A consensus CaMK IV-responsive RNA sequence mediates regulation of alternative exons in neurons

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ABSTRACT

Neurons make extensive use of alternative pre-mRNA splicing to regulate gene expression and diversify physiological responses. We showed previously in a pituitary cell line that the Ca^{++}/calmodulin-dependent protein kinase CaMK IV specifically repressed splicing of the BK channel STREX exon. This repression is dependent on a CaMK IV-responsive RNA element (CaRRE) within the STREX 3′ splice site. Here, we report that similar Ca^{++} regulation of splicing, mediated by L-type calcium channels and CaM kinase IV, occurs in cultured neurons and in the brain. We identify a critical CaRRE motif (CACATNRTTAT) that is essential for conferring CaMK IV repression on an otherwise constitutive exon. Additional Ca^{++}-regulated exons that carry this consensus sequence are also identified in the human genome. Thus, the Ca^{++}/CaMK IV pathway in neurons controls the alternative splicing of a group of exons through this short CaRRE consensus sequence. The functions of some of these exons imply that splicing control through the CaMK IV pathway will alter neuronal activity.

Keywords: CaMK IV-responsive RNA element; alternative splicing; neuronal genes

INTRODUCTION

Ca^{++}-regulated gene expression is important for a variety of cellular functions, including long-term changes in neuronal activities (West et al. 2001, 2002). A key step in the expression of many neuronal genes is the alternative splicing of their pre-mRNA (Grabowski and Black 2001; Black 2003), which is often regulated by extracellular stimuli (Stamm 2002; Shin and Manley 2004). Membrane depolarization and the CaMK IV pathway have been shown to repress the splicing of a group of alternative exons (Vallano et al. 1999; Xie and Black 2001). However, the component factors and target elements underlying this regulatory pathway remain unclear.

Alternative exons in mammalian cells are controlled by combinations of *cis*-acting pre-mRNA elements (Smith and Valcarcel 2000; Black 2003). These elements are either intronic or exonic and act as either enhancers or silencers. These elements are often bound by specific RNA binding proteins that assemble onto the pre-mRNA to either induce spliceosome assembly at new splice sites or block its assembly at other sites. A given exon is usually affected by multiple proteins having both positive and negative effects on splicing. The best characterized splicing activator proteins are members of the SR protein family (Fu 1995; Graveley 2000), which bind to exonic splicing enhancers (ESEs). Well-known repressor proteins include hnRNP A1 and PTB (Wagner and Garcia-Blanco 2001; Black 2003; Chabot et al. 2003), which bind to silencer elements to block spliceosome assembly. The combined expression of these factors is thought to lead to stable differences in exon inclusion between different cell types. In contrast to these systems, there are other exons whose splicing changes in response to extracellular stimuli or growth conditions (Stamm 2002; Black 2003; Shin and Manley 2004). The sequence elements and proteins that mediate a splicing response to these stimuli are largely unknown.

Several systems have been used to examine inducible alternative splicing (Stamm 2002; Black 2003; Shin and Manley 2004). Particular exons in the cell surface protein genes *CD44* and *CD45* are regulated by the protein kinase C pathway (Konig et al. 1998; Lynch and Weiss 2000). For CD44, the pathway downstream of PKC is known to require the ERK (extracellular-signal-regulated kinase) kinase, ERK phosphorylation of Sam68, and specific exonic sequence elements (Weg-Remers et al. 2001; Matter et al.

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2002). For CD45 exons, a consensus RNA element was isolated that is sufficient to respond to PKC stimulation when transferred to another exon (Rothrock et al. 2003).

In neurons, several exons are known to respond to membrane depolarization and other stimuli (Daoud et al. 1999; Vallano et al. 1999; Xie and Black 2001; Stamm 2002; Mu et al. 2003). We previously developed a system to examine this regulation using the excitable pituitary cell line GH_3 (Xie and Black 2001). We showed that depolarization of GH_3 cells repressed the STREX exon of the BK potassium channel gene in a CaM kinase-dependent manner. Through coexpression of activated kinases with splicing reporter genes, we showed that this exon repression required CaM kinase IV. Using this system, we identified the upstream 3′ splice site of the STREX exon as sufficient to mediate CaMK IV-dependent repression of a heterologous exon. We also showed that exons 5 and 21 of the NMDA receptor type I (NR1) were also repressed by CaMK IV.

In this report, we show that depolarization-induced splicing repression is not limited to GH_3 cells, but also occurs in neurons. This inducible splicing repression again requires calcium signaling. Moreover, we show that the CaRRE is not part of the splice site itself, but is a special element placed between the polypyrimidine tract and the AG of the 3′ splice site. We identify several other exons that carry this element and are regulated by CaMK IV.

RESULTS

Regulation of the splicing of STREX and other exons in cerebellar neurons by Ca^{++} signals

We showed previously that membrane depolarization in GH_3 cells repressed the splicing of the STREX exon of BK channels. The CaM kinase inhibitor KN93 blocked splicing repression, indicating a role for the CaM kinase pathway in this effect (Xie and Black 2001). In neurons, the depolarization-induced activation of CaMK IV requires calcium entry through L-type Ca^{++} channels (Marshall et al. 2003). To demonstrate the regulation of STREX in neurons and test the involvement of L-type Ca^{++} channels in its repression, we cultured primary cerebellar neurons and tested the effect of Ca^{++} channel blockers or antagonists on STREX splicing.

