, cotransfection of 1 μg CLK2; lane 3, cotransfection of 2 μg CLK2. (B) Statistical evaluation of at least three independent experiments. The percentage exclusion of exon 10 is indicated. Error bars indicate standard deviations of at least four independent experiments.

mCLK1–4 Promote Exon 10 Skipping in a Heterologous Context

We previously demonstrated that both flanking exons 9 and 11 are involved in fine-tuning regulation of exon 10 (Gao *et al.,* 2000). We therefore asked whether CLKs could still promote exon 10 skipping in a heterologous background. To test this, we employed a construct where exon 10 is flanked by tat exons (Hasegawa *et al.,* 1999). As shown in Fig. 3A, CLK2 promotes exon 10 skipping in this context as well ($F = 19.84$, $df = 2$, $P = 0.002$). However, CLK2 increases exon 10 skipping from 25 to 45% ($P = 0.03$) (Fig. 3B), which is a small effect when compared to the tau construct containing the natural flanking exons (Fig. 2C). In addition, higher amounts of CLK2 (μg) must be transfected to achieve this effect (Figs. 3A and 3B). Similar results were observed with the other CLKs (1, 3, and 4) and constructs flanked by constitutive insulin exons (Gao *et al.,* 2000) (data not shown). We conclude that CLKs induce skipping of exon 10 regardless of genomic context. However, the natural flanking sequences promote the ability of CLKs to cause exon 10 skipping, which is in agreement with our earlier findings (Gao *et al.,* 2000).

mCLK2 Can Revert the Alternative Splicing Pattern of Most FTDP-17 Mutations Which Enhance Exon 10 Usage

Next, we wished to determine whether CLK2 can revert the missplicing observed in mutations of tau exon 10 associated with FTDP-17. Since the effect of CLK2 is stronger when exon 10 is flanked by the natural tau exons 9 and 11, several of the FTDP-17 mutations were created in the SV9/10L/11 minigene background (Gao *et al.,* 2000). All of these mutations changed exonic or intronic sequence elements involved in splicing. They were N279K, which creates a purine-rich enhancer; L284L, a silent mutation that destroys a tat-like silencer; S305N, a mutation that improves U1 snRNA binding and possibly destabilizes the hairpin found at the 5′ splice site of exon 10; and finally the intronic mutation M16 and its compensatory mutation C16, which destroy and respectively restore this hairpin (Fig. 1B). With the exception of S305N, expressing these constructs in the presence of CLK2 promoted exon 10 skipping that was similar to the effect seen with the wild-type tau minigene SV9/10L/11. In contrast, the catalytically inactive variant CLK2KR (Nayler *et al.,* 1997) had no effect, indicating that skipping of exon 10 is mediated by phosphorylation. As expected, CLK2 had the strongest effect on the L284L mutation, where an exonic silencer sequence was destroyed, and restored exon 10 skipping to levels similar to those of the wt construct ($P = 0.848$). The only mutation that did not change its splicing pattern in response to CLK2 was S305N ($F = 0.02$, $df = 2$, $P = 0.980$) bearing an improved 5′ splice site of exon 10 (Figs. 4A and 4B). Even transfection of 4 μg CLK2 did not induce exon 10 skipping (data not shown). The mutation is located at position −2 of the 5′ splice site of exon 10, where a guanine is only rarely found (Stamm *et al.,* 1994, 2000) and thus improves the 5′ splice site, so that exon 10 is now constitutively used and no longer subject to regulation. Similar effects of splice site improvement were seen in other neuronal exons (Kuo *et al.,* 1991; Stamm *et al.,* 1999). We conclude that the phosphorylation induced by CLK2 can compensate for defects in regulatory elements of tau exon 10, as long as the exon can still be alternatively regulated. Furthermore, the change in splicing is most likely the result of the phosphorylation activity and not due to a sequestration of proteins by CLKs, as the CLK2KR mutants have no effect.

