Regulation of Alternative Pre-mRNA Splicing by Depolarization/CaMKIV

by

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ABSTRACT

Alternative pre-mRNA splicing is often controlled by cell signals (1-3). Membrane depolarization/calcium (Ca^{2+}) signaling controls alternative splicing of a group of genes in neurons and endocrine cells (4-9), with important implications in memory formation or secretion of hormones and neurotransmitters (10-15). However, the underlying molecular basis remains largely unknown.

In rat GH_3 pituitary cells, BK potassium channels control cellular electrical firing, which is critical for the release of growth hormone and prolactin. Inclusion of the STREX exon of the *Slo1* gene encoding the channel α subunit is repressed by the Ca^{2+}/calmodulin-dependent kinase IV (CaMKIV) upon depolarization (4). We isolated CaMKIV-responsive RNA elements (CaRREs) from a library of 13-nucleotide random sequences through *in vivo* selection in HEK293T cells. Most elements are CA-rich or A-rich, with the heterogeneous nuclear ribonucleoprotein (hnRNP) L as a binding factor. This is consistent with the finding that CA-rich elements and hnRNP L are targeted by CaMKIV in the regulation of splicing (16).

In further efforts to directly link the kinase with hnRNP L, we showed that hnRNP L is essential for the full repression of STREX by depolarization and that a highly conserved CaMKIV target serine (Ser513) of L is required. Ser513 phosphorylation enhanced L binding to the STREX CaRRE1, leading to reduced binding of the constitutive factor U2AF65 to the 3' splice site of STREX. Mutation of Ser513 abolished both activities. Therefore, hnRNP L mediates the repression of STREX by depolarization through modulation of a key step in spliceosomal assembly.

We further identified hnRNP L, L-like (LL) and PTB as repressors of STREX and other depolarization-regulated exons with differential effects. Moreover, a full response of STREX to depolarization is mediated by combinations of hnRNP L and LL or PTB. Another depolarization-responsive exon, the exon 18 of the neuregulin 1 gene, is also controlled in a similar way, with the hnRNP L Ser513 required as well.

This work provides the first direct link between the Ca^{2+} signaling and a specific serine of a regulatory splicing factor. Elucidation of the underlying molecular mechanisms would likely help us understand the fine-tuning of hormone secretion and memory formation.

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DEDICATION

This work is dedicated to my wife, Wenyan, our parents, Tianli and Jianzhong, Fengying and Qu, and my daughter, Ellie.

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ABBREVIATIONS

AHP	afterhyperpolarization
APS	ammonium persulfate
ASF/SF2	alternative splicing factor/splicing factor 2
BPS	branch point sequence
CA1	cornu ammonis 1
CaMK	Ca^{2+}/calmodulin-dependent protein kinase
cAMP	adenosine 3',5'-cyclic monophosphate
CaRRE	CaMKIV-responsive RNA element
ChIP	chromatin immunoprecipitation
CCCH	Cys–Cys–Cys–His
CHX	cycloheximide
CIAP	calf intestinal alkaline phosphatase
CLIP	Cross-Linking and ImmunoPrecipitation
CO_2	carbon dioxide
CREB	Ca^{2+}/cAMP Responsive Element Binding Protein
CTCF	CCCTC-binding factor
DMEM	Dulbecco's Modified Eagle Medium
DNMT	DNA methyltransferase
DOC	deoxycholic acid
DTT	dithiothreitol
EDA	extra domain A
EDI	extra domain I

EDTA	ethylenediamine tetraacetic acid
EJC	exon junction complex
ESE	exonic splicing enhancer
eNOS	endothelial nitric oxide synthase
ERK	extracellular signal–regulated kinase
FBS	fetal bovine serum
FGFR	fibroblast growth factor receptor
Fox-1/A2BP1	Feminizing gene 1 on X/Ataxin 2-Binding Protein 1
GH	growth hormone
H89	N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinoline sulfonamide
HAT	histone acetyltransferase
HDAC	histone deacetylase
hnRNP	heterogeneous nuclear ribonucleoprotein
hnRNP LL	hnRNP L-like
IMDM	Iscove's Modified Dulbecco's Medium
KH	K-homology
KI	K interactive
KMT	lysine methyltransferase
KNS	K nuclear shuttling domain
KRG buffer	Krebs-Ringer glucose buffer
LTD	long-term depression
LTP	long-term potentiation

MAPK	mitogen-activated protein kinase
MBNL	Muscleblind-like
MED23	mediator complex subunit 23
miRNA	microRNA
MRG 15	MORF-related gene 15
pre-mRNA	precursor messenger RNA
NCAM	Neural Cell Adhesion Molecule
NCS	newborn calf serum
NMD	nonsense mediated RNA decay
NMDAR	N-methyl-D-aspartate-sensitive glutamate receptor
Nrxn	Neurexin
NRS	nuclear retention signal
NSCLC	non–small cell lung cancer
PEG8000	Poly(ethylene glycol), M.W. 8000
PKA	cAMP-dependent protein kinase/Protein kinase A
PKC	Protein kinase C
PMSF	Phenylmethanesulfonyl fluoride
RNA Pol II	RNA polymerase II
PP2A	Protein phosphatase 2A
PTB	polypyrimidine tract binding protein
PTC	premature termination codon
Py tract	Polypyrimidine tract
RBD	RNA binding domain

rhMLV	rhesus-adapted Murine Leukemia Virus LTR
RIPA	Radio immunoprecipitation assay
RNAi	RNA interference
RRM	RNA recognition motif
RS-rich domain	arginine/serine-rich domain
RT-PCR	Reverse transcription-polymerase chain reaction
Sam68	Src-associated in mitosis, 68 kDa protein
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SELEX	systematic evolution of ligands by exponential enrichment
SF1/BBP	splicing factor 1/branch point binding protein
shRNA	Short hairpin RNA
siRNA	small interference RNA
Snap25	25-kD synaptosomal-associated protein
SNARE	Soluble NSF (Nethylmaleimide-sensitive fusion protein) accessory protein (SNAP) receptor
snoRNA	small nucleolar RNA
snRNP	small nuclear ribonucleoprotein
SRSF	serine-arginine rich splicing factor
STAR	Signal transduction and activation of RNA
STREX	stress axis-regulated exon
TBS	Tris-buffered saline
TPA	12-O-tetradecanoylphorbol-13-acetate
Tra2	Transformer 2

TSA	trichostatin A
U2AF	U2 auxiliary factor
UTR	untranslated region
VGCC	voltage-gated Ca^{2+} channel
µg	microgram
µl	microliter
µm	micrometer
UV	ultraviolet

CHAPTER I

Literature Review

1. Precursor messenger RNA (pre-mRNA) splicing

In higher eukaryotes, only a small portion of most protein-coding genes can be expressed. The selective expression is through several known mechanisms including the usage of alternative transcription start sites and 3' ends (17,18), as well as the major player pre-mRNA splicing (19).

Splicing is the process by which stretches of sequences within a gene, called introns, are removed from pre-mRNA and the expressed regions, call exons, are joined, through two transesterification reactions. In the first reaction at the 5' end of an intron (5' splice site/donor site), the phosphodiester bond joining the exon and intron nucleotides is cleaved through nucleophilic attack by the 2'-hydroxyl group (OH) of an intronic adenosine (branch point) at the 3' intron end (3' splice site/receptor site) (19). This reaction results in the ligation of the adenosine to the first nucleotide of the intron via a 2'-5'-phosphodiester bond, forming the characteristic lariat structure. Thereafter, the 3'-OH freed at the end of the upstream exon attacks the 3' splice site, leading to the intron release from the RNA transcript and ligation of two exons. Due to their critical roles in splicing, the 5' splice site (most conserved is the GU dinucleotide) and the 3' splice site consisting of the branch point sequence (BPS), the polypyrimidine tract (Py tract) and the 3' AG at the intron end (19) are characteristic elements of an intron, or in other words, essential pre-mRNA elements determining where to splice.