In nondepolarizing media, cerebellar granule neurons undergo extensive cell death (Vallano et al. 1999; See et al. 2001). Hence, we cultured these cells in the presence of 25 mM KCl, where they survive well and differentiate into highly connected neuronal populations (Fig. 1A). Under these depolarizing conditions, the STREX exon is mostly excluded from the BK channel mRNA (Fig. 1B, lanes 2,3). In untreated samples, STREX-included transcripts comprise ~17% of BK channel mRNA as measured by semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR, 24 cycles of PCR). Cells treated with the CaM kinase inhibitor KN93 undergo massive cell death, making it impossible to test the effect of this drug (Fig. 1A; See et al. 2001). In contrast, cells treated with drugs that block or antagonize specific Ca^{++} channels survived well, and the level of STREX splicing was measured in these cells (Fig. 1A). Neither the N-type calcium channel blocker ω-Conotoxin GVIA (Reynolds et al. 1986) nor the NR1 inhibitor AP5 (Davis et al. 1992) significantly affected STREX splicing under these conditions (Fig. 1B, lanes 6,7). In contrast, treatment with nifedipine, an L-type Ca^{++} channel blocker, consistently doubled STREX inclusion to ~34% (Fig. 1B, lanes 4,5). Thus, calcium signals modulate STREX exon inclusion in cerebellar neurons, as was seen in GH_3 pituitary cells. In cerebellum, this regulation is mediated by L-type Ca^{++} channels.

To confirm that calcium signaling represses STREX splicing in brain tissues, we examined RNA from the cerebella of CaMK IV knockout mice (Ho et al. 2000). Wild-type mice express abundant CaMK IV protein as detected in Western blots of total cerebellar protein (Fig. 1C, lane 1, top). The kinase activity of CaMK IV was monitored by the presence of phosphorylated Ser133 of CREB (Sun et al. 1994), a direct endogenous target of the enzyme, using an anti-phospho-CREB antibody. In CaMK IV heterozygous mice, CaMK IV protein is hardly seen and CREB Ser133 phosphorylation is undetectable, indicating a significant loss of activity in the heterozygotes. Both CaMK IV and CREB phosphorylation are undetectable in homozygous null mouse tissue (data not shown).

In wild-type cerebellum, the STREX exon is mostly repressed. STREX exon-included transcripts of the BK channel are <1% of the total in semi-quantitative PCR (24 cycles). In contrast, in the CaMK IV heterozygotes, STREX inclusion increased 16-fold to ~9%, while the total transcript level stayed constant relative to the GAPDH mRNA. RNA samples from the CaMK IV (−/−) homozygous knockout mice showed much lower levels of BK channel and other mRNAs relative to the total isolated RNA (data not shown). These mRNAs were either expressed at lower levels or were unstable in the knockout cerebellum (data not shown). This prevented accurate measurement of exon inclusion in the homozygous knockout compared to the heterozygote or wild-type tissues. We thus focused on comparing the wild-type with the heterozygous knockout tissues, which were highly reduced in CaMK IV protein and activity. From this comparison, it is clear that the CaMK IV pathway does indeed contribute to the repression of STREX splicing in cerebellar neurons.

The PCR primers used in these experiments span three positions of alternative splicing in the BK channel transcript (Tseng-Crank et al. 1994; Xie and McCobb 1998). There are six alternative exons that could be differentially included into these positions (Fig. 1D). Interestingly, only the inclusion of the STREX exon was increased by the blockade of L-type calcium channels in cerebellar neurons (Fig. 1B) or by the reduction of CaMK IV in the CaMK IV heterozygous mice