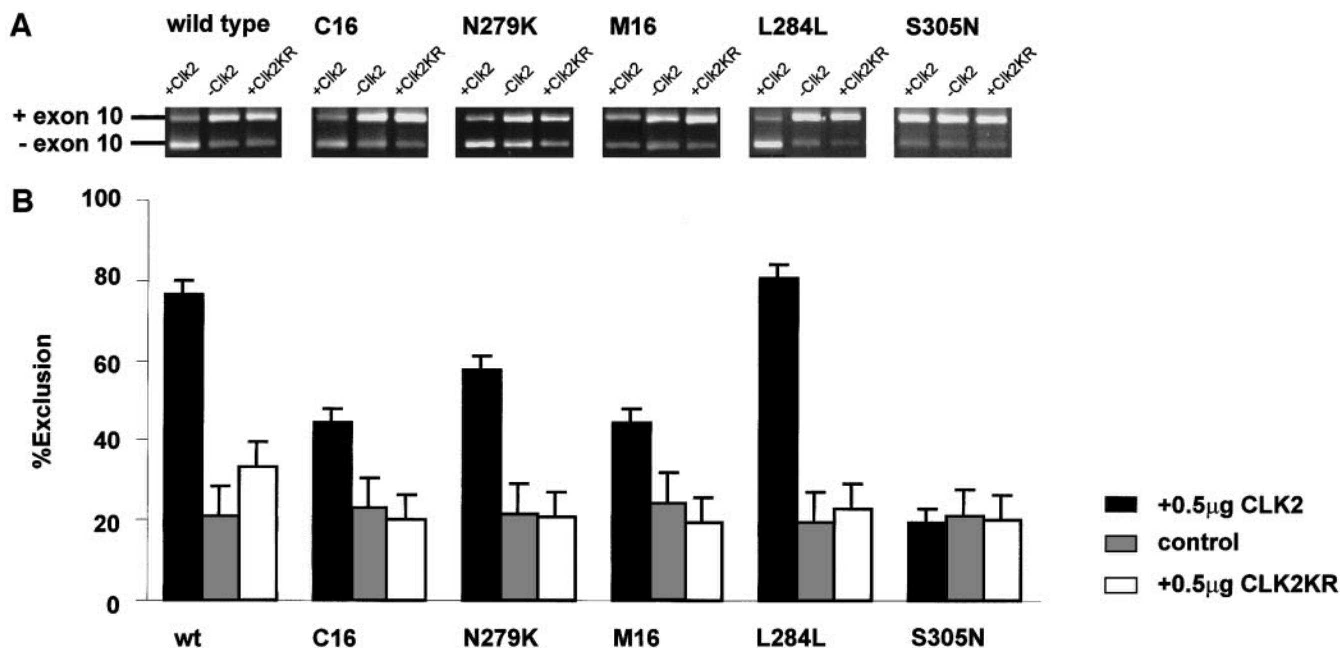

FIG. 4. Influence of mCLK2 on exon 10 mutants. (A) One microgram of the SV9/10L/11 minigene (wt) with the appropriate mutants was cotransfected with 0.5 μg of the mCLK2 expression construct into HEK293 cells. The sequences of the mutations are shown in Fig. 1. As a control, 0.25 μg of mCLK2KR was used. CLK2KR is catalytically inactive due to a lysine to arginine exchange at its ATP-binding site (Nayler *et al.,* 1997). (B) Statistical evaluation of at least three independent experiments. Black bars indicate percentage exon 10 exclusion in the presence of 0.5 μg mCLK2, gray bars show exon 10 exclusion in the absence of mCLK2, and white bars show exon 10 exclusion in the presence of the catalytically inactive form mCLK2KR. Error bars indicate standard deviations.

Exon 10 Is Regulated by SRPK1

Another kinase that phosphorylates SR proteins *in vitro* and regulates spliceosome assembly and intranuclear distribution of splicing factors is SRPK1 (Gui *et al.,* 1994; Papoutsopoulou *et al.,* 1999). To determine whether SRPK1-mediated phosphorylation of splicing factors has an effect similar to that of CLK2, we tested SRPK1 in cotransfection experiments with the SV9/10L/11 minigene. As can be seen in Fig. 5, SRPK1 stimulates exon 10 skipping (F = 17.51, df = 2, P = 0.03). However, the effect is not as strong as that observed with CLK2 (P = 0.026) (Fig. 7).