Selection of splice sites largely depends on assembly of spliceosome on RNA transcripts. The spliceosome is a large, highly dynamic protein-RNA complex composed

of five small nuclear ribonucleoprotein (snRNP) particles and auxiliary proteins ranging in number from about 90 to 300, depending on species (20-22). The assembly of spliceosome on nascent transcripts involves extensive RNA-RNA, protein-RNA and protein-protein interactions leading to stepwise formation of spliceosomal complexes (19,23). This assembly is initiated by interactions between spliceosome components and essential RNA elements in introns. During the process, the 5' splice site interacts with the U1 snRNP via at least in part base-paring between the splice site and the U1 snRNA. At the 3' intron end, the 3' splice site is recognized by the 35-kDa subunit of U2 auxiliary factor (U2AF35) contacting the 3' AG and the 65-kDa U2AF65 binding the upstream Py tract, as well as the SF1/BBP (splicing factor 1/branch point binding protein) interacting with the BPS. These interactions at both intron ends result in the formation of the E complex. The E complex proceeds with the engagement of the U2 snRNP at the branch point via RNA-RNA base-pairing stabilized by protein components of U2 snRNP, and consequently replacement of SF1/BBP, leading to the formation of the A complex. The subsequent recruitment of the U4/U6-U5 tri-snRNP generates the B complex that catalyzes the first step of splicing upon activation through conformational changes and release of U1 and U4. Following reorganizations of components eventually convert the activated B complex into the C complex that catalyzes the second step of splicing.

Through these procedures, a spliceosome assembles as a cross-intron complex, which appears to apply mostly to short introns or pre-mRNAs containing a single intron (24). However, genes of higher eukaryotes contain multiple introns that are mostly long, making it a question of how spliceosome components communicate across a long intron during the early steps of assembly. In this case, spliceosome components first assemble

across an exon (average is 50~250 bp in humans (25)), referred to as exon definition (19). This procedure essentially includes recognition of the downstream 5' splice site by U1 snRNP and binding of the upstream 3' splice site by U2AF and U2 snRNP respectively, and then bridging of the two complexes by the serine-arginine rich (SR) proteins (See 2.3.3). To initiate splicing reaction, the cross-exon complex must be converted into the cross-intron complex, so called intron definition (25), of which the underlying mechanism is not fully-understood yet. However, recent studies indicate the exon junction complex (EJC) better known for post-splicing mRNA processing is an essential component mediating long intron splicing (26,27), though involvement of heterogeneous nuclear ribonucleoproteins (hnRNPs) is also suggested (28,29).

Besides the canonical spliceosome, there is also a minor spliceosome that includes four distinct snRNPs (U11, U12, U4atac and U6atac) and also the U5 snRNP (30). Introns spliced by the minor spliceosome have longer 5' splice site consensus sequences and BPS, and usually lack the Py tract (30,31). Due to the essential role of the U12 snRNP in excision of these introns, they are called U12-type introns, accordingly the typical introns that require U2 snRNP are U2-type introns. The minor spliceosome can also cooperate with the major spliceosome when the U2- and U12- type introns are in the same pre-mRNA (32,33).

Although the essential elements in pre-mRNA play important roles in splicing control, it is unlikely that their interplays with spliceosome components will explain all the splicing events in higher eukaryotic cells, even just considering the length of most introns. It is rational to speculate extensive involvements of regulatory RNA elements and protein/RNA factors outside of the essential elements and spliceosome components.

This is even more obvious when it comes to alternative splicing, the selective removal of introns and usage of exons.

2. Alternative pre-mRNA splicing

Alternative pre-mRNA splicing refers to selective usage of an exon or exons in a pre-mRNA through modulating choices of splice sites, by which more than one mRNA isoforms, called splice variants, are produced from a single pre-mRNA.

Alternative splicing contributes greatly to the proteomic diversity of higher eukaryotes. A gene used to be defined as a genetic unit encoding one protein. However, genome sequencing results from various species revealed that the total numbers of protein-coding genes in organisms do not reflect their biological complexity. For example, the estimated number of human genes is about 20,000~25,000 (34), only about four times the number found in the budding yeast *Saccharomyces cerevisiae*, and similar to that of the *Arabidopsis thaliana* (35). These observations indicate that mechanisms other than gene numbers must act to fulfill the needs during evolution of complex metazoans. Such mechanisms include alternative usages of DNA promoters/transcription start sites (17,36) and 3' ends of RNA (18), RNA editing (37), alternative translational initiation (38), and protein splicing (39) in rare cases. However, alternative pre-mRNA splicing likely plays the most critical role in producing proteomic diversity (19). The updated estimate is that more than 90% of human genes undergo alternative splicing (40,41).

The advantage to utilize alternative splicing instead of huge numbers of genes is manifested at least in several aspects (42-45). Firstly, it enables cells to rapidly produce new proteins/new functions (42) without initiating new transcription events that may take

much longer if new components such as more transcription factors are needed. Secondly, even if just modifications of current transcription factors are required, it may still need more modifying enzymes, such as kinase/phosphatase, acetyltransferase/deacetylase and methyltransferase/demethylase, or at least altered localization and activity of enzymes. Thirdly, if chromatin remodeling is required, related protein factors may need to be produced or modified first. Fourthly, if one gene encodes only one protein, then many more genes are needed. Assuming protein isoforms share most of their exons, then there will be many copies of a same exon, which seems not necessary, and more importantly, may cause frequent unwanted genomic recombination (43,44). Besides, to ensure proper expression of specific isoforms from different genes, it is possible that promoters and transcription factors need to be diversified greatly if gene number is the only determinant. Finally, alternative splicing will benefit cells by providing a cost-effective strategy for rapid re-organization of genetic information in needs of development or adaptation to environmental changes without changing the DNA template (42,45), which provides an important protective mechanism to the genome.

Alternative splicing is more extensively used in higher eukaryotes than in lower eukaryotes (46,47), which can be partially evidenced by the number of genes subjected to alternative splicing, the occurrence of introns in genes and numbers of regulatory factors. More importantly, there is much more extensive alternative splicing usage in the nervous and the endocrine systems (40,48,49) , which applies global and intricate controls of cells. So, is alternative splicing an indication of the evolution of life, especially the development of high intelligence?

2.1 Patterns of alternative splicing

Identification of alternative splicing events depends on analyses of released sequences of genomes and transcripts combined with experimental examinations (35). Several patterns of alternative splicing have been characterized (19). The most prevalent one is the cassette exon that is either included or excluded from mRNA. There are also mutually exclusive exons produced by picking up only one from two or more adjacent exons at a time. In some cases, an exon can have alternative 5' or 3' splice sites. Another pattern, called intron intention, produces mRNA transcripts containing retained introns. Mechanisms controlling these modes of alternative splicing mainly relate to choice of splice sites. Alternative exons can also be introduced by differential usage of transcriptional promoters or by alternative 3' end processing, suggesting involvement of other machineries of gene expression.

Recently, systematic analysis of alternative splicing events through global-profiling techniques, such as hybridization-based methods, exon array and RNA-Seq (high-throughput sequencing of cDNA fragments) (35,50), further demonstrated the abundance of alternative splicing (35,40,41). Besides, these techniques greatly facilitate identification of differentially used alternative exons and numerous new splice variants (51-53).