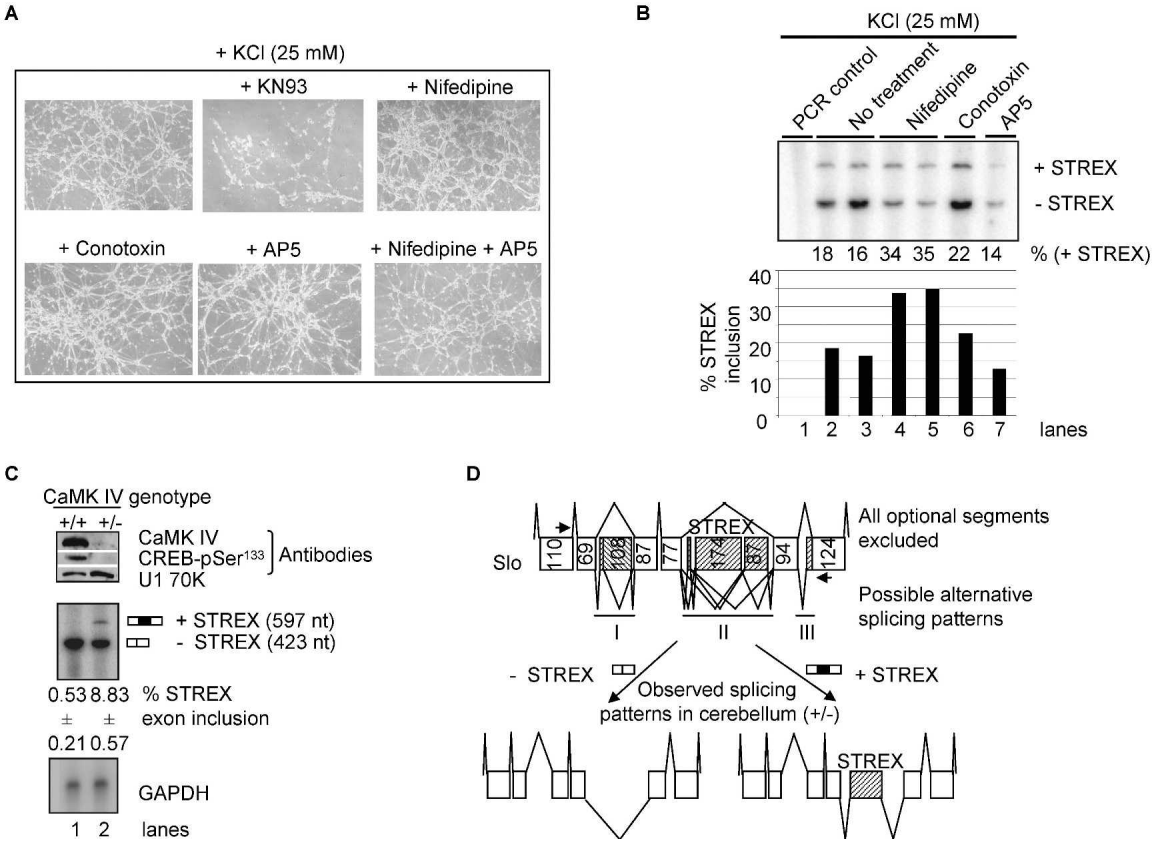

FIGURE 1. Regulation of STREX splicing by Ca^{++} signals in cerebellar neurons. (*A*) Phase images of primary cerebellar cultures grown in the presence of 25 mM KCl and various channel blocker or antagonists. (*B*) *Slo* gene RT-PCR products from RNA samples of rat primary cerebellar neuron culture. Cells were cultured in depolarizing concentrations of KCl (25 mM) without treatment (lanes *2*,*3*) or treated with nifedipine (an L-type calcium channel blocker; lanes *4*,*5*) or ω-Conotoxin GVIA (an N-type calcium channel blocker; lane *6*) or AP5 (an NMDA receptor antagonist; lane *7*) for 12 h before RNA extraction. The bands for the STREX exon-included or -excluded products are indicated to the *right*. The percent exon inclusion is indicated *below* the gel and graphed at the *bottom*. The gel shown is representative of two independent experiments. (*C*) Changes in endogenous STREX exon splicing upon reduction of CaMK IV activity in CaMK IV (+/−) cerebellar tissue. (*Top* panel) Characterization of CaMK IV activity on Western blots of cerebellar proteins from CaMK IV wild-type (+/+) and heterozygous (+/−) mice. Antibodies probed for the presence of CaMK IV protein, phospho-Ser^{133}-CREB, and U1 70K protein (protein loading control) are indicated to the *right*. (*Middle* panel) RT-PCR products of the *Slo* gene from the RNA samples of the mouse cerebella. GAPDH was used as an RNA loading control (*bottom*). (*D*) Diagram of the STREX exon region of the *Slo* gene. PCR primers (arrows) spanned three positions of alternative splicing (I, II, and III), with the length of exons indicated *inside* the exon boxes, except three small exons with lengths of 12, 9, and 24 nt (from *left* to *right* at positions I, II, and III, respectively).

(Fig. 1C). This indicates that the CaMK IV repression of alternative splicing is specific for certain Slo exons and is not due to a general effect on all alternative exons.

We showed previously that exons 5 and 21 of NR1 can also be regulated by CaMK IV (Xie and Black 2001). Membrane depolarization and L-type Ca^{++} channel activation were also shown to regulate the splicing of the NR1 exon 5 in cultured cerebellar neurons (Vallano et al. 1999). In addition, a variant exon of SNAP25a was reported to be regulated by membrane depolarization (Hepp et al. 2001; Sorensen et al. 2003). We examined the splicing of these exons in the CaMK IV heterozygous knockout mice. Notably, these exons were also affected by the loss of CaMK IV, but not always in the same direction. The inclusion of NR1 exon 5 was decreased in the CaMK IV (+/−) samples (Fig. 2A, lanes 1,2), instead of increasing as predicted from previous results (Xie and Black 2001; see Discussion). In contrast, the splicing of NR1 exon 21 increased in the CaMK IV heterozygous (+/−) cerebella (lanes 3,4). This is consistent with its repression by transiently expressed CaMK IV in HEK cells (Xie and Black 2001). A control alternative exon from PTB was little changed in the CaMK IV heterozygote (lanes 5,6).

The SNAP25a and 25b mRNA isoforms contain mutually exclusive exons of the same size (Bark 1993; Bark and Wilson 1994; Fig. 2B). To distinguish these exons on the gel, we digested the RT-PCR products with restriction enzymes that cleave either the SNAP25a exon (NdeI) or the SNAP25b exon (AvaII). The inclusion of the SNAP25a exon was undetectable in wild-type mice, as the PCR product was not cleavable with NdeI. In contrast, this exon

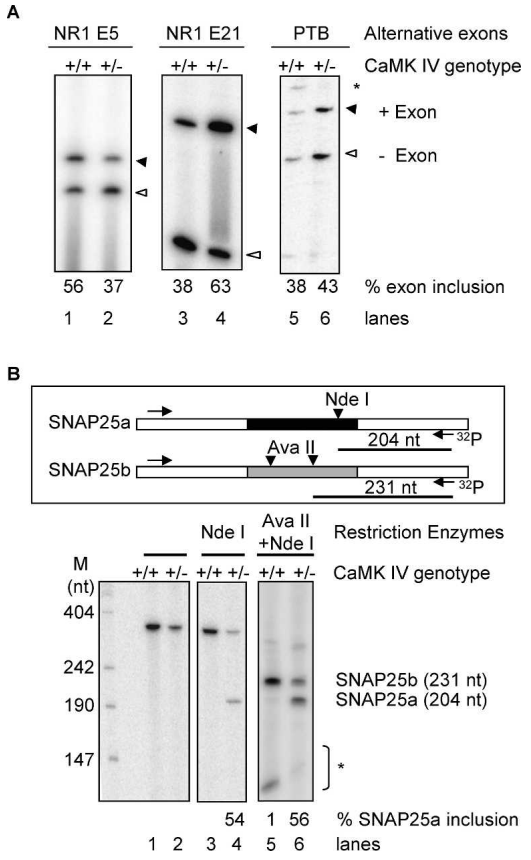

FIGURE 2. Alternative splicing of other neuronal exons in the cerebella of CaMK IV knockout mice. (*A*) RT-PCR products encompassing exon 5 or exon 21 of the NR1 or the PTB alternatively spliced region from cerebellar RNA samples of wild-type and heterozygous CaMK IV null mice. The exon included and excluded products are indicated to the *right* and the percent exon inclusion *below*. (*B*) Splicing of the SNAP25a exon in cerebella of the CaMK IV mutant mice. The positions of the restriction sites specific for the SNAP25a or b exons are diagramed *above* the respective RT-PCR fragments in the *upper* panel (not to scale). The RT-PCR products are ^{32}P-labeled at the 5′ end of the reverse primer (arrows). The exons are resolved by digesting the PCR products with the restriction enzymes indicated *above* the lanes (*lower* panel). The expected product sizes are indicated to the *right*. The asterisk indicates a product of unknown identity.

dramatically increased in the heterozygous mice to ~55% of the total, as seen in both the NdeI (lane 4) and double digest samples (lane 6).