Regulation of Tau Exon 10 by CDK5

CLK2 is a nuclear kinase. Its subnuclear localization and activity are regulated by phosphorylation of serine 141, which is conserved among all CLK family members (Nayler *et al.,* 1998b). It is likely that this serine residue is the target of upstream kinases that remain to be determined. Recently, it was suggested that CDK5 (or NCLK), the major cyclin-dependent kinase in the brain, is constitutively active in neurons affected with Alzheimer's disease. It is activated by p35, which was shown to be cleaved to the constitutively active form of p25 in Alzheimer's disease. CDK5 controls other proteins, such as DARPP-32, which was shown to regulate a serine/threonine kinase and a serine/threonine phosphatase (Bibb *et al.,* 1999; Patrick *et al.,* 1999). In addition, CDK5 is found in hyperphosphorylated neurofilaments in Parkinson's disease (Nakamura *et al.,* 1997). Since CDK5 has multiple cellular substrates (Bibb *et al.,* 1999; Mandelkow, 1999) and is structurally related to CLK2, we asked whether it can influence exon 10 splicing. We analyzed the effect of CDK5 together with its activators p35 and p25 on the SV9/10L/11 minigene. As shown in Fig. 6, transfection of CDK5 alone (F = 0.124, df = 2, P = 0.886) or together with p35 (F = 0.937, df = 2, P = 0.442) had no significant effect on exon 10 usage. However, in the presence of the constitutive activator p25, CDK5/p25 promotes exon 10 skipping (F = 5.184, df = 2, P = 0.049). This effect is weaker than the one observed with CLK2, although about 10 times more DNA was transfected. We conclude that CDK5 can barely alter the alternative splicing patterns of tau exon 10 *in vivo* even if it is activated by p25.

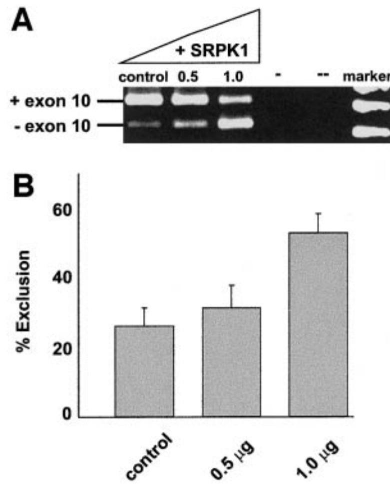

FIG. 5. Influence of SRPK1 on exon 10. (A) One microgram of the SV9/10L/11 minigene was transfected with increasing amounts of an SRPK1 expression vector. – and –– are control reactions without cDNA template and reverse transcription, respectively. (B) Statistical evaluation of three independent experiments. Numbers indicate micrograms of mCLK2 transfected.

DISCUSSION

Missplicing of tau exon 10 is associated with about half of the known cases of FDTP-17 (Spillantini *et al.,* 2000b) and with the pathology of various other neurodegenerative diseases (Spillantini and Goedert, 1998). Several mutations associated with FTDP-17 that promote exon 10 inclusion have been described. Since exon 10 encodes an additional microtubule-binding site, the enhanced expression of these isoforms could result in tau molecules that are more prone to aggregation and formation of filamentous lesions (Hasegawa *et al.,* 1999). Here we show that the splicing of tau can be influenced toward exon 10 exclusion in cell culture systems by overexpressing the serine/threonine kinases CLK1–4. Importantly, the RNA-processing pathways of mutations in exonic elements can be altered toward exon 10 skipping. In contrast, the S305N mutation that improves the 5′ splice site of exon 10 and possibly destabilizes the stem–loop structure behaves like a constitutive exon and can no longer be regulated.

