RBM4 INTERACTS WITH AN INTRONIC ELEMENT AND STIMULATES TAU EXON 10 INCLUSION

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Running Title: Splicing Regulation and Neurodegeneration

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Running title: Alternative splicing regulation of tau exon 10

Tau protein, which binds to and stabilizes microtubules, is critical for neuronal survival and function. In the human brain, tau pre-mRNA splicing is regulated to maintain a delicate balance of exon 10-containing and exon 10-skipping isoforms. Splicing mutations affecting tau exon 10 alternative splicing lead to tauopathies, a group of neurodegenerative disorders including dementia. Molecular mechanisms regulating tau alternative splicing remain to be elucidated. In this study, we have developed an expression cloning strategy to identify splicing factors that stimulate tau exon 10 inclusion. Using this expression cloning approach, we have identified a previously unknown tau exon 10 splicing regulator, RNA binding motif protein 4 (RBM4). In cells transfected with a tau minigene, RBM4 over-expression leads to an increased inclusion of exon 10, whereas RBM4 down-regulation decreases exon 10 inclusion. The activity of RBM4 in stimulating tau exon 10 inclusion is abolished by mutations in its RNA binding domain. A putative intronic splicing enhancer located in intron 10 of the tau gene is required for the splicing stimulatory activity of RBM4. Immunohistological analyses reveal that RBM4 is expressed in the human brain regions affected in tauopathy, including the hippocampus and frontal cortex. Our study demonstrates that RBM4 is involved in tau exon 10 alternative splicing. Our work also suggests that down-regulating tau exon 10 splicing activators such as RBM4 may be of therapeutic potential in tauopathies involving excessive tau exon 10 inclusion.

Microtubule associated protein Tau (MAPT) regulates the organization and stability of microtubules (MTs) in the neurons. In humans, the Tau protein is encoded by a single gene on chromosome 17. The tau gene is expressed at a high level in neurons and at lower levels in glia and certain non-neuronal cells. Involved in maintaining cell morphology, axonal extension and vesicle transport, Tau is critical for the formation and function of neurons (1-3, for recent reviews, see 4-9). The expression of the tau gene is under complex regulation at multiple steps, including both post-transcriptional and post-translational levels. In the human brain, six tau isoforms are expressed as a result of alternative splicing of exons 2, 3 and 10 (10-12). Alternative splicing of exon 10 (Ex10), which encodes for one of the four MT-binding domains, gives rise to tau isoforms containing either four MT-binding repeats (Tau4R, Ex10+) or three MT-binding repeats (Tau3R, Ex10-). In the adult human brain, the regulated splicing of exon10 results in a ratio of Tau4R to Tau3R as approximately one.

Genetic studies have revealed a number of mutations in the human tau gene in patients with tauopathy. More than 30 different mutations have been associated with frontotemporal dementia with parkinsonism linked to chromosome-17 (FTDP-17) (13-15). This is an autosomal dominant disorder with clinically heterogeneous manifestations that include behavioral, cognitive and motor abnormalities. FTDP-17 mutations can be classified into two groups, missense mutations that affect Tau protein activity and splicing mutations that alter the ratio of distinct tau splicing
isoforms (for recent reviews, see 4-9). Almost all splicing mutations characterized so far affect the regulation of tau exon 10 splicing. In vitro experiments suggest that Tau4R and Tau3R proteins bind and stabilize MTs in different manners (16-18). This delicate balance between exon10+ to exon10- tau isoforms is crucial for neuronal function in learning and memory. However, the underlying mechanism remains to be elucidated [(19),(20),(13) ; reviewed in(5)].

A large number of genes in the human genome utilize alternative splicing to generate functionally distinct gene products. Understanding how these alternative splicing events are regulated is an important issue in functional genomics. In past two decades, a number of alternative splicing regulators have been identified. Many of these trans-acting factors were initially identified using biochemical approaches (reviewed in (21),(22),(23). In this study, we have developed an expression cloning approach using a tau exon 10 splicing GFP reporter, Tau4R-GFP, in which GFP expression was dependent on the tau exon 10 inclusion. Using this system to screen a human brain cDNA library, we have identified a previously unknown tau exon10 splicing activator, RBM4, a protein recently shown to play a role in alternative splicing of alpha-tropomyosin (24). Our experiments show that overexpression of RBM4 stimulates tau exon 10 inclusion, and RNAi-mediated knock-down of RBM4 expression in transfected cells reduces tau exon inclusion. Immunohistochemical staining indicates that RBM4 is expressed in the brain regions involved in tauopathy. Our results support a role of RBM4 in regulating tau exon 10 splicing.