Thus, a group of alternative exons is specifically regulated in the cerebellum by CaMK IV. We wanted to examine whether a common element confers this repression on some of these exons. For this, we used the STREX 3′SS to precisely define the CaMK IV-responsive RNA element.

An 11-nt motif essential for CaMK IV repression of alternative splicing

We previously showed that the upstream 3′ splice sites of STREX and NR1 exon 5 were sufficient to confer inducible repression by CaMK IV on a heterologous exon (Xie and Black 2001). These sequences were 54 and 61 nucleotides (nt) long and contained all of the features needed for the splice sites to function. Thus, we needed to define the CaRRE within these sequences more precisely.

The entire STREX splice site is highly conserved between mammalian species. However, comparing the 3′ splice site of the mouse STREX exon (mSTREX) with that of the fugu fish (fSTREX) showed blocks of similar and dissimilar sequences (Fig. 3A). Interestingly, when this fSTREX 3′ splice site was transferred to a test exon (DUP175) (Xie and Black 2001), it did not confer CaMK IV repression on the reporter exon (Fig. 3B,C, lanes 5,6). Thus, the inducible repression of the mSTREX 3′ splice site must require nucleotides that are different in the corresponding fSTREX sequence. The STREX 3′ splice site can be roughly divided into three regions: the branch point (BP), the polypyrimidine tract (Py), and a spacer between the Py and the 3′ AG (Fig. 3A). The BP and Py are mostly conserved between mouse and fugu. The unusual spacer sequences in these splice sites that separate the Py and the 3′ AG are not conserved in sequence and contain most of the differences between the two sites. The contribution of each region to the CaMK IV response was addressed by creating hybrid splice sites in the reporter

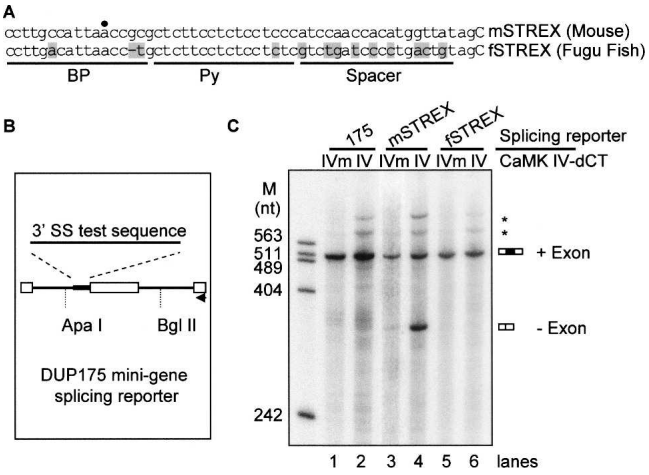

FIGURE 3. Comparison of the CaMK IV response between the *fugu* fish and mouse STREX 3′ splice site sequences. (*A*) Alignment of the mouse and fugu STREX exon 3′ splice sites. Fugu nucleotides different from mSTREX are shaded. The black dot indicates the presumptive branch point. (*B*) Mini-gene splicing reporter constructs used in this and following assays. The pDUP175 vector contains a 175-nt constitutive exon in the middle (Xie and Black 2001). The mSTREX or fSTREX 3′ splice sites or hybrid mutants were used to replace the 3′ splice site of DUP175 in cotransfections with constitutive CaMK IV or inactive mutant CaMK IV. (*C*) Primer extension products from HEK 293T cells after transfection with DUP175, DUP175ST (mSTREX) (Xie and Black 2001), or DUP175-fSTREX, together with CaMK IV-dCT-K75E (IVm) or CaMK IV-dCT (IV). Size markers and extension products from the exon-included or -excluded mRNAs are indicated to the sides. The asterisks indicate primer extension products from partially spliced or full-length pre-mRNA. The gel is representative of three or more independent transfections of each construct.

gene and testing whether the site behaved like the fSTREX (no response) or mSTREX (inducible repression; Fig. 3C).

To identify the RNA motif essential for the CaMK IV effect, we changed the nonconserved fSTREX nucleotides sequentially back to the mSTREX sequence and tested the mutant sites' ability to respond to CaMK IV (Fig. 4). Replacement of the fugu branch point with mouse sequence did not restore CaRRE function (Fig. 4A, fSTREX-M1, and 4B, lanes 1,2), suggesting that the nonconserved nucleotides in this region do not affect the mCaRRE function. In contrast, the nucleotides in the spacer region had a much stronger effect on CaRRE activity. Replacement of nucleotides in the 5′ half of the fSTREX spacer with the corresponding mSTREX nucleotides gave a moderate increase in exon repression by CaMK IV (Fig. 4A, fSTREX-M3, and 4B, lanes 5,6). More strikingly, replacements that recreated the 3′ half of the mouse spacer region allowed much stronger repression by CaMK IV (Fig. 4A, fSTREX-M6 to M9, and 4B, lanes 11–18). Thus, the spacer region is a key element for the CaMK IV response. Replacement of smaller portions of the fugu spacer with mouse nucleotides narrowed down the CaRRE still further. Notably when two A residues in the mouse sequence were replaced with C, the CaMK IV response was nearly eliminated (compare fSTREX-M9 with fSTREX-M10). The most active sequence contains the mouse STREX sequence from −15 to −3 relative to