2.2 Effect of alternative splicing on protein products

Through changing combinations of protein sequences, alternative splicing not only contributes to protein complexity, but also modifies and diversifies protein functions. Moreover, alternative splicing is also a mechanism for cellular protein homeostasis

through introducing premature termination codons (PTCs) that causes nonsense-mediated mRNA decay (NMD) (54). Recently, the profound effects of alternative splicing on protein functions have been well reviewed (55). Table 1 lists some examples of regulated protein functions by alternative splicing. Notably, alternative splicing produces multiple effects for some proteins, such as Tra2.

Table 1. Protein functions modified through alternative splicing

Functions affected	**Example of protein**
Ca^{2+} sensitivity	*Slo* (4,56)
Enzymatic activity	CD45, Fyn (57,58)
Ligand/receptor binding	CD44, dopamine D3 receptor, ErbB4, TSH receptor (59-62)
Protein localization	Rbfox1, ErbB4, Nizp1 (8,61,63)
Protein stability	c-Fos, IRF-1, PKCδ (64-66)
Pre-mature stop codon	Tra2, Sxl (19)
Reduced function or non-functional isoforms	Tra2, Caspase9b, REST (NRSF) (67-69)
Dominant negative isoform	Caspase9b, dMax, Fox (68,70,71)
Not-translated	Msl2 (19)
Antagonistic functions	Bcl-x_L and Bcl-x_S, Caspase 2S and 2L, Caspase 9a and 9b (72-74)

2.3 Control of alternative splicing

Generally, alternative splicing is controlled by manipulation of splice site selections by the spliceosome through interactions among various regulatory factors (19). Mainly two layers of controls are involved: modulation of the splicing machinery and effects from chromatin remodeling accompanied by epigenetic modifications of DNA and histones and cotranscriptional mechanisms (75). In many cases, the two most

downstream components, regulatory cis-acting RNA elements and trans-acting splicing factors, are indispensable.

2.3.1 Regulatory RNA elements

Although the GU and AG dinucleotides at both splice sites are critical for splicing of many introns, they are parts of longer consensus sequences of splice sites and unlikely sufficient by themselves to guide splicing (76). On the other hand, there are also introns that are not marked by the canonical 5'/3' splice site sequences (30). Therefore, more RNA sequences outside of the essential elements are required to ensure precise assembly of spliceosome on target RNA. The critical information is encoded by regulatory cis-RNA elements that mark primary RNA transcripts with signs of splicing or no splicing.

Numerous regulatory RNA elements have been identified through mutagenesis analysis or large-scale screening (77-81). Most of them appear general, while those tissue-specific or cell-signaling responsive ones are less well characterized. Regulatory elements are typically categorized into 4 classes based on their locations in pre-mRNA and their effects on splicing, including exonic splicing enhancer (ESE), exonic splicing silencer (ESS), intronic splicing enhancer (ISE), and intronic splicing silencer (ISS). However, this classification system may not be highly definitive in some cases because some elements can be bound by factors with opposite effects or show location-dependent effects (82-84), resulting in either splicing promotion or repression. This suggests that RNA elements can be dual-functional and that information carried by them can be translated in different ways depending on the binding factors or the context they reside in. Recently, a special type of composite regulatory element was reported (85). This type of element appears as a combination of the 3' and 5' splice sites, and induces an effect

analogous to splicing of mutually exclusive exons but with no intron. The presence of these unique elements suggests a highly diverse and complicated regulation of splicing.

Interestingly, it seems that the importance or regulatable extent of the two splice sites may not be equal (19,86). A large-scale sequencing based on the whole-exome in 29 myelodysplasia patient samples identified mutations in 5 genes encoding splicing factors, including U2AF35 and SF3B1 that are mainly involved in early steps of spliceosome assembly at the 3' splice site (86). The fact that three of the four essential pre-mRNA elements, the BPS, the Py tract and the 3' AG are located in the 3' splice site of a typical intron (19) is also a good suggestion that regulation at the 3' end may be more intensive. However, more extensive investigations are needed to determine whether and how prevalent this effect is.

2.3.2 Effects of pre-mRNA secondary structure

Pre-mRNA secondary structures also play roles in splicing control. There have been several lines of evidence showing either inhibiting or facilitating effects of secondary structures on splicing, as described in a recent review (87).

The essential splicing elements can form intramolecular base pairings in secondary structures, which prevent recruitments of U1 and U2 snRNPs or binding of auxiliary factors such as U2AF (87). On the other hand, structures around these sites can also facilitate assembly of the splicing apparatus. In this case, 5' and 3' splice sites are brought closer to each other, or the BPS and the 3' splice site separated by a longer spacer are in close proximity (87). A well-known example is the exon cluster 6 of the Drosophila Down Syndrome Cell Adhesion Molecule (Dscam) gene that can produce 48 mutually exclusive exons (88). It was proposed that choice of an exon variant is

determined by interactions between a 66-nt docking sequence near the 5' end of the intron upstream of the exon cluster 6 and a selector sequence upstream of each exon variant. This brings the 5' splice site close to each 3' splice site and likely releases the splicing repressor bound to each downstream exon (88). This model was experimentally verified using a BAC containing the full-length Dscam gene (89), and further confirmed in another system using the mutually exclusive exon cluster 5 of 14-3-3ξ (90). Moreover, the authors also identified and experimentally demonstrated similar regulatory structural codes in the exon clusters 4 and 9 of the Dscam and the myosin heavy chain (Mhc) gene of Drosophila. These revealed an unusual mechanism for the control and fine-tuning of mutually exclusive splicing. In other models, cryptic splice sites are used due to disruption of RNA structures that cover the cryptic splice sites (91,92).

RNA secondary structures can also modulate function of regulatory cis-acting RNA elements. One example is the extra domain A (EDA) exon of the fibronectin gene (93,94). This exon contains an ESE likely bound by SR proteins. When the ESE is presented by a stem-loop structure to SR proteins, the EDA exon is then included. Alterations of the secondary structure may cause the ESE unavailable and thus disrupt splicing of the EDA exon (93,94). Splicing of the microtubule-associated protein tau (MAPT) exon 10 (E10) is regulated by multiple exonic and intronic elements (95). Mutations of the element in the 5' splice site of the downstream intron 10 disrupt the original stem-loop structure and lead to increased level of E10 (96,97), which associates with the development of frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17) (98). A recent study identified the polypyrimidine tract binding protein (PTB) associated splicing factor (PSF) as an suppressor of the tau E10

through interacting with the stem–loop structure (99). Similar examples of splicing factors recognizing secondary structures are also found in other studies (100).

2.3.3 Regulatory splicing factors

Directional information carried by cis-acting RNA elements is mostly read by regulatory trans-acting splicing factors, which then signal positively or negatively to the splicing machinery and thus determine whether to use a specific site. The majority of them are RNA binding proteins including two major families, the SR proteins and hnRNPs (19). A common structural feature among most members of these two families is their RNA recognition motifs (RRMs) (101,102), the best-known and most prevalent RNA binding domain (RBD), of which typical function is for protein-RNA interactions. The major structural characteristic distinguishing the two families is their domains responsible for protein-protein interactions (101,102). SR proteins feature their arginine/serine (RS)-rich domains, while hnRNP proteins harbor diverse domains. However, the functional difference between RRMs and protein-protein interaction domains in splicing may not be that strict because RRMs can mediate protein-protein interactions and RS domains can also bind RNA (103,104). Besides, it seems splicing activities of splicing factors not necessarily reside in RBDs, at least for some splicing factors. For example, the Muscleblind-like (MBNL) proteins 1 and 3 regulate splicing through a region downstream of their N-terminal RBDs because removal of this region abolished their splicing activities but essentially did not change their RNA binding affinity (105). These suggest diversified effects of functional domains of splicing factors.