**EXPERIMENTAL PROCEDURES**

*Expression cloning using the tau reporter gene.*

We prepared a human adult brain cDNA library using the adult brain mRNA purchased from Clontech. The quality of this cDNA library was tested using RT-PCR and small-scale screening before the library was subdivided into 400 cDNA pools. It was estimated that each pool contained approximately 5000 clones. Each of these 400 primary pools was amplified for 6-8 hours at 37°C after being carefully plated on 20x20cm bacterial plates. cDNAs were prepared from each pool using an optimized medium-scale plasmid preparation method combining polyethylene glycol precipitation with the endotoxin-free DNA preparation kit (Qiagen) to ensure the high quality DNA preparations for transfection. The typical transfection efficiency is approximately 50-60%, as assessed by GFP expression in the control cells transfected with a GFP plasmid. For the first round of transfection, 40 combined pools (combined from 10 primary pools in a grid fashion to allow easy identification of individual positive primary pools) of cDNAs were transfected in cells stably expressing the corresponding reporter genes. Using this grid design, 400 cDNA pools were screened in 40 primary transfections in 15 cm tissue culture dishes and further screened in subsequent rounds of transfections.

Because HEK293 cells had a high efficiency of transfection and a low background GFP expression, we chose this cell line for preparing a stable cell line expressing the Tau reporter gene. Stable HEK293 cells expressing Tau4R-GFP reporter were selected with G418 and used for expression of cDNA clones following transfection with pooled cDNAs from the adult brain cDNA library. During the first two rounds of transfection, it appeared more efficient to visually examine individual transfected culture plates under an inverted fluorescent microscope than using a cell sorter. The primary positive pools that gave significant activation of GFP expression were identified, and cDNAs in these pools were further divided. For each primary positive pool, 20 sub-pools were prepared and individually transfected into stable cells expressing the Tau4R-GFP reporter gene in 10cm dishes. The positive sub-pools were again identified using inverted fluorescent microscopy and subdivided for another round of transfection until individual single cDNA clones were isolated. After 7 rounds of retransfection, we were able to isolate single cDNA clones that have significant effects on tau exon 10 splicing.

**Plasmids and antibodies.**

Mammalian expression plasmids for HA-tagged wild type or mutant RBM4 (27) and for raver were described previously(40). The plasmid expressing RNAi for RMB4 was described (24). Monoclonal anti-HA antibody was purchased from...
Covance. Affinity purified polyclonal anti-RBM4 prepared as described previously (27).

**Transfections and Reverse Transcription (RT)-PCR.**

HEK293 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Transfection was carried out using a previously described calcium phosphate precipitation procedure with 1–3 μg of DNA (41). Cells were harvested 48 hours after transfection, and the RNA was extracted using the RNAeasy kit (Qiagen). In RNAi experiments, lipofectamine 2000 (Invitrogen) was used according to manufacturer’s instruction. Splicing products of the expressed tau minigenes were detected using RT-PCR as previously described (41). RT-PCR was also performed to detect the endogenous Bcl-xL and gamma-actin genes using the corresponding specific primers. The products were analyzed by electrophoresis using 6% polyacrylamide gels.

**RNA-protein interactions.**

The RNA probes were prepared by in vitro transcription in the presence of 32P-UTP using T7 RNA polymerase from linearized DNA templates as described (26). The RNA probes were gel-purified and used for UV-crosslinking assay. The recombinant RBM4 protein was expressed as a 6xHis-tagged protein and purified from E. coli as described by Lai et al (27). For UV cross-linking, approximately 20 femtomoles of RNA probe was incubated at 30°C with 1ug of purified RBM4 or 30ul cell lysate in a 40ul reaction mixture under in vitro splicing conditions (26). The samples were irradiated on ice under 254-nm light for 10 min. The probes were digested in equal volumes of RNase A (5mg/ml) (Sigma) at 30°C for 20 min. For immunoprecipitation, following the RNase treatment, samples were incubated with HA.11 anti-HA monoclonal antibody (Covance) at 4°C for 2 hours. Protein A/G-agarose beads were then added with further incubation and gentle rocking. Following washing, the RNA-bound proteins immunoprecipitated were eluted and resolved on SDS-PAGE followed by autoradiography.

**Immunohistochemical staining**

Human brain samples from autopsy material were fixed in formalin and paraffin embedded. Six micron thick sections from the frontal and hippocampal regions were used for immunohistochemical staining. The Santa-Cruz staining kit for rabbit polyclonal antibody was used to perform immunostaining with the affinity-purified rabbit anti-RBM4 antibody. Antigen retrieval was carried out in the presence of 1 mM EDTA or TE, pH9.0. Primary antibody was used at 1:1000 dilution. Immunostaining was carried out following the instruction manual, color development performed with DAB and counterstaining with hematoxyline.