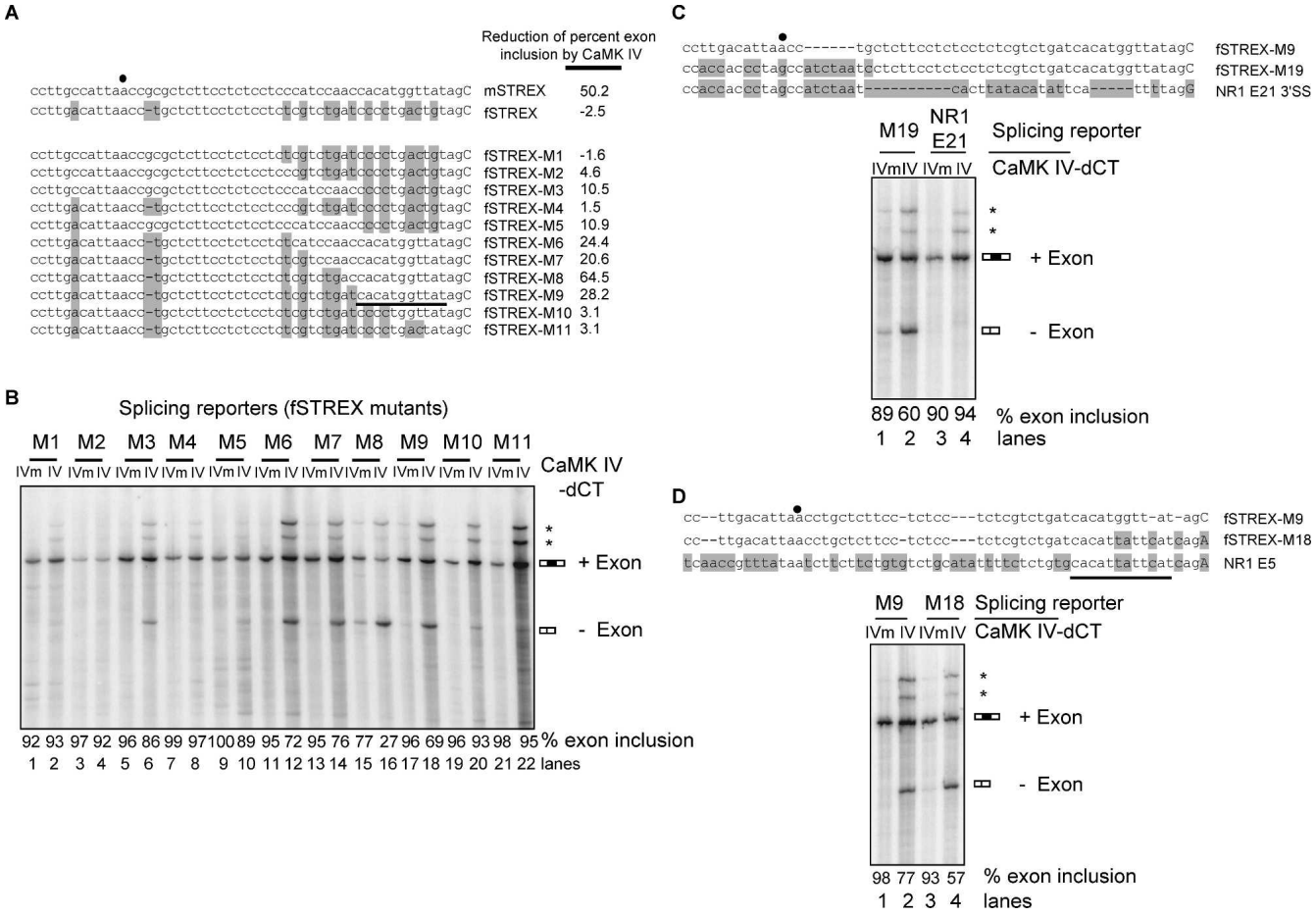

FIGURE 4. The CaRRE sequence is located between the polypyrimidine tract and the 3′ splice site AG in mSTREX. (*A*) Hybrid mutants (M1–M11) where nucleotides in the fugu splice site were sequentially changed to match mSTREX are shown. The reduction of exon inclusion for each construct is indicated at the *right*. This is relative to the percent inclusion in the presence of the inactive CaMK IVm as measured in *B*. The nucleotides different from the mSTREX sequence are shaded. The minimal region required for mCaRRE function is underlined. (*B*) Primer extension products from HEK 293T cells after transfection with the hybrid mutant minigenes, together with CaMK IVm or IV. The gel is labeled as in Figure 3C. (*C*) The branch point region does not affect the CaRRE response to CaMK IV. Shown *above* is an alignment of fSTREX-M9, fSTREX-M19, and the 3′ splice site of NR1 Exon 21. In fSTREX-M19, the branch point region is replaced with that of the NR1 E21, a 3′ splice site that is not responsive to CaMK IV. The nucleotides different from the fSTREX-M9 are shaded. *Below*: Primer extension products from HEK 293T cells after transfection with the minigenes indicated at the *top*, together with CaMK IVm or IV. The products resulting from the inclusion or exclusion of the central test exon are indicated to the *right*. (*D*) The CaRRE sequence is exchangeable with the CaRRE of NR1 E5. *Above*: An alignment of fSTREX-M9 and the NR1 E5 3′ splice site, with the additional mutant fSTREX-M18, in which the CaRRE region is replaced with the sequence of NR1 E5. *Below*: Primer extension products from HEK 293T cells after transfection with the mini-genes in the *upper* panel, together with CaMK IVm or IV.

the 3′ splice site (5′-ACCACATGGTTAT-3′) and yielded a 64% reduction in exon inclusion in response to CaMK IV (Fig. 4A, fSTREX-M8, and 4B, lanes 15,16). Nucleotide changes within this motif nearly abolished the response (Fig. 4A, fSTREX-M10 and -M11, and 4B, lanes 19–22). This identifies the CaRRE nucleotides within the spacer sequence as required in responding to CaMK IV.