The exact molecular mechanism of CLK action remains to be determined. The action of CLKs on splice site selection is selective, since only tau exon 10 and SRp20 exon 4 (Stoss *et al.,* 1999), but not four other minigenes tested, were affected. Since CLKs do not bind RNA, the effect is most likely indirect. For example, splicing factors could be released from the speckles, where they are stored. It has been demonstrated that CLKs regulate the intranuclear localization of SR proteins (Duncan *et al.,* 1998; Nayler *et al.,* 1998b) and that phosphorylation of the RS domain is necessary for targeting SR proteins to sites of transcription (Misteli, 2000; Misteli *et al.,* 1998). To test this hypothesis, we compared the effect of several proteins that are phosphorylated by CLKs *in vitro* with the CLK effect on exon 10 regulation. All these factors induced exon 10 skipping, although more DNA needed to be transfected to achieve a change in regulation similar to that of the CLKs. Furthermore, quantification demonstrated that mCLK2 was the kinase with the strongest effect on exon 10 regulation (Fig. 7). This indicates that kinases with similar biochemical properties have different specificities *in vivo,* by mechanisms that remain to be explored. Since none of the SR proteins tested has a similar strong effect (Fig. 7), we suspect that several different SR proteins regulate tau exon 10 *in vivo.* One explanation could be that CLK2 releases a specific subset of splicing regulatory proteins from speckles in a composition or

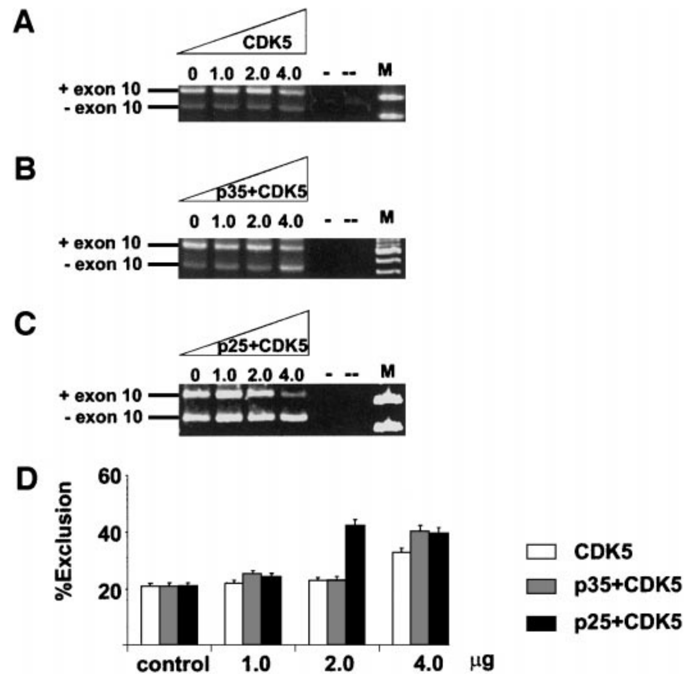

FIG. 6. Influence of CDK5 on exon 10. (A) One microgram of the SV9/10L/11 minigene was transfected with increasing amounts of CDK5 expression construct into HEK293 cells. (B) One microgram of the SV9/10L/11 minigene was transfected with increasing amounts of CDK5 expression construct in the presence of 1 μg p35 expression construct. (C) One microgram of the SV9/10L/11 minigene was transfected with increasing amounts of CDK5 expression construct in the presence of 1 μg p25 expression construct. (D) Statistical evaluation of three independent experiments.