**RESULTS**

**Establishing a GFP-reporter-based strategy for cloning trans-acting splicing regulators that stimulate exon 10 inclusion**

In our previous studies, a human tau minigene has been established that recapitulates tau exon 10 alternative splicing in the human brain (25, 26). In this minigene system, alternative inclusion or exclusion of the exon 10 leads to the formation of Tau4R and Tau3R respectively. To develop a system for identifying splicing activators that enhance tau exon 10 inclusion, we prepared a tau exon10-splicing GFP reporter (Tau4R-GFP) by fusing the tau minigene with a cDNA fragment encoding green fluorescent protein (GFP, Fig.1). This Tau4R-GFP reporter gene was constructed by adding a translation start codon ATG upstream of exon 10 so that the tau mini-gene construct produced a fusion protein containing tau peptide fragment fused to GFP (Fig. 1A). One base pair was inserted by site-directed mutagenesis in the exon 10 so that exon 10 and exon 11 were not in the same frame following splicing. This Tau4R-GFP reporter gene enabled us to use GFP expression as an indicator for exon 10 inclusion. When the Tau4R-GFP reporter gene was transfected into Hela or HEK293 cells, the tau minigene underwent splicing in a similar pattern as the wild type tau gene, producing both Tau3R and Tau4R splicing isoforms. No aberrant splicing, such as activation of cryptic splice sites, was detected (data not shown). The Tau4R-GFP reporter showed a low background of GFP
expression in HEK293 cells, facilitating the expression cloning screening as described below.

Identifying trans-acting factors enhancing tau exon 10 inclusion

We have been interested in identifying protein factors important for regulating tau exon 10 alternative splicing. By examining their biochemical function and molecular mechanisms, we wish to understand how these splicing regulators control the balance of different tau splicing isoforms. To search for splicing factors that stimulate tau exon 10 splicing, we made use of the Tau4R-GFP reporter gene cassette (Fig.1A). We developed a progressive subdivision and screening protocol (Fig.1B) to screen a human brain cDNA library (for details see Experimental Procedures). Following 7 rounds of re-subdivision and re-transfection, seven individual cDNA clones that influence tau exon 10 splicing were isolated. Five cDNA clones are still under characterization. Sequencing analyses of the remaining cDNAs and databank searches showed that two cDNA clones encode a RNA binding protein known as RNA binding motif protein 4, RBM4 (27). RBM4 protein will be the focus of this report.

Confirming the activity of RBM4 in regulating tau exon 10 splicing.

To validate the screen and rule out potential indirect effects on the GFP reporter, we examined the effect of RBM4 on tau exon10 splicing using the original tau exon 9-10-11 splicing substrate (25). Another RRM-containing splicing factor, Raver (40), was also tested. The tau minigene was co-transfected with either control vector or RBM4- or Raver- expression plasmid (Fig 3). Following transfection, Tau exon 10 alternative splicing was then measured using RT-PCR. Although the wild-type and mutant RBM4 proteins were expressed at a similar level (Fig. 4B and Fig.4D), the stimulation of tau exon 10 splicing was observed only in cells transfected with the wild-type but not mutant RBM4 (Fig. 4A). The quantification of tau splicing isoforms in these co-transfection coupled RT-PCR experiments indicated that the ratio of exon 10-inclusion to exon 10-skipping tau isoforms (Ex10+/Ex10-) was significantly increased in cells transfected with the wild-type RBM4 as compared with those of the vector control or cells expressing the RRM mutant RBM4 (p<0.001).  On the other hand, overexpression of the RRM mutant RBM4 protein did not affect tau exon 10 splicing, similar to the vector control (Fig.4E). These experiments demonstrate that the RNA recognition domain of RBM4 is required for its stimulation of exon 10 inclusion, suggesting that RBM4 may regulate tau exon 10 splicing by interacting with tau pre-mRNA.

RBM4 interacts with an intronic element in tau pre-mRNA.