As shown above, the nonconserved nucleotides of the STREX BP region are not essential for the CaMK IV effect on splicing. To test the conserved nucleotides in this region, we replaced this part of fSTREX-M9 with the corresponding region from the 3′SS of the NR1 exon 21 (Fig. 4C), which is not responsive to CaMK IV (Xie and Black 2001). This replacement did not alter the CaMK IV response (Fig. 4C, fSTREX-M19, lanes 1,2). Thus, in contrast to the spacer region, the branch point region of mSTREX is not essential for CaRRE response to CaMK IV.

The 3′ splice site of NR1 exon 5 is also sufficient to confer CaMK IV-dependent repression on an otherwise constitutive exon (Xie and Black 2001). Comparison of the minimal STREX CaRRE sequence with the NR1 exon 5 splice site indicates that they share the sequence CACATNRTT(C)AT (Fig. 4D). Changing the 11-nt mouse sequence in fSTREX-M9 to match the corresponding region of NR1 exon 5 conferred even stronger repression by CaMK IV (Fig. 4D, fSTREX-M18). Thus, in responding to CaMK IV, NR1 exon 5 apparently uses the same 11-nt motif as STREX, in the same location between the polypyrimidine tract and the AG.

The 11-nt CaRRE motif is also found in other exons

Not all exons that respond to CaMK IV contain CaRREs within their 3′ splice sites. As shown previously, the NR1 exon 21 responds to CaMK IV through CaRRE elements outside of the upstream 3′ splice site (Xie and Black 2001; J.-A. Lee and D.L. Black, unpubl. data). The 3′ splice site of the SNAP25a exon is dissimilar to STREX. Although it contains two copies of CACAY in its upstream intron (tttcaaattctgtttcacatagtcatttctcatgttctgttggagacccccaaaaaattcattccacactgtcatccctttgtcctaaccagA), these elements are not in a similar location to the STREX or NMDA exon 5 elements. The core of the CaRRE sequence is CACAT in the mouse STREX and NR1 exon 5 elements, but the human STREX exon carries the sequence CACAC instead. If the CaRRE is a common feature in calcium regulation of splicing, then it should be found in other exons subject to CaMK IV-dependent repression.

Examining the last 20 nt of all the annotated introns in the human genome database, we searched for matches to the CaRRE motif. In this way, we identified 69 exons containing the element CACAYNNTTAT, placed in an equivalent position relative to the exon as the STREX and E5 elements. Of these, 11 exons could be confirmed as alternative exons based on known mRNA and EST data (Table 1). To confirm that these 3′ splice sites could respond to CaMK IV, we transferred the 3′ splice sites of the PABPC1, ZFY, and MAX exons to the equivalent position upstream of the constitutive DUP175 exon. As seen with the STREX splice site, these splice sites on their own led to some increased exon skipping (Fig. 5, lanes 3,5,7). Importantly, as with STREX, coexpression of CaMK IV led to substantially increased repression of these hybrid exons (Fig. 5, lanes 4,6,8). Thus, these new exons carrying the CaRRE are also repressed by CaMK IV.

DISCUSSION

In this report, we show that the alternative splicing of STREX is controlled by the CaMK IV pathway in neurons, as was seen previously in GH_3 cells. In neurons, this repression requires L-type calcium channel activation. Consistent with these results, we find that disruption of the CaMK IV pathway in mice also alters the splicing of CaMK IV responsive exons. In the CaMK IV heterozygous mutant mice, which are drastically reduced in CaMK IV expression and activity, several exons known to be affected by depolarization show changes in splicing. As with other knockouts, it is difficult to attribute a particular change in gene expression to a direct effect of the mutated gene. The cerebella of these mice have developed with reduced levels of a key regulatory molecule, CaMK IV, and have likely compensated for this missing factor in multiple ways. Nevertheless, it is clear that in neurons and the brain, calcium signaling mediates important changes in splicing.

We next identified an 11-nt CaRRE motif as the key feature mediating the CaMK IV-dependent repression of STREX exon inclusion. We find this motif in the 3′ splice sites of several other alternative exons. Like STREX, these 3′ splice sites have the CaRRE just upstream from the AG. Also like STREX, these sites confer repression by coexpressed CaMK IV, when transferred to a reporter exon. Thus, the CaRRE motif contributes to the regulation of multiple alternative exons by CaMK IV.

Alternative exons are generally controlled by multiple sequence elements and regulatory factors. Similar to transcriptional regulation, the responsiveness of a particular exon to its assemblage of factors will in turn depend on multiple signaling pathways. In the CaMK IV cotransfection assay, we have isolated a 3′ splice site from the rest of the regulated exon. Exons whose splice sites respond to CaMK IV coexpression with the DUP reporter may not be solely dependent on the CaRRE. In fact for the STREX exon, we find that the CaRRE is an essential feature of the regulation, but not the only contributing element (Xie and Black 2001). For other exons, extensive mutagenesis analyses will be required to demonstrate the role of the CaRRE relative to other elements.

Splicing regulators can either enhance or repress an exon, depending on their sequence context and cellular environment (Jin et al. 2003; Dredge et al. 2005; El Ibrahim et al. 2005; Hui et al. 2005; Kanopka et al. 1996). Thus, it is possible that

TABLE 1. Matches to the CaRRE sequence CACAYNNTTAT found upstream of alternative exons in the human genome