SR proteins are found in all metazoans and plants. So far, 12 SR proteins have been identified in humans, designated as SR splicing factor (SRSF) 1~12 according to a recent

nomenclature system (106). Each human SR protein contains one or two N-terminal RRMs and a downstream RS domain except the SRSF10 that carries an extra RS domain preceding its RRM. In addition to the canonical SRSFs, there are also SR-like splicing factors containing RS-domains, for example, the regulatory factors human transformer 2 (hTra2) α and β, as well as some constitutive factors including U2AF35, U2AF65 and U1-70K (107). Some SR-related splicing factors contain RS domains but no typical RRMs and they bind RNA with distinct domains. More complete lists of these additional SR proteins and their functions can be found in recent reviews (101,107). The prevalence of RS domains in splicing-related proteins indicates their important roles. Indeed, it is suggested that RS domains in SR proteins are critical functional modules for proper recognition of weak splicing signals (101,107) and likely a major target for regulation (108). SR proteins are essential for both constitutive and alternative splicing (101). Generally, SR proteins are recognized as splicing activators (101), however, some of them can also repress splicing depending on their posttranslational modifications and locations of their elements (109-111).

Another family is the hnRNPs that actually belong to the first studied RNA binding protein family (112). They were initially identified as proteins bound to heterogeneous RNA molecules that are recognized as pre-mRNA later on (113). There are more than 20 canonical hnRNPs, named from hnRNP A to U. Unlike SR proteins, hnRNP proteins are structurally diverse (102). Most members share the RRM-type RBDs, but a few of them possess the K-homology (KH) domain, such as hnRNPs E and K. Extra domains in hnRNPs are more diversified, such as the RGG motif (arginine-glycine-glycine rich), the glycine-rich domain, the proline-rich domain, the basic leucine zipper (bZIP)-like

domain, the nuclear retention signal (NRS), the K nuclear shuttling domain (KNS) and the K interactive (KI) domain (102). These domains have different functions including protein-protein interactions. In contrast to the SR proteins, hnRNPs are generally recognized as splicing repressors that can directly interfere with assembly of spliceosome on pre-mRNA or antagonize effects of splicing activators (102). However, there are also examples where hnRNPs promoted splicing (114,115), suggesting the importance of context in determining directions of splicing.

Besides the two classical families, there are more RNA binding proteins identified as splicing factors. Many of them contain RRMs as well, such as the neuron-specific splicing factorsNova1/2 and the Feminizing gene 1 on X (Fox) proteins (84,116,117). Some have non-typical RBDs, for example, the KH domains in members of the signal transduction activators of RNA (STAR) family such as the Src-associated in mitosis 68 kDa protein (Sam68) (118) and the Cys–Cys–Cys–His (CCCH)-type zinc-finger RBDs of the MBNL proteins (105).

RNA targets of regulatory splicing factors can be isolated from disease-related mutations or systematically identified through screening strategies such as systematic evolution of ligands by exponential enrichment (SELEX) or loss-of-function analysis combined with microarray or CLIP (Cross-Linking and ImmunoPrecipitation) (82,114,119-123). Characterization of target elements helps understanding of the regulatory mechanisms of alternative splicing, prediction of genome-wide targets as well as systematic analysis of regulatory networks.

However, from the nature of RNA binding proteins, it should be noted that many splicing factors also play a role in other steps of RNA metabolism, including packaging

of nascent transcripts, RNA 3' end processing, mRNA transport and stability, as well as translation (124-129). Some even participate in the maintenance of genomic stability and transcription as well (130,131), suggesting a link between transcription and alternative splicing.

Very interestingly, splicing regulatory factors may not necessarily be proteins because some types of small RNA are also implicated in regulation of splicing. For example, the small nucleolar RNA (snoRNA) HBII-52 shows complementarity in its antisense element to the exon Vb of the serotonin receptor 5-HT_{2C}R. Binding of HBII-52 to this exon repressed its splicing (132). Further studies in mouse demonstrated that shorter HBII-52 RNAs lacking the snoRNA stem produced by posttranscriptional processing bound hnRNPs instead of assembling into snoRNP, and were involved in splice site selection (133). Moreover, binding of small interference RNAs (siRNAs) to intron or exon can promote splicing, which requires methylation of histone H3 and lower processivity of RNA polymerase II (Pol II) (134). These discoveries expand the family of splicing regulators and suggest new mechanisms of splicing regulation.

2.3.4 Control of regulatory splicing factors

To ensure proper control of alternative splicing, splicing factors are regulated by diverse mechanisms, which essentially include control at the protein level, switch of isoforms, change of localization and coregulators, as well as modulation of activity (3).

The above regulations of splicing factors can be gene expression associated. For example, protein levels of splicing factors can be modulated through transcriptional (135) and posttranscriptional controls. The latter includes production of a splice variant that can induces NMD (136), downregulation by microRNAs (miRNAs) (137,138) and regulation

of mRNA stability (3). For some of the other mechanisms, it is quite often that combined effects are applied to the control of splicing factors. For instance, different splice variants of a splicing factor may have differential localization and activities (8).

Splicing factors can also be regulated through posttranslational modifications, which lead to relocalization, changes of protein stability, switching to different partners, as well as altered activities (3,109,111). Such modifications include, but are not limited to, phosphorylation, methylation and acetylation. For example, RNA binding affinity of splicing factors can be modulated by phosphorylation. Phosphorylation of the RS domain of SRSF1 (previously known as ASF/SF2) not only enhanced its interaction with the constitutive splicing factor U1 70k, but also abolished its RNA binding *in vitro* (103). Sam68 bound its target RNA less efficiently when phosphorylated by the extracellular signal–regulated kinase (ERK) through the Ras signaling pathway both *in vitro* and *in vivo* (139). This modification did not affect its interaction with U2AF proteins but attenuated 3’ splice site recognition by U2AF65 (139). In these examples, phosphorylation reduced RNA binding affinity of these splicing factors, while the opposite effect is less reported. It was shown that phosphorylation of SRSF1 by PKA promoted its interaction with tau pre-mRNA (140). Moreover, PKA phosphorylation at the SRSF1 Ser119 possibly enhanced its RNA binding *in vitro* (141). RNA binding affinity of splicing factors can also be regulated by acetylation and methylation. Sam68 is acetylated in human mammary epithelial cell lines, and more strikingly, in breast cancer cell lines (142). Importantly, the increased acetylation of Sam68 associates with enhanced RNA binding affinity. However, methylation of its arginine in the RG (arginine-glycine) repeats dramatically reduced its poly(U) binding ability *in vitro* (143).

2.3.5 Coupling of alternative splicing with transcription and chromatin status

As two close steps in gene expression, alternative splicing and transcription can be coupled (144). Currently, two major models are described (144). One model focuses on the recruitment of splicing factors by the transcription machinery through the carboxyl-terminal domain (CTD) of Pol II; the other emphasizes the kinetics of Pol II by suggesting that the rate of Pol II affects outcomes of alternative splicing. Both models have supportive evidence with the kinetic model receiving more attention. Moreover, cotranscriptional control of splicing involves chromatin remodeling through epigenetic modifications, such as DNA methylation, histone acetylation, methylation and phosphorylation (75,145). These modifications alter accessibility of chromatin to transcription, affect transcription processivity, differentially mark introns and exons (146,147), and even participate in recruitment of splicing factors (145,148). Roles of these mechanisms in the coupling of alternative splicing with chromatin/transcription have been summarized recently (75,149-152). Here, I will give some examples of how these connections are built up through DNA binding proteins as well as splicing factors.