The observation that the RNA recognition motif of RBM4 is necessary for its splicing stimulatory activity in tau exon 10 splicing prompted us to test whether RBM4 interacts with tau pre-mRNA. A UV-crosslinking coupled immunoprecipitation assay was performed using 32-P labeled tau pre-mRNA and protein extracts
prepared from HEK293 cells transfected with HA-tagged RBM4 (HA-RBM4) or the vector control. Following UV crosslinking, immunoprecipitation was carried out using a monoclonal anti-HA antibody, and immunoprecipitation products were analyzed by SDS-PAGE followed by autoradiography. A 40kDa band was detected in the reactions containing HA-RBM4 (Fig. 5A, lane 2) but not in the vector control (Fig. 5A, lane 1), indicating that RBM4 interacts with tau pre-mRNA. To map the site of RBM4 interaction, different regions of tau pre-mRNA were tested. UV crosslinking experiments using RNA fragments corresponding to different regions of tau pre-mRNA suggest RBM4 interacts with an intronic region downstream of exon 10 in the tau pre-mRNA. To test the specificity of the interaction between RBM4 and the tau intronic region, the UV-crosslinking assay was carried out in the presence of excess amount of unlabeled RNA, either the tau pre-mRNA or non-specific control RNA as competitors. The RBM4 binding signal was significantly reduced in the presence of the unlabeled tau RNA containing the 260-nucleotide intronic region (Fig. 5E, lane 2), as compared with the reaction in the absence of competitive RNA (Fig. 5E, lane 1) or with that in the presence of unlabeled non-specific control RNA (Fig. 5E, lane 3). These experiments demonstrate that RBM4 specifically interacts with tau pre-mRNA in the intronic region downstream of the regulated exon 10.

**Mutation of the intronic RBM4 binding site reduces the activity of RBM4 in stimulating tau exon 10 inclusion.**

The UV crosslinking experiments described above show that RBM4 interacts with tau pre-mRNA in the intronic region downstream of the 5’ splice site of exon 10. Sequence analysis indicates that a pyrimidine-rich element (UCCUUCUUG) is located 100 nucleotide downstream of the 5’ splice site in this intronic region. This element shares sequence similarity to the RBM4 binding site identified in alpha-tropomyosin pre-mRNA (24). To examine the potential role of this putative RBM4 binding site, we performed site-specific mutagenesis and prepared a mutant tau minigene by changing the pyrimidine nucleotides in the UCCUUCUUG element to purine nucleotides. The mutant tau pre-mRNA (tau-mRBMBS) was then tested for its interaction with purified RBM4 protein. As compared with the wild-type tau pre-mRNA, the mutant tau transcript showed significantly reduced binding to RBM4 protein (Fig. 5F, compare lane 3 with lane 2).

To test the functional significance of this intronic RBM4 binding site in tau exon 10 splicing, we compared tau exon 10 splicing of the wild type and the mutant tau pre-mRNA in the cells co-transfected with the RBM4 expression plasmid. RBM4 overexpression was confirmed both at the RNA and at the protein levels (Fig. 6B and 6D, with actin level as the internal control shown in Fig. 6C). The mutant tau pre-mRNA showed a significantly reduced response to RBM4
than the wild-type tau splicing substrate (Fig. 6A, compare lane 1 and lane 2). Although the splicing stimulatory activity of RBM4 was not completely eliminated, the effect of the mutation in the RBM4 binding site on RBM4-mediated exon 10 inclusion was consistently observed. Figure 6E shows the quantification of ratios of Exon 10+/Exon10- tau transcripts from 6 independent experiments. When the intronic UCCUUCUUG was mutated, the stimulatory activity of exon 10 inclusion by RBM4 overexpression was reduced, changing the ratio of exon10+/exon10- from approximately 6 with the wild-type tau splicing substrate to 2 with the mutant tau pre-mRNA (Fig. 6E). The residual stimulatory activity by RBM4 on the mutant tau pre-mRNA is consistent with the observation that RBM4 binding was not completely abolished in the mutant tau pre-mRNA (Fig 5E, lane 3).

Knockdown of RBM4 by RNAi decreases tau exon 10 inclusion, suggesting a role of endogenous RBM4 in stimulating exon 10 inclusion

To test the potential role of the endogenous RBM4 in regulating exon 10 splicing, we used RNA interference (RNAi). RBM4-specific RNAi or control was co-transfected using lipofectamine along with the tau minigene into HEK293 cells. For reasons not clear yet, cells transfected under these conditions showed an increase in Tau4R even in mock or control transfections (data not shown, see Fig. 7B, lane 1). The effect of the RNAi was evaluated at both RNA and protein levels. RT-PCR with RBM4-specific primers and western blotting with anti-RBM4 antibody showed that the RBM4-specific RNAi transfection led to approximately 70-80% reduction in RBM4 expression level (RNA in Fig 7C and protein in Fig 7E respectively, compare lane 1 with lane 2). We examined tau exon 10 splicing by measuring the ratio of exon 10 splicing isoforms (Ex10+/Ex10-) following the quantitative RT-PCR as described before. RBM4 downregulation by RNAi led to a decrease in the inclusion of exon 10 (Fig 7A compare lane 1 and 2). The results from six independent experiments were quantified. RNAi knockdown of RBM4 led to an approximately 7-fold decrease in exon 10 inclusion as compared to the control RNAi (Fig 7F). This effect was specific because alternative splicing of other genes examined was not affected, including Bcl-x (Fig.7B). This observation suggests relative specificity of RBM4 in regulation of tau exon 10 alternative splicing. These results indicate that the endogenous RBM4 protein plays a role in activating tau exon 10 inclusion.