Ch.	No. of hits	No. of matches[a]	3′ Splice site sequence	GenBank No.	Protein features/function
1	3				
2	2				
3	3				
4	3	1	aaaactttctctttgacccacattcttaaatggctgcAG	NT_016354	DNA binding domain
5	4				
6	9	1	gactttataagcgtttttctcccccacactttaaacAG	NM_030752	T-complex polypeptide 1
7	5				
8	4	2	ttttcccagttaatgtatattcacatctttaaaatgcAG	NM_002568.1	Poly(A) binding protein, cytoplasmic 1
			aaaaatgatagacgcctaattcacactgttaaattttAG	XM_043070	LYsine-Rlch CEACAM1 co-isolated
9	0				
10	7				
11	3	2	tctctgacacacatacaccgacccacacgtttatctcAG	NM_017547	Electron transport
			tagggtgaagctgggtacctgacctgcccacactcttAG	NM_004517	Signaling, neurite growth
12	2	1	ctctgtctttttgacttgttgacacaccattacgctcAG	NM_022792	Matrix metalloproteinase
13	3				
14	3	1	ttgtgaaattaattgcttttccacatgtttatttactAG	NM_145114.1	Cell apoptosis (MAX)
15	4				
16	1				
17	2				
18	1				
19	1				
x	7	2	cagccatttcatctttttccctactgcacacccttacAG	NM_003334.1	Ubiquitin-activating enzyme E 1
			ttagctctcttaactgttgcacatatttatttaaactAG	NM_024597.1	Serine/threonine protein kinase
y	2	1	taatgttgtgtaattctgtgttttaatgcacattgttAG	NM_003411.1	Zinc finger protein (ZFY)
Total	69	11			

Ch., Chromosome; [a]matches upstream of alternative exons.

the CaRRE in some contexts is not always a repressor element. This may be what is seen in the responses of NR1 exon 5. The NR1 exon 5 reporter showed exon repression when CaMK IV was overexpressed by transfection, but the endogenous NR1 exon 5 also showed decreased splicing in the CaMK IV knockout mice. In contrast, although NR1 exon 21 was repressed by overexpressed CaMK IV, this exon increased in splicing in the knockout mice. As with STREX, this would be predicted if CaMK IV were repressing its splicing. However unlike STREX and exon 5, the responsive element for exon 21 is not in the 3′ splice site. Interestingly, NR1 E5 and E21 were previously shown to exhibit converse inclusion patterns in different brain regions (Zhang et al. 2002). These results make clear that the response of a particular exon to a stimulus is likely to be more complex than can be mediated by a single regulatory element and its binding factor.

In addition to NR1 exon 21, we have found another exon, FGFR2 EIIIb (Avivi et al. 1993; Carstens et al. 2000), that does not contain a CaRRE within its 3′ splice site, but which is still repressible by overexpressed CaMK IV (Xie and Black 2001; data not shown). These two exons apparently contain different elements positioned within the exon sequence itself (Xie and Black 2001; J.-A. Lee, J. Xie, and D.L. Black, unpubl. observations). In understanding these responses, it will be essential to identify all of the different regulatory elements leading to a response as well as dissecting the different kinds of stimuli that induce it and how they intersect.

With the caveats described above, the identification of the CaRRE and the demonstration of its activity in neurons are important steps in understanding the mechanisms of CaMK IV-mediated repression of splicing. In constitutive 3′ splice sites, the polypyrimidine tract is usually immediately upstream of the essential AG (Burge and Sharp 1999; Black 2003). This is important for the stable binding of the splicing factor U2AF65/35 (Merendino et al. 1999; Wu et al. 1999; Zorio and Blumenthal 1999). In some regulated splice sites, the polypyrimidine tract is separated from the AG by a relatively large region of regulatory RNA (Smith and Valcarcel 2000). The STREX exon presents the intermediate case of a short, apparently single element separating the two essential features of the splice site. The binding of a factor to this element is likely to interfere with the interactions of U2AF65 and U2AF35 with their target sequences. Thus, one model for the repression is recruitment of a CaMK IV-activated factor to this site. Whether this is a new factor synthesized in response to CaMK IV or a factor whose binding is modulated by the stimulus is not yet clear.

In addition to the CaRRE binding factor, there may be cofactors involved in the splicing repression. There are several binding elements for the splicing repressor PTB in the STREX polypyrimidine tract (one UCUU and two CUCU;

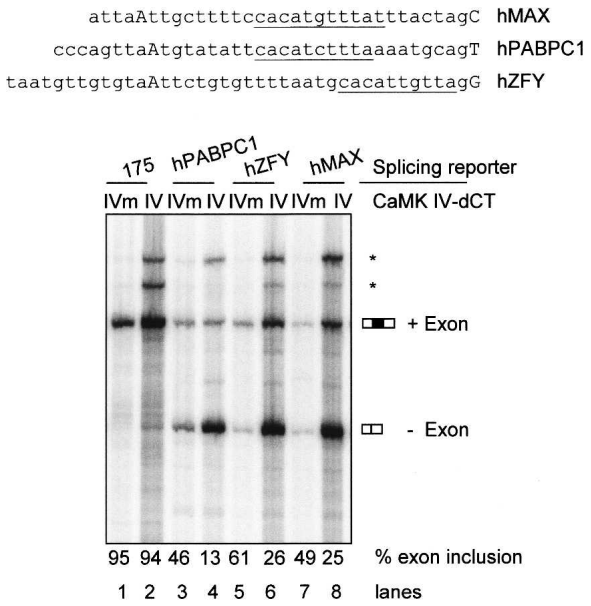

FIGURE 5. CaMK IV regulation of additional alternative 3′ splice sites carrying a CaRRE sequence. The 3′ splice sites of CaRRE-containing alternative exons shown *above* were placed upstream of the DUP175 test exon. These splicing reporters were transfected into HEK293T cells, together with CaMK IVm or IV. RNA was isolated and assayed by primer extension. The exon-included or -excluded products are labeled to the *right*, and percent exon inclusion is indicated *below*. This gel is representative of three independent transfections. Note that CaMK IV sometimes boosts expression from the CMV-driven reporter gene, compared to the mutant enzyme. This effect is inconsistent and we have shown that this does not affect the splicing pattern of the reporter (J.-A. Lee and D.L. Black, data not shown).