The zinc finger DNA binding protein CCCTC-binding factor (CTCF), a structural component of long-range interactions of the genome and a transcription insulator (153,154), is critical for differential uses of CD45 E5 during B cell maturation. CTCF binds the genomic region of the CD45 E5 in a way negatively correlated to DNA methylation of E5 (155). In naive B cells whose methylation within E5 is low, CTCF binding resulted in pause of RNA pol II at E5, which led to E5 inclusion. During maturation, CTCF binding to E5 reduced due to increased methylation and resulted in

exon skipping. Thus, transcription processivity alterations modulated by DNA methylation and a DNA binding protein determine splicing outcomes.

Chromatin associated proteins can also couple chromatin modifications with alternative splicing (145). Mutually exclusive exons IIIb and IIIc of the fibroblast growth factor receptor 2 (FGFR2) are differentially included in epithelial cells and mesenchymal cells. Trimethylated H3K36 (H3K36m3) preferentially enriched within the EIIIb region in mesenchymal cells recruits the splicing repressor PTB to the 3' splice site of EIIIb through a histone tail binding protein MRG 15, which causes EIIIb repression (145).

On the other hand, splicing factors can actively regulate transcription rate through histone acetylation. For example, the RNA binding Hu proteins can interact with the RNA Pol II core complex in mouse primary cerebellar neurons (156). In embryonic stem cell-derived neurons, this interaction resulted in increased elongation rate of transcription in regions surrounding alternative exons E23a~E28 of the Nf1 gene and E6 of the Fas gene (157,158), which led to skipping of Nf1 E23a and Fas E6. The elevated elongation rate correlates with histone hyperacetylation in the regions of regulated exons likely due to inhibition of the histone deacetylase 2 (HDAC2) by Hu. Importantly, the Hu-mediated histone acetylation requires its target RNA elements, the AU-rich sequence upstream of E23a (156). Thus, a regulatory splicing factor can modulate alternative splicing through tuning of transcription rate via histone acetylation.

Interestingly, alternative splicing can also be modulated by altered Pol II elongation through siRNA. In this model, efficiency of Pol II elongation was reduced by siRNA targeting sequences surrounding E33 at the fibronectin EDI site, which promoted E33 inclusion. This effect requires Argonaute proteins and involves the formation of

heterochromatin by methylation at two lysine sites of histone H3 at target sites and the heterochromatin-associated protein HP1α (134).

2.3.6 Cell signaling control of alternative splicing

Considering its selective and differential nature, alternative splicing must be controlled by "signals" to ensure proper production of protein isoforms during development, in specific tissues or sexes. These "signals" most likely represent more systematic and long-term control of alternative splicing. Here I will focus mainly on signals that are more transient and specific in regulating certain alternative splicing events.

Cell signaling plays important roles in the control of alternative splicing. In many cases, cell signaling controlled splicing involves at least certain extracellular stimulus, kinases or phosphatases, as well as regulatory splicing factors and RNA elements (135,159,160). Therefore, one mechanism of cell signal-induced splicing shifts is associated with modulation of splicing components, such as phosphorylation of or protein level changes of splicing factors (135,161,162). Examples of cell signal controlled alternative splicing whose components have been characterized in relatively more details are listed in table 2. These include signals controlling neuronal activity, immunological activation, cell growth/death and hematopoiesis (Table 2).

Some of the stimuli activate downstream signaling pathways, at least partly, via second messengers (Table 2). For instance, depolarization can induce both Ca^{2+}- and cyclic AMP (cAMP)-dependent signaling pathways; forskolin can activate cAMP-dependent pathways. Second messengers amplify signals initiated by extracellular stimuli and activate multiple downstream pathways which control splicing of more genes. In

some cases, cross-talk among and within second messenger systems provides combinatorial control and fine-tuning of splicing (163,164).

Cell signaling can also modulate splicing through induced secondary RNA structures. For example, spermidine, a type of polyamine with effective translation-promoting effects, stimulates protein synthesis through multiple mechanisms, which requires its binding to the loop region of stem-loop structures in mRNA or tRNA (165). These interactions induce structural changes of RNA (165,166), which is essential for initiation of the synthesis of the bacterial oligopeptide-binding protein OppA and formation of the Ile-tRNA in rat liver (165). Moreover, spermidine also induced conformational changes of a 34-nt stem–loop RNA derived from the U6 snRNA, an essential component of splicing (166). Moreover, spermidine and other polyamines promoted inclusion of an NMD-inducing exon of the spermidine/spermine N1-acetyltransferase (SSAT) (167), a key enzyme mediating the conversion between spermidine and spermine. This regulation provides a feedback control of polyamine metabolism. Therefore, regulated RNA secondary structure is also a layer of alternative splicing control.

Moreover, cotranscriptional splicing can be induced by cell signals as well. For example, inclusion of the extra domain I (EDI) exon of the fibronectin gene increased in response to low-dose ultraviolet irradiation (UV) independent of p53 (168). This is associated with inhibition of transcriptional elongation caused by hyperphosphorylation of the RNA Pol II CTD (168).

Cell signal-regulated alternative splicing not only provides exquisite controls on gene expression but also modulates important cellular functions. For example, cell signal-

induced compositional changes between splice variants with antagonistic functions affect cell growth and death, such as Bcl-xL (antiapoptotic) and Bcl-xS (proapoptotic), caspase 9a (proapoptotic) and 9b (antiapoptotic), PKC δI (proapoptotic) and δVIII (antiapoptotic) (Table 2).

Table 2. Cell signaling-controlled alternative splicing

Gene name	Target exon	Effect on exon inclusion	Function of exon	Stimulus	Signaling pathway	Splicing factor involved	Change of splicing factor by signal	Effect of splicing factor change	Cell/ tissue	Species	Ref
Bcl-xL	E2	Promotion	Promotion of growth		Fyn	Sam68, hnRNP A1	Phosphorylation of Sam68	Reduction of RNA binding	HEK293	H	(169)
		Promotion				SRSF9 (SRp30c)			HeLa	H	(170)
Bcl-xS	E2	Promotion	Proapoptotic	Ceramide	PP1				A549	H	(171)
		Promotion		staurosporine	Inhibition of PKC				HEK293	H	(72)
		Promotion		Ro-31-8220	PP1				MDA-231 and BT20	H	(172)
		Promotion		emetine dihydrochloride	PP1				C33A, MCF-7 PC3		(173)
CaMKK 2	E16	Promotion	Promotion of neurite branching	Forskolin	PKA				B35	M	(164)
Caspase 9	Exon3~6	Promotion	Proapoptotic	Ceramide	PP1	SRSF1 (ASF/SF2, SRp30a)	Likely dephosphorylation		A549	H	(174)
		Repression	Antiapoptotic		PI3K/Akt	SRSF1 (ASF/SF2, SRp30a)	Phosphorylation		NSCLC cells	H	(160)
		Repression	Antiapoptotic			hnRNP L	Phosphorylation		NSCLC cells	H	(68)
CD44	E5	Repression	Extracellular domain	T cell activation	PKC, Ras	hnRNP A1			T cells		(175)
		Promotion		T cell activation	Ras-ERK	Sam68, Srm160	Phosphorylation of Sam68 by ERK	Enhancing binding to pre-mRNA	EL4	M	(161)
	Cassette exons	Repression		Tamoxifen		ESRP1	Decreased protein level		HMLE	H	(162)
CD45	Exons 4~6	Repression	T cell differentiation	T cell activation		hnRNP LL	Increased hnRNP LL level		T cells		(135)
		Derepression		PMA	GSK3	PSF	Phosphorylation of T687	Binding to TRAP35/	T cells (JS1)	M	(176)
Neurexin 1α	E20	Repression	May affect ligand binding	25 mM KCl	CaMKIV	Sam68	Phosphorylation	Unknown	Cerebellar culture	M	(9)
NMDA R1	E5	Repression	Agonist binding and other pharmacological properties	25-50 mM KCl	CaMKIV						(4)
		Promotion		50 mM KCl		Fox-1/A2BP1	skipping of exon 19	Increased nuclear isoform	P19	M	(8)
	E21	Repression	Membrane delivery	25-50 mM KCl	CaMKIV						(7)
		Repression		25-50 mM KCl		hnRNP A1	Increased nuclear level	Enhanced RNA binding	Cortical culture		(6)
PKC δ	E10	Promotion	Disruption of caspase-3 recognition of PKC,	Retinoic acid (RA)		SRSF2 (SC35)	Increased SC35 level		Primary hippocampa	H	(177)