RBM4 expression in the human neurons in the frontal cortex and hippocampus

To begin to explore the potential involvement of RBM4 in tauopathy, we examined RBM4 expression in the human brain, particularly in the regions affected in tauopathy patients. Immunohistochemical staining of postmortem adult human brain samples was carried out using an affinity-purified anti-RBM4 antibody. As shown in Figure 8, the frontal cortex (panels A and B) and hippocampal (panels C and D) sections showed strong signals, whereas such staining signal was not detectable in the negative controls either in the absence of the anti-RBM4 antibody (panel E) or in the presence of the antibody preparation after absorption with RBM4 protein (not shown). RBM4 immunostaining signals were detectable in both neurons and glial cells including layers II neurons in the frontal cortex (marked by arrows in Fig. 8A and Fig. 8B) and CA1 pyramidal neurons in the hippocampus (as indicated by arrow-heads in Fig. 8C and Fig. 8D). The signal was stronger in the nucleus than in the cytoplasm. Together with the activity of RBM4 in stimulating tau exon 10 inclusion, RBM4 expression in neurons suggests a possible role of RBM4 as a regulator of tau exon 10 alternative splicing in the human brain.

Discussion

Pre-mRNA splicing is the most upstream event in the post-transcriptional RNA processing. It is estimated that over 65% of human genes undergo alternative splicing to form functionally distinct gene products (28). Alternative splicing is an extremely powerful mechanism for generating proteomic diversity. Accumulating evidence supports an important role of aberrant splicing in the pathogenesis of human diseases. Cis-acting splicing mutations that affect either splice sites or splicing regulatory elements lead to the formation of defective or toxic gene products. Such splicing mutations can cause human diseases by four types of mechanisms: exon skipping, intron retention, cryptic splice site activation or imbalance of
different splicing isoforms (for recent reviews, see (21),(22),(23)). The importance of maintaining the delicate balance of different splicing isoforms has only begun to be appreciated. A number of model systems have been established to investigate the cis-regulatory elements and trans-acting factors controlling alternative splicing events. However, relatively little is known about mechanisms regulating alternative splicing of genes associated with human pathogenesis.

Most of splicing regulators have been identified using biochemical approaches. So far there has been no report on using an expression cloning approach to systematically screen for alternative splicing regulators. Our study demonstrates that such an expression cloning approach based on alternative splicing-dependent GFP-reporter can be successfully used to identify alternative splicing regulator(s). Further improvement of this expression cloning strategy may lead to development of high-throughput systems for identifying genes important for splicing regulation. For example, a luciferase reporter constructed from our Tau-GFP reporter is being used to develop a high throughput system for tau exon 10 splicing regulation (C. Donahue, K Kosik and JYW, unpublished study).

RBM4 was initially identified in a yeast two-hybrid screen for proteins interacting with transportin-SR2 (27). Mammalian RBM4 contains, at its amino-terminus, two RNA recognition motifs (RRMs) and a CCHC-type zinc finger. At the C-terminal region of RBM4 are three alanine-rich stretches. RBM4 has been shown to promote the inclusion of skeletal muscle exon of alpha-tropomyosin by binding to a CU-rich intronic element. Domain dissection experiments show that the RNA recognition motif of RBM4 is required for splice site modulation, whereas the CAD domain was required for trafficking to the nucleus ((27), (24)). In alpha-tropomyosin alternative splicing, RBM4 competes with PTB for common or overlapping intronic elements and activates the inclusion of skeletal muscle-specific exon (24).

In our study, RBM4 was identified by its functional activity in stimulating exon 10 inclusion during the expression cloning using the tau exon 10-alternative splicing-dependent GFP reporter. Both overexpression (Fig 3 and Fig 4) and RNAi-mediated down-regulation (Fig. 7) in transfected cells using the tau minigene support a role of RBM4 in enhancing tau exon 10 inclusion. The RNA-binding domain of RBM4 is required for its stimulatory activity on tau exon 10 inclusion, because mutating the conserved aromatic amino acid residues in the RNA recognition motif eliminated such splicing enhancement (Fig.4). This is consistent with the previous observation that the same RRM mutant was inactive in stimulating alternative splicing in vitro (27).