Fig. 3; Chan and Black 1995; Singh et al. 1995; Chou et al. 2000). Mutations in the polypyrimidine tract that would be predicted to improve U2AF binding and reduce PTB also reduce the CaMK IV splicing repression (Xie and Black 2001). The PTB elements alone are not sufficient to confer a CaMK IV response (Fig. 3A, fSTREXM1-M5, and 3B, lanes 1–10). Similarly, the 3′ splice site of the *c-Src* N1 exon, a known PTB binding site (Chan and Black 1997; Chou et al. 2000), induced only slight repression of the DUP175 exon in response to CaMK IV (<10%, data not shown). Thus, it is unlikely that PTB alone is sufficient to mediate the CaMK IV effect, but it could cooperate with the CaRRE in repressing splicing. A cofactor for PTB-mediated repression of a smooth muscle exon, Raver1, was recently identified (Gromak et al. 2003). It will be interesting to investigate whether similar PTB corepressors, which could modulate PTB activity in particular contexts, exist for other exons.

In addition to understanding the mechanisms of splicing regulation, the identification of the CaRRE will help us understand the role this regulation plays in cell biology. There are likely many targets of this regulation. Our search of human exons carrying this element is clearly an underestimate of its frequency. For example, the search did not identify the STREX or NR1 E5 exons. This may be due to the annotation software missing some alternative exons. As we refine the functional sequence of the CaRRE, additional searches will likely identify more exons and allow us to assess whether exons in particular cellular processes are controlled by this element. Since CaMK IV is expressed in other tissues besides the brain (Means et al. 1991), the CaRRE motif is likely to affect multiple cellular functions. For example, PABPC1 is involved in cytoplasmic mRNA metabolism (translation and decay), and MAX is involved in cell growth/apoptosis (Grandori et al. 2000). On the other hand, STREX, NR1 exons 5 and 21, and SNAP 25a are all potentially involved in the plastic changes in neuronal activity that are known to occur in response to the calcium signaling pathway. By identifying a larger set of these exons, we may be able to discern an underlying logic in the whole ensemble of exons being regulated.

There are many important questions regarding both the mechanisms of inducible splicing regulation and its role in cell biology. To these ends, the precise characterization of the CaRRE will allow careful analyses of its binding factors. The demonstration of its responsiveness to calcium signaling in primary neurons and the brain will allow the examination of the role of additional molecules in this pathway using inhibiting drugs and other approaches. In future experiments, we will need to follow the Ca^{++} signaling pathway from the L-type calcium channel, through CaMK IV, to the CaRRE. To understand the role of this process in neuronal cell biology, we will need to examine the whole ensemble of exons being changed by a stimulus.

MATERIALS AND METHODS

Primary cerebellar neuron culture (Ryan et al. 1993; Sippy et al. 2003)

Briefly, neonatal (day 1 at birth) rat cerebella were trypsinized and dissociated neurons were plated in neural basal media supplemented with insulin, transferrin, and B27 (Invitrogen) on Matri-gel-coated six-well plates. KCl (25 mM) and arabinoside-C were added to the media to keep cells from apoptosis and prevent the growth of nonneuronal dividing cells, respectively. Cells were treated with ion channel blockers or antagonists after 1 wk in vitro.

Targeted mutagenesis

PCR with synthesized mutant primer sequences was used to make the mutations with Pfu DNA polymerase. Mutant plasmid inserts were confirmed by sequencing.

Primer extension assay of mini-gene splicing reporters

This is done with a protocol modified from the previously published one (Modafferi and Black 1997). HEK 293T cells were used for its better growth and attachment to the plates and retaining the CaMK IV effect on splicing. Typically, cells grown in six-well plates were transfected with the mini-genes for about 20 h, harvested, and lysed

for cytoplasmic RNA preparation. Half the RNA was used in a 10 μL RT mix and denatured at 85°C for 5 min before annealing at 55°C with ^{32}P-labeled DNA oligo DUP1 for 1 h. Then the mix was spun down and 0.25 μL Superscript II was added and incubated at 45°C–48°C for 45 min. The resulting primer extension product was phenol extracted, precipitated, and loaded onto 8% denaturing PAGE gel. The gel was dried, exposed, and scanned in a PhosphorImager. All the primer extension gels are representative of at least two independent experiments of each sample.

Database search

The mouse STREX peptide sequence was used to search the Fugu genome database (http://fugu.hgmp.mrc.ac.uk/blast/) with TBLASTN to identify a STREX homolog of fugu fish. The genomic sequence preceding this peptide-encoding sequence is taken as the 3′SS of fugu STREX sequence.

The CaRRE consensus sequence was used to search the annotated human genome sequence or a subset of the human genome containing alternative exons and the flanking intron sequences kindly provided by Christopher Lee (Modrek et al. 2001). Pattern matching with the CaRRE consensus sequences was done by scripts written in Perl using the BioPerl modules (http://www.bioperl.org/). The identified 3′SSs were used in the UCSC human BLAT search (http://www.genome.ucsc.edu/cgi-bin/hgBlat?command=start&org=human) to identify downstream alternative exons. Those with at least one EST or mRNA that does not contain the downstream exons but still with common sequences flanking them are considered as alternative exons.

RT-PCR from CaMK IV knockout mouse tissue

Mouse cerebella were ground with pestles in 1.5-mL tubes in RLT buffer (supplied in the RNeasy kit; Qiagen). The resulting lysates were then extracted for total RNA following the supplier's protocol for animal tissues. About 200 ng RNA used for 20 μL of reverse transcription (RT) reaction containing Superscript II (Invitrogen) and oligo dT_{18}. One half microliter of the RT product was used for a 12.5 μL PCR reaction with an initial incubation at 95°C for 5 min followed by 24 cycles, each comprising 95°C, 30 sec; 53°C, 30 sec; 72°C, 30 sec. In these PCR reactions, antisense primers of each gene were end labeled with [γ-^{32}P]ATP by T_4 polynucleotide kinase. PCR products were resolved on 8% denaturing polyacrylamide gels and scanned by a phosphorimager (Molecular Dynamics or Bio-Rad).

Gel quantification

Band intensities of ^{32}P-labeled primer extension or RT-PCR products were quantified using ImageQuant 5.2 software from Molecular Dynamics or Quantity One-4.5.0 from Bio-Rad, depending on the scanner used.

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