			prosurvival						l neuron		
Slo	STREX	Repression	Increase of repetitive firing	Stress hormone (hypophysectomy)			N /A	Enhancing RNA binding?		R	(178)
		Repression	Higher sensitivity to Ca2+, voltage and glucocorticoid, inhibition by PKA and oxidation	25-50 mM KCl	CaMKIV	hnRNP L	Unknown		GH_3	R	(4,16)
		Promotion		25-50 mM KCl		Unknown	N /A		Cortical culture	R	(6)
Tau	E10	Promotion	Microtubule binding	forskolin	PKA-Cα	SRSF1	Phosphorylation of RS domains and RRM		HEK293T Brain	H R	(140)

Species: H, human; M, mouse; R, rat

3. Regulation of alternative splicing by membrane depolarization

Alternative pre-mRNA splicing can be regulated by changes in neuronal activity, such as those induced by cell membrane depolarization. Membrane depolarization is the process by which the intracellular side of membrane potential changes from negative to positive compared to its resting status due to influx of positively charged ions, or in rare case efflux of Cl^- (179,180). It triggers activation of various cell signaling pathways and controls numerous cellular processes and physiological functions (181,182). At the molecular level, this cellular activity induces extensive alterations in gene expression, mostly known at the level of transcription and protein modifications (183-186). In the past decade, its role in the regulation of alternative splicing has been receiving more attention, with better understood models mostly coming from the endocrine and nervous systems (4-9). Depolarization induces splicing changes mainly through Ca^{2+} signaling. Since recent reviews have described Ca^{2+}-regulated alternative splicing events, Ca^{2+}-responsive RNA elements and involved splicing factors (56,187), some general features and mechanisms underlying the control of alternative splicing by depolarization will be discussed here.

3.1 General characteristics of depolarization-regulated splicing

Depolarization induces cell signaling through Ca^{2+} and other second messengers; however, currently identified target exons are mainly controlled by Ca^{2+} signaling. The role of other second messengers such as the cAMP and the phosphatidylinositol are largely unknown, although a few studies suggest the involvement of the cAMP pathway (6,188).

Investigations of individual exons, database search for depolarization-responsive RNA elements, as well as microarray analyses identified numerous alternative exons regulated by depolarization (4-7,9,11,189,190). Most of the exons are encoded by genes functioning in calcium homeostasis, transportation of ions, intracellular signaling, vesicular transportation and synaptic functions, as well as metabolism and cell apoptosis. The genes encode ion channels, receptors, kinase, protein phosphatase, neuroendocrine secretory proteins, synaptic adhesion proteins, metabolic enzymes (microarray) and DNA/RNA binding proteins. Thus, depolarization-regulated splicing likely controls many cellular and physiological functions such as secretion of hormones and adaptation to a stimulus.

Data from exon array analysis also showed that depolarization induces changes in gene expression at whole transcript level and exon level, but dominated by the latter (189). Genes that exhibited splicing shifts are temporally regulated (189). Those changed at early time points and lasted to the late stage of depolarization are mostly Ca^{2+}-related genes. Similar effects were seen for genes encoding kinases and related proteins, which also showed expression changes at early time points. Changes in the expression of genes involved in metabolism, cell growth and apoptosis are following, while those regulating transcription and RNA processing even later. These seemingly sequential changes suggest finely temporal control of gene expression in response to depolarization, which appears fitting well with cell functioning and adaptation to stimuli.

Another interesting feature is that splicing changes induced by depolarization are reversible (7,8). For example, recovery of P19 cells in non-depolarizing media following KCl-treatment can restore splicing of E21 of the N-methyl-D-aspartate (NMDA)-

sensitive glutamate receptor 1 (NMDAR1) to its resting pattern (7). This suggests that splicing-related modulations by depolarization can be transient and stimulus-dependent. Moreover, splicing of some regulated exons can go back to resting patterns in a time-dependent manner during chronic depolarization, such as the NMDAR1 E5 (7). The mechanism underlying its "auto" recovery involves shifting of isoforms of the splicing factor Fox-1/A2BP1 by depolarization (8), which may reflect active adaptation to dynamic changes of environment. Details will be discussed in section 3.2.

Responsiveness of alternative exons can be depolarizing strength-dependent, such as KCl concentration or duration of treatment, in a gene/exon-dependent manner (7). For example, the NMDAR1 E21 was repressed more during prolonged incubation in depolarizing media (6 h vs 24 h) (7), while reduction of the stress axis-regulated exon (STREX) of the *Slo* gene showed no obvious change with KCl-treatments ranging from 6 h to 3 d (Observations by Guodong Liu).

3.2 Mechanisms underlying depolarization-regulated alternative splicing

Currently, two mechanisms are emerging; one of them is direct targeting of regulatory splicing components and the other coupled to chromatin remodeling and transcription. However, examples for each are limited, leaving the mechanisms largely unknown.

3.2.1 Depolarization induces splicing shifts through modulation of regulatory splicing components

Dissection of components transducing depolarization signals to splicing has identified the requirement for the L-type voltage-gated calcium channels (VGCCs) or the NMDARs by using pharmacologic inhibitors such as nifedipine, MK801 and AP5

(4,6,7). Elevation of cytosolic Ca^{2+} through the VGCCs or the NMDARs triggers downstream signal transductions mainly through the Ca^{2+}/calmodulin-dependent protein kinase (CaMK) pathway as confirmed by kinase inhibitors KN93 and its non-active analog KN92 (4,6,7). The CaMK pathway then induces splicing changes through modulating the interplay between regulatory RNA elements and splicing factors (4-7,9), which will be the major focus of this section.

By far, the best-characterized examples of depolarization-regulated splicing are controlled by the CaMKIV pathway (4-7,9). CaMKIV is a member of the Ca^{2+}/calmodulin-dependent Ser/Thr kinase family, mainly expressed in brain and thymus, but also detected in testis, spleen and ovary as well (191-199). CaMKIV is activated by depolarization through Ca^{2+}/calmodulin and needs CaMK kinases (CaMKK1 and/or 2) for full activation through phosphorylation of its Thr^{200} (in human; in mouse, Thr^{196}) (200,201). It is also negatively regulated by phosphatases such as calcineurin (202) and PP2A (203,204). CaMKIV is predominantly located in nuclei (205,206) and is well-known for its role in transcriptional control and protein modification through phosphorylation (207-215). However, its role in alternative splicing is just emerging during the past decade.

CaMKIV activated by membrane depolarization represses inclusion of many alternative exons including the STREX of the *Slo1* gene, E5 and E21 of the NMDAR1 gene, and E20 of the Neurexin 1 (Nrxn1) gene (4,5,7,9). Specific regulatory RNA elements have been characterized, while reported splicing factors are limited.

A pioneering example of depolarization-regulated alternative splicing through CaMKIV came from studies of the *Slo1* gene encoding the big conductance voltage- and

Ca^{2+}-activated potassium (BK) channel α subunit. This gene has, at least, 10 alternative exons (56). Disruption of the hormonal stress axis by hypophysectomy (pituitary removal) led to repression of two exons in the Ca^{2+}- sensitive domain at the COOH-terminal in chromaffin cells, with the longer exon of 174 nt named STREX (178).