Our UV-crosslinking experiments suggest that RBM4 interacts with an intronic region downstream of the 5’ splice site of exon 10. Further mutagenesis experiment indicates a CU-rich element in this region as a RBM4 binding site that is functionally important for the splicing stimulatory activity of RBM4. Mutating the pyrimidine into purine residues in this RBM4 binding site significantly reduced the interaction between RBM4 and tau pre-mRNA (Fig. 5). The same mutation also remarkably decreased the stimulation of exon 10 inclusion by RBM4 (Fig.6). Therefore, our study not only identified a new intronic splicing enhancer element for tau exon 10 splicing, but also uncovered an important splicing regulator, RBM4, that interacts with this intronic splicing enhancer element.

Our immunocytochemical staining experiments indicate that human RBM4 is expressed in the neurons, including the hippocampus and the frontal cortex, regions affected in tauopathy. Interestingly, a previous study reported an association of RBM4 with Down’s syndrome in the fetal brain using a proteomics approach, suggesting that RBM4 is important for neuronal function (31). It has also been reported that RBM4 is phosphorylated by cyclin A1-cdk2 complex (32). It is conceivable that cellular stresses could affect the activity of RBM4 and influence its regulation of tau exon 10 splicing, possibly contributing to the pathogenesis of a subset of tauopathy.

Tauopathy is one of most common forms of neurodegeneration. This is a group of genetically and phenotypically heterogeneous diseases, including Alzheimer’s Disease (AD), Down’s Syndrome (DS), several variants of Prion Diseases, progressive supranuclear palsy (PSP), amyotrophic lateral sclerosis (ALS), Pick’s Disease (PiD), corticobasal degeneration (CBD), frontotemporal dementia (FTD). The formation of
filamentous Tau protein-containing inclusions in the affected brain regions is the common neuropathological feature of these diseases. The underlying molecular defects for the majority of these diseases remain to be elucidated. Mutations in tau gene have been associated with only a subset of tauopathy, namely, FTDP-17. Splicing mutations in human tau gene, including all intronic mutations and several exonic mutations, are associated with changes in Tau 4R/3R ratio. Studies of these splicing mutations in the tau gene in FTDP-17 patients demonstrates the importance of controlling the balance of different Tau isoforms by alternative splicing for normal brain function (for recent reviews see (6); (9)). Identification of critical alternative splicing regulators for tau gene expression will not only help in our understanding of regulatory mechanisms of tau alternative splicing but also provide important information on how to correct the tau splicing defects in cells carrying FTDP-17 intronic mutations. For example if certain proteins are identified which can reverse the Tau4R/Tau3R ratio to the normal 1:1 ratio in FTDP-17 mutant cells, it will provide potentially useful information for future design of treatment strategy for the FTDP-17 patients carrying tau splicing mutations.

Several cis-acting regulatory elements have been identified to influence tau exon 10 alternative splicing, including a purine-rich element inside exon 10, a stem-loop at the 5’ splice site of exon 10, and intronic splicing silencer and enhancer elements inside or near exon 10 (25, 33-37, reviewed in 7-9). Our study indicates that an intronic pyrimidine-rich element located 100 nucleotides downstream of the 5’ splice site of exon 10 also plays a role in tau exon 10 splicing.

It remains to be elucidated what transacting splicing factors in the brain regulate tau exon 10 alternative splicing. A number of known splicing regulators have been tested in a chimeric tau mini-gene. In this chimeric tau system, SRp30c, SRp55, SRp75, 9G8, U2AF, PTB and hnRNP repress tau exon 10 inclusion, whereas CELF3 and CELF4 activate exon 10 splicing ((38), (39)). Their effects on tau pre-mRNA splicing in the context of native tau exon 9 and exon 11 splice sites remain to be examined. We have established a tau minigene system using human tau gene that contains the native tau gene splice sites (25). Using the minigene and biochemical approaches, we identified tra2beta as a SR-rich protein that binds to the purine-rich exonic splicing enhancer element and stimulates tau exon 10 splicing (26). A recent study showed that both Tra2beta and ASF/SF2 interact with this exon 10 purine-rich enhancer and play a role in exon 10 splicing (37). Here we report the role of RBM4, a previously unknown tau splicing regulator, in stimulating tau exon 10 inclusion. We further identified the intronic pyrimidine-rich intronic splicing enhancer element downstream of exon 10 and showed that this intronic element is required for the stimulatory activity of RBM4 in tau exon 10 inclusion. It will be interesting to test whether RBM4 is associated with the development of tauopathy, in particular, among patients without mutations in the tau gene.