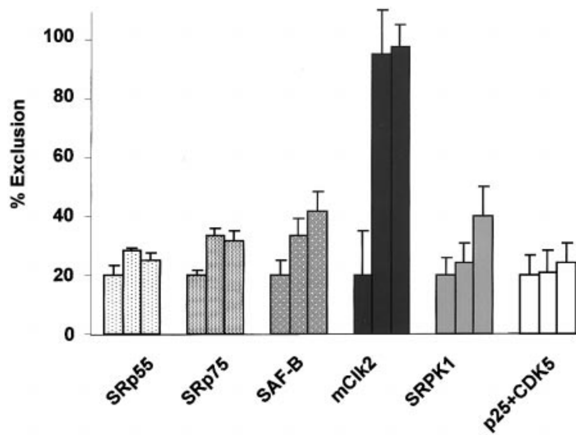

FIG. 7. Comparison of cotransfection experiments. The bars show the percentage inclusion of cotransfection experiments employing the SV9/10L/11 minigene and SR proteins SRp55 and SRp75 (Gao *et al.*, 2000; Manley and Tacke, 1996); scaffold attachment factor B (Nayler *et al.*, 1998c); and mCLK2, SRPK1, and CDK5 (Figs. 2, 5, 6). The bars represent 0, 0.5, and 1 μg of transfected splicing factors.

phosphorylation state (Prasad *et al.,* 1999) that is optimal for exclusion of tau exon 10. Another possibility is that CLKs change the ratios of splicing factor isoforms, which have different effects on splice site selection. For example, SRp20 produces isoforms that either include or exclude an RS domain (Jumaa *et al.,* 1997; Jumaa and Nielsen, 1997) and CLK2 promotes formation of the RS domain-containing form (Stoss *et al.,* 1999). Since most splicing factors are subject to regulation by alternative splicing (Nayler *et al.,* 1998a), CLKs might regulate other RS domain-containing proteins as well. Finally, CLKs themselves are regulated by alternative splicing, but their regulatory transacting factors remain to be determined (Duncan *et al.,* 1998). Thus, our data show how a complicated network of regulatory proteins can be altered by phosphorylation, resulting in specific changes in pre-mRNA-processing pathways.

We found the effect of CLKs more pronounced when tau exon 10 was in its natural rather than in a heterologous context. This is in agreement with our earlier findings (Gao *et al.,* 2000) and strongly suggests that splicing of exon 10 is regulated by more elements than have been assumed so far (D'Souza *et al.,* 1999). A dependence of alternative exon regulation on flanking exons was also observed in other systems, such as tropomyosin exons 6 and 7 (Guo and Helfman, 1993), tau exon 6 (Wei and Andreadis, 1998), fibronectin (Muro *et al.,* 1999), and myosin light chain (Gallego and Nadal, 1990). Our results caution that the exclusive analysis of exon 10 variants in exon trap vectors might give erroneous results.

Selective deposition of only some tau isoforms is observed in a number of sporadic neurodegenerative diseases, such as progressive supranuclear palsy, corticobasal degeneration, and Pick's disease (Spillantini and Goedert, 1998). These diseases are frequently associated with missplicing of exon 10 (Chambers *et al.,* 1999). It is possible that the change in isoform expression is caused by differences in the concentration of transacting factors that regulate pre-mRNA processing. These variations are the likely cause for the tissue-specific action of mutated alleles (Nissim-Rafinia *et al.,* 2000) and probably account for pleiotrophic phenotypes of some mutations in a population (Rave-Harel *et al.,* 1997). We therefore speculate that changes in the concentration of proteins involved in pre-mRNA processing may contribute to tauopathies by affecting alternative splicing.

Most of these regulatory proteins are released from their intranuclear storage sites by phosphorylation, which would coincide with the different phosphorylation status seen in most of these diseases (Mandelkow and Mandelkow, 1998). With a better understanding of the molecular mechanisms regulating alternative splicing it is becoming clearer that defects in pre-mRNA splicing have been underestimated as a factor in human disease (Daoud *et al.,* 2000; Philips and Cooper, 2000; Stoss *et al.,* 2000). A number of studies show that missplicing events causing disease can be changed in cell culture systems (Friedman *et al.,* 1999; Hofmann *et al.,* 2000; Karras *et al.,* 2000; Schmajuk *et al.,* 1999; Wilton *et al.,* 1999), suggesting that changing splice site selection, e.g., through low-molecular-weight substances affecting phosphorylation, could be a new therapeutic principle.