Dissection of regulatory elements essential for repression of the STREX identified the first CaMKIV-responsive RNA element (CaRRE) in the 3' splice site upstream of the exon. The minimum sequence of this element is CACAUGGUUAU (for human, the 5^{th} nt is C) (Table 3), just preceding the 3' AG of the intron end. Interestingly, CaRRE-like elements were identified from other CaMKIV-targeted exons as well, including E5 and E21 of the NMDAR1 (4,5,7). These elements are located either in a position similar to that of the STREX (E5) or in the exon (E21). These three elements share the critical CACAY motif (Y is a pyrimidine) at their 5' ends (Table 3) and are called type 1 CaRRE (CaRRE1). Another purine-rich element showing little similarity to CaRRE1 (Table 3) is isolated at the 5' end of the NMDAR1 E21 and is called CaRRE2 (7).

Table 3. Known and potential members of the CaRRE family

Type	Sequence	Location	Regulated exon/gene	Reference
CaRRE1	CACAUGGUUAU	Intron	STREX/Slo	(5)
	CACAUUAUUCAU	Intron	E5/NMDAR1	(7)
	CACAUUUAGGGC	Exon	E21/ NMDAR 1	
CaRRE2	GUGGUAGAGCAG	Exon	E21/ NMDAR 1	
Other	UAGG	Exon	E21/ NMDAR 1	(6)
	GGGG	Intron	E21/ NMDAR 1	
	UAAA/AU-rich	Intron	E20/Nrxn	(9)

Effects of CaRRE1 variants depend on their sequence context, copy number and location (7). Besides, CaRRE1 and CaRRE2 may cooperate with each other because introduction of both CaRREs of the NMDAR1 E21 led to a complete skipping of a heterologous exon even without CaMKIV, which is otherwise constitutively included (7). Similar cooperation is also seen for the CaRRE1 and an exonic polypyrimidine sequence of the STREX (4). Interestingly, the communication between CaRRE1 and CaRRE2 seems sequence context-dependent as well because replacement of the CaRRE1 in the NMDAR1 E21 with the one for E5 only partially restored exon repression (5,7). Although it can function in either the 3' splice site or in exon, the CaRRE1 near the intronic 3' AG shows stronger effect (7), which may suggest mechanisms for finely tuning of splicing in response to depolarization/CaMKIV.

Searches in the human genome database or the mouse alternative exon datasets found many more alternative exons containing or flanked by CaRRE like elements (5,7). Some of them are repressed by both depolarization and CaMKIV, such as E5a of the Snap25 (25-kD synaptosomal-associated protein) (5,216-218). Importantly, the CaRRE like elements conferred CaMKIV-repression of heterologous exons in splicing reporters (4,5,7,16). These suggest the CaRREs may represent a category of elements that are critical for depolarization-regulated exons. However, the presence of CaRREs may not be always predictive of directions of splicing. For example, inclusion of STREX is decreased by KCl-depolarization in both pituitary GH_3 cells and cerebellar neurons (4,5), but increased in primary cortical culture (6). Similarly, the NMDAR1 E5 is repressed by CaMKIV in KCl-stimulated GH_3 cells (4), but further decreased in primary cerebellar neurons from CaMKIV heterozygous knockout mice (5). By contrast, inclusion of the

NMDAR1 E21 increased as expected in the same knockout mice. These also suggest cell-context effect and complex control of the exons by multiple regulators.

Regulatory factors mediating depolarization-regulated splicing through CaRRE1 and 2 are not clear yet. One candidate regulator is hnRNP L, which is based on the CA repeats in the CaRRE1 of STREX and will be discussed later.

Besides the CaRRE1 and CaRRE2, a few known regulatory RNA elements also mediate depolarization-induced splicing likely through CaMKIV and other splicing factors. These elements include two UAGG motifs in the NMDAR1 E21 (Note that this exon was called C1 exon by the authors) (6) and the UAA(A) motifs in the flanking introns of the Nrxn1α E20 (9).

The two UAGG motifs are in the 3' half of the NMDAR1 E21. Mutation at either motif clearly reduced E21 repression by overexpressed CaMKIV in splicing reporter assays (7), suggesting the UAGG motifs as potential targets of CaMKIV. The effect of the UAGGs is likely mediated by hnRNP A1 (6,188). Depolarization induced stronger binding of hnRNP A1 to the RNA probe containing UAGG motifs, which is possibly due to increased nuclear protein level (6). However, role of phosphorylation of hnRNP A1 is also implicated (188). Therefore, it is possible that depolarization induces repression of E21 partly through enhanced hnRNP A1 binding to its target sites.

Neurexins are synaptic adhesion molecules encoded by three genes, Nrxn1~3 (219,220). The Nrxn1α E20 is flanked by intronic AU-rich sequences containing UAAA motifs that are Sam68 high affinity sequence (221). It is repressed by KCl-depolarization through CaMKIV and Sam68 (9). Interestingly, phosphorylation of the Sam68 Ser20, a potential target for CaMKs (R-X-X-S/T), dramatically increased upon depolarization (9),

suggesting CaMKIV regulates splicing of Nrxn1α E20 by targeting Ser20 of Sam68. However, a direct functional link between the phospho-Ser20 and splicing is still missing. Depolarization also induced repression of the Nrxn2α E11 and the Nrxn3α exons 11 and 20 through the PKC/Rho-associated protein kinase (ROCK) pathway that can modulate Ca^{2+} sensitivity of the contractile system (11,190). However, whether this regulation is independent of CaMKIV and what splicing factors are involved are unknown.

Therefore, the depolarization/CaMK pathway can regulate alternative splicing likely through modulations of nuclear abundance and/or phosphorylation status of regulatory splicing factors.

The variant isoforms of a splicing factor can also be regulated by depolarization to control splicing of other transcripts. In differentiated mouse P19 cells, the NMDAR1 E5 was repressed by CaMKIV in the first 6 hours of incubation in depolarizing-KCl culture media and then started to increase during extended incubation (8). Recovery of its level is mediated by the splicing factor Fox-1/A2BP1 through an intronic motif UGCAUG (84,222-225) close to the 5' splice site of E5. Fox-1 is a neuronal splicing factor whose target genes play important roles in neuronal development (223,225,226). Its alternative exon E19 is repressed by depolarization through the CaMK pathway, generating a nuclear isoform of the Fox-1. Subsequent accumulation of the nuclear Fox-1 overcomes repression of the NMDAR1 E5 by CaMKIV and leads to recovery of its inclusion (8). Interestingly, inclusion of the NMDAR1 E5 decreased in the CaMKIV heterozygous knockout mice (5), an effect opposite to that in GH_3 cells, which may be explained by cell-context effect. However, another possibility is that repression of the Fox-1 E19 is disrupted due to insufficient CaMKIV activity in the knockout mice. Consequently, the

cytoplasmic isoforms of Fox-1 dominate its nuclear isoforms, which may lessen the promoting strength for the NMDAR1 E5 and eventually lead to its further repression. Nevertheless, these provide a model, in which reversible splicing is mediated through splicing switch and protein relocalization of a regulatory splicing factor induced by depolarization. This also suggests a mechanism for cells, particularly for neurons, to keep homeostasis of proteins with important functions.