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FOOTNOTES

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FIGURE LEGENDS

Figure 1. Panel A. A schematic diagram of the Tau4R-GFP reporter constructs. The tau splicing reporter gene is designed in such a way that GFP is only expressed when the exon 10 is included (for detailed description of the reporter gene construction, see Experimental Procedures). Panel B is a diagrammatic representation of the cDNA library screening protocol based on GFP expression following progressive division and subdivision of cDNA pools and transfection of cDNA pools into the stable cell line expressing Tau4R-GFP reporter.

Figure 2. The expression of Tau-GFP reporter during the cDNA library screening. The stable HEK293 cell line expressing the Tau4R-GFP reporter gene was used for screening cDNA library. A progressive subdividing-retransfecting method was used to identify cDNAs encoding potential regulators of tau alternative splicing, as described in Experimental Procedures. GFP fluorescence was monitored by fluorescent microscopy following the transfection of pools of cDNAs prepared from the cDNA library. Panels A1 to A3 are phase-contrast images, whereas panels B1 to B3 are their corresponding fluorescent microscopic images. These experiments show progressive enrichment of GFP expressing cells following the primary (Panel B1), the third round (Panel B2) and the sixth round (Panel B3) of subdivision and retransfection of the positive cDNA pools.

Figure 3. Over-expression of RBM4 protein increases the inclusion of tau exon 10 in transfected cells. The corresponding cDNA plasmids expressing either the vector control (lane1), RBM4 (lane2) or Raver (lane3) were co-transfected with Tau minigene tauEx9-10-11 into HEK293 cells. Panel A, tau exon 10 alternative splicing was detected using RT-PCR. Panel B shows the expression level of RBM4 transcript in the corresponding groups using RT-PCR with RBM4 specific primers. Panel C, the levels of actin transcript in the corresponding groups, showing comparable amounts of RNA used in each reaction. Panel D reveals RBM4 proteins levels in the corresponding groups as detected by Western blotting using the anti-RBM4 antibody.

Figure 4. The RNA recognition domain of RBM4 is essential for its activation of tau exon 10 inclusion. The tau minigene was co-transfected with either the vector control (lane 1) or cDNA expression plasmid encoding the wild-type RBM4 (lane2) or the mutant RBM4 (RBM4mRRM, lane3). Panel A is the tau exon 10 splicing pattern as detected by RT-PCR. Panel B shows the level of RBM4 transcripts as detected
by RT-PCR for each group. Panel C shows the actin transcript levels as detected by RT-PCR for internal controls. Panel D shows the level of RBM4 protein in each group as detected by Western blotting using a specific anti-RBM4 antibody. Panel E, Quantification of tau exon 10 splicing data derived from six independent experiments. The graph shows average ratio Ex10+/Ex10- transcripts ± SE (***= p<.001).

Figure 5. RBM4 interacts with an intronic element downstream of the 5’ splice site of exon 10 in tau pre-mRNA. UV crosslinking experiments were performed using either protein extracts prepared from cells transfected with HA-RBM4 expression plasmid (panels A-C) or purified recombinant RBM4 protein (panel D). Tau pre-mRNA or its fragments, as depicted, were radiolabeled and used in the crosslinking assay. Following UV crosslinking and RNase treatment, the reactions were analyzed using autoradiography following SDS-PAGE. Panels A and B. Different tau pre-mRNA transcripts (lane 2) or control RNA transcripts (lane 1) were incubated with cell lysates containing HA-RBM4. Following UV crosslinking and RNase digestion, RBM4 was immunoprecipitated using a monoclonal anti-HA antibody. Immunoprecipitates were analyzed on SDS-PAGE followed by autoradiography. Panel C. Radiolabelled RNA fragment containing the putative RBM4 binding site was incubated with protein extracts containing HA-tagged wild-type RBM4 (lane 1), vector-control (lane 2), or HA-tagged RBM4-mRRM mutant (lane 3). Panel D. Recombinant RBM4 protein was incubated with the radio-labeled control RNA (Lane 1) or the tau intronic RNA fragment containing the putative RBM4-binding element (lane 2) under splicing conditions followed by UV crosslinking. Panel E. The UV cross-linking experiment was performed with recombinant RBM4 protein and P32-labeled RNA transcript containing the tau intronic RBM4-binding element under splicing condition in the absence of competitor RNA (lane 1) or in the presence of 100 fold molar excess of unlabeled tau RNA containing the RBM4-binding element (lane2) or in the presence of 100 fold molar excess of unlabeled control RNA (lane 3). Panel F. The UV cross-linking experiment was carried out using recombinant RBM4 protein with radio-labeled control transcript (lanel), or with a RNA transcript containing either the wild-type (lane 2) or mutant (lane 3) RBM4-binding element under the splicing condition. Autoradiographs of gels following SDS-PAGE of the UV crosslinking reaction products are shown in all panels.