EXPERIMENTAL METHODS

Cloning and Mutagenesis

Standard cloning techniques were used. Human tau genomic fragments were cloned into pSVIRB. SV9/10L/11 (tau minigene) contains the 3′ 161 nucleotides of tau exon 9 and 1.5 kb of its downstream intron fused to insulin exon 2, tau exon 10, and the proximal portions of its flanking introns (471 bp upstream and 408 bp downstream) and the 5′ 68 nucleotides of tau exon 11 and 324 nucleotides of its upstream intron fused to insulin exon 3 (Gao *et al.,* 2000). Mutations (C16 double mutant, combining M5 and M16, N279K, M16 intronic,

L284L, S305N) were introduced using the QuickChange site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions, except that the *Dpn*I digestion was overnight.

The pSPL3 wt construct contains tau exon 10 including 10 nucleotides at the 3′ intron boundary and 85 nucleotides at the 5′ boundary flanked by tat exons (Hasegawa *et al.,* 1999). CLK1–4 constructs (Nayler *et al.,* 1997, 1998b) were described earlier. Eukaryotic expression of other splicing regulator cDNAs (SRp55, SRp75, ASF/SF2, SAF-B, SRPK1) was under control of the CMV promoter.

In Vivo Splicing Assays

In vivo splicing was performed essentially as described (Stoss *et al.,* 1999). Briefly, 1 μg of the reporter gene was transfected together with increasing amounts (0, 0.5, 1 μg) of transacting factor constructs in HEK293 cells using the calcium phosphate method. Empty vector (pEGFP-C2, Clontech) was added to ensure that equal amounts of DNA were transfected. Transfection was performed at 37°C in 3% CO_2 overnight. RNA was isolated 17–24 h after transfection using an RNeasy mini kit (Qiagen), following the manufacturer's instructions. RNA was eluted in 40 μl RNase-free H_2O. For reverse transcription, 2 μl of isolated RNA was mixed with 5 pmol antisense minigene-specific primer in 2 μl 5× RT buffer, 1 μl 100 mM DTT, 1 μl 10 mM dNTP, 3 μl H_2O, 0.25 μl RNase inhibitor, and 0.25 μl H^- reverse transcriptase. The tubes were incubated for 45 min in a 42°C water bath.

Two microliters of the RT reaction was mixed with 2.5 pmol of sense and antisense primer each, 10× PCR buffer, 200 mM dNTP, 2 mM $MgCl_2$, and 0.1 μl of *Taq* polymerase.

For the tau SV9/10L/11 minigene, reverse transcription followed by PCR using the primers sense INS1 (cag cta cag tcg gaa acc atc agc aag cag) and antisense INS3 (cac ctc cag tgc caa ggt ctg aag gtc acc) was performed. PCR conditions were initial denaturation for 2 min at 94°C; 30 cycles: 30 s denaturation at 94°C, annealing at 55°C for 1 min, extension at 72°C for 1 min, after 30 cycles a final extension at 72°C for 20 min, and cooling to 4°C in a Biometra trio block thermocycler (Gao *et al.,* 2000).

For the PSL3-wt minigene the following primers were used: pSL3 forward, cgagctc gct tgt tca ctc atc ctt ttt; and pSL3 reverse, cga gct cgc agt gtc cgc aag tgt a. PCR conditions were identical to those for SV9/10L/11 amplification.

Quantification of the Results

PCR products were resolved on a 2% agarose gel and stained with ethidium bromide. Bands were quantified directly from the gel with the Herolab EASY system using Image master VDS and analysis software. The percentage exclusion is the percentage of spliced RNA that contains the exon (IOD of the peak of exclusion band/IOD of the peak of inclusion band + IOD of the peak of exclusion band) × 100. Mean values (± standard deviation) were calculated from three independent experiments each. Statistical analysis was performed by ANOVA followed by the Scheffé F test for comparison between samples. $P < 0.05$ was regarded as significant.

Western blot analysis was performed as described (Hartmann *et al.,* 1999).

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