Figure 6. Mutation in the RBM4 putative binding site in tau pre-mRNA affects the exon 10 splicing stimulating activity of RBM4. The expression plasmid for RBM4 (lane 1 and 2) or the vector control (lane 3) was co-transfected into HEK293 cells with either the wild-type tau minigene (lane1) or the mutant tau minigene (tauEx9-11mRBM4BS) in which the putative RBM4-binding site had been mutated (lane 2 and3). Panel A. Tau exon 10 alternative splicing was assayed using RT-PCR. Panel B. The level of RBM4 transcript was detected by RT-PCR with RBM4 specific primers. Panel C is the level of actin transcript as detected by RT-PCR as the internal control. Panel E shows the levels of RBM4 protein expression detected by Western blotting using specific anti-RBM4 antibody. Panel F is the quantification of results derived from six independent experiments. The graph shows the average ratio Ex10+/Ex10- transcripts ± SE (***= p<.001).

Figure 7. RNAi mediated-knockdown of RBM4 decreases tau exon10 inclusion. RNAi specific for human RBM4 (lane2) or control RNAi (lane1) was co-transfected with Tau minigene TauEx9+11(wt) into HEK293 cells as described in the Experimental procedures. RNA preparations or protein lysates were made 48 hours post-transfection and analyzed by RT-PCR or Western blotting. Panel A shows tau exon10 alternative splicing isoforms as detected by RT-PCR. Panel B is the alternative splicing pattern of Bcl-x detected by RT-PCR using Bcl-x specific primers. Panel C is the level of RBM4 RNA detected by RT-PCR. Panel D is the level of actin transcript as detected by RT-PCR as the internal control. Panel E shows the level of RBM4 protein expression detected by Western blotting using specific anti-RBM4 antibody. Panel F is the quantification of tau Ex10 splicing isoforms as shown in panel A. The results are derived from six independent experiments. The graph shows the average ratio Ex10+/Ex10- transcripts ± SE (***= p<.001)
Figure 8. RBM4 expression in human frontal cortex and hippocampus was detected by immunohistochemical staining. Adult human brain sections of frontal cortex (Panels A and B, 150x and 600x magnification respectively) or hippocampus [panels C (x300), D (x600), E (x300)] were used. Panel E shows a negative control, staining in the absence of the primary antibody. The frontal cortical sections and hippocampal sections were used for immunohistochemical staining with the affinity purified anti-RBM4 antibody. RBM4 showed strong signals in the frontal cortex neurons, including layers II neurons (marked by arrows) and scattered signals in CA1 pyramidal neurons in the hippocampus (as indicated by arrow-heads) in the brain. The signal was stronger in the nucleus in comparison to the cytoplasm.
Figure 1

A

Out of frame: GFP off

In frame: GFP on

B

Tau splicing minigene reporter line

Transfect with cDNA pools

Select for pools with activated GFP expression

Subdivide positive cDNA pools & retransfect

Isolate cDNA & Sequence

GFP Positive Single cDNA

×N
Figure 2
Figure 3
Figure 4

A 1 2 3
Ex10+  Ex10-

B
RBM4

C
ACTIN

D
RBM4

E

Ex10+/Ex10-

Ctrl RBM4 RBM4 mRRM
Figure 5
Figure 6

A

Ex10+

Ex10-

B

RBM4

C

ACTIN

D

RBM4

E

Ex10+/Ex10-

|   | Tau | Wt | Mt | Mt | Mt |
|---|-----|----|----|----|----|
| RBM4 | +   | +  | +  | -  |    |

**Graph E:**

- **Ex10+/Ex10-** levels across different conditions.
- Statistical significance indicated by asterisks (***).
Figure 7

A

Ex10+
Ex10-

B

Bcl-xL
Bcl-xS

C

RBM4

D

ACTIN

E

1
2

RBM4

F

Ex10+/Ex10-

Control RNAi
RBM4-RNAi

***
Figure 8
RBM4 interacts with an intronic element and stimulates tau exon 10 inclusion
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