These examples indicate that depolarization changes splicing mainly through the CaMK pathway, particularly CaMKIV. However, other studies suggest that the PKA pathway is also involved (6,188). H89, an inhibitor of PKA downstream of the cAMP signaling, reduced hnRNP A1 binding to the NMDAR1 E21 depolarization-induced repression of the exon in primary cortical cultures (6). Overexpressed PKA but not its dominant-negative mutant induced repression of E21 in a splicing reporter minigene (188). Therefore, it is likely that PKA also regulates splicing of this exon. Moreover, binding of hnRNP A1 to a high affinity RNA probe was enhanced upon its phosphorylation by PKA *in vitro* (188). The Ser199 of hnRNP A1 is a phosphorylation target of several kinases including PKA and PKCζ (227,228). Its phosphorylation was increased by overexpressed PKA in neuroblastoma cells or by KCl-depolarization in primary neurons (188). It is also implicated that phosphorylation of the Ser199 may increase hnRNP A1 protein stability and RNA binding activity (229). Therefore, it is possible that PKA regulates E21 splicing by enhancing hnRNP A1 activity through phosphorylation of its Ser199. However, the direct functional link between Ser199 phosphorylation and RNA binding as well as splicing remains to be verified. Interestingly, the effect of PKA seems downstream of CaMKIV because coexpression of

constitutively-active CaMKIV and the PKA mutant caused less exon repression compared to coexpression with active PKA (188). However, whether it happens under depolarization is not known. On the other hand, a more specific PKA inhibitor KT5720 had little effect on depolarization induced repression of E21 (6). Therefore, the role of PKA in depolarization-stimulated splicing remains to be verified.

In summary, a major signaling pathway in depolarization-regulated splicing is the CaMKIV pathway that modulates splicing through various regulatory elements. Identified splicing factors are few and can be regulated by depolarization through various mechanisms, likely including phosphorylation, protein level control, shifts of protein isoforms and changes of protein localization. Roles of other signaling pathways downstream of depolarization and other splicing factors are under study.

3.2.2 Role of chromatin and transcription

Neuronal activities, such as membrane depolarization, regulate gene expression by not only posttranslational modification and recruitment of transcription factors (230-234) but also remodeling of chromatin structures through modifications of histones (182,235).

A recent study on the neural cell adhesion molecule (NCAM) gene demonstrated such a connection between depolarization-induced chromatin remodeling and alternative splicing (236). Inclusion of the NCAM E18 decreased upon KCl-depolarization in neuroblastoma N2a cells and primary hippocampal neurons, which was not affected by the CaMK inhibitor KN93 in the presence of the protein synthesis inhibitor cycloheximide (CHX). However, the repression of E18 is abolished upon inhibition of transcription, suggesting association of splicing with transcription. This link is strengthened by a recombinant “slow” RNA Pol II that promoted E18 inclusion in

minigenes, which appears to fit with the kinetic model that elongation rate of RNA pol II inversely regulates splicing (237). Therefore, E18 repression is associated with processivity of RNA pol II. This is related to modifications of chromatin by depolarization-induced local hyperacetylation of H3K9 in the chromatin region spanning from E17 to the end of E19 end as shown by native chromatin immunoprecipitation (nChIP) (236). This is also supported by inhibition of HDAC with trichostatin A (TSA), which mimicked the depolarization-repression of E18. Moreover, increased level of H3K36m3, which preferentially marks expressed exons (146), was observed in the same area (236). These changes are expected to cause relaxation of flanking chromatin, which is thought to be more compact under the resting condition based on the nCHIP results (236), and increased accessibility of this region to transcription. Consequently, accelerated transcription at this region is expected to skip E18.

However, similar increases in histone acetylation and methylation were observed in the upstream E17 as well (236), which is also supposed to induce higher accessibility of this region to transcription. Then, why is E18 skipped but not E17? One possibility could be that transcription is not fully accelerated at the E17 region and splicing can still catch up with the transcribed region. On the other hand, although repression of the NCAM E18 by depolarization was not affected by CaMK inhibitors along with protein synthesis inhibitors, the response was partially reduced by CaMK inhibitors alone (236), suggesting other mechanisms may be involved as well. Interestingly, H3K36m3 can regulate chromatin status-coupled splicing by recruiting splicing factors. As discussed, H3K36m3 in the FGFR2 EIIIb region triggers exclusion of EIIIb through PTB recruited via a histone binding protein in mesenchymal cells (145). It might be interesting to

investigate whether depolarization-induced H3K36m3 in the NCAM gene could recruit some splicing repressor(s).

3.2.3 Regulation by a candidate factor hnRNP L

Of particular interest to us is whether hnRNP L is a mediator of depolarization-regulated splicing and how.

HnRNP L was initially identified from HeLa cells as a hnRNP component of 64~68 kDa separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (238). HnRNP L contains four RRMs, two N-terminal glycine-rich domains, as well as two proline-rich domains upstream of RRM3 (102). It binds preferentially to CA repeats or CA-rich sequences (82,239). Its RRM2 is critical for binding both CA repeats (CA>10) and CA-rich sequences, while RRMs 3 and 4 prefer CA-rich sequences (240). It is also found that the region containing RRM2 is required for protein-protein interactions (241).

HnRNP L is a multi-function factor participating in several processes of RNA metabolism, including transcription (242), splicing, polyadenylation (poly (A)) site selection (243,244), nuclear export of intronless RNA (243,245), mRNA stability (239,246,247) and translation (124). It has been implicated in cancer and spermatogenesis (248,249), which likely correlates with its role in regulating alternative splicing and mRNA stability of growth/apoptosis-related genes (68,250,251). Recently, it is also found that hnRNP L is involved in differentiation and migration of T cells during development in knockout mice (252).

A better characterized function of hnRNP L is as a splicing regulator. The first link between hnRNP L and splicing was established based on its role in the splicing of the

human endothelial nitric oxide synthase (eNOS) gene that contains CA repeats of variable lengths ($(CA)_{14–44}$) in the intron 13 (114). HnRNP L is the major protein UV cross-linked to a RNA probe carrying $(CA)_{32}$ repeats and promoted *in vitro* splicing of a constructed eNOS RNA substrates containing intronic $(CA)_{32}$ (114). Thereafter, a number of CA-repeat/CA-rich sequence containing genes were identified as targets of hnRNP L in alternative splicing (16,82,244,250,253). The hnRNP L-like (hnRNP LL) protein, a paralogue of hnRNP L, also functions as a splicing regulator (135,254). Interestingly, the two paralogues are essential splicing factors in signal-induced splicing of several genes, such as the CD45 exons 4-6 (135,159).

Recently, hnRNP L was also implicated as a regulator of depolarization-induced splicing (16). Our laboratory demonstrated that hnRNP L binds the mouse CaRRE1 for the STREX in nuclear extracts in a CA-dependent way and is an essential component in CaMKIV-induced splicing through CA repeats (16). Moreover, hnRNP L is a candidate regulator for the depolarization-induced repression of STREX. Considering the high prevalence of CA repeats in the human genome (82) and the CA-rich contents of many CaRRE1s (5,7), it is possible that hnRNP L regulates splicing of a range of genes responsive to depolarization.

Interestingly, hnRNP L is also suggested in cotranscriptional alternative splicing (255,256). It was identified as an essential component of the human histone methyltransferase 3a (KMT3a/Set2) complex *in vivo* (255). The KMT3a mediates methylation of the histone H3 lysine 36 (H3K36) which is associated with transcriptional activation (146) and alternative splicing (236). Downregulation of hnRNP L resulted in reduction of intragenic H3K36me3 level (256). Moreover, hnRNP L interacts with the

Mediator complex subunit 23 (MED23) (256), a component of the Mediator complex that regulates transcription. This can modulate hnRNP L interacting with promoters of the genes whose alternative exons are regulated by L and MED23 synergistically, suggesting that the two factors can coordinately regulate alternative splicing in a promoter-dependent way (256). Therefore, hnRNP L can function as a linker between transcription and splicing. Since the cotranscriptional splicing of the NCAM E18 also associates with increased H3K36me3 level (236), it thus would be interesting to investigate if hnRNP L could mediate depolarization-induced cotranscriptional splicing.

Mechanisms underlying hnRNP L regulated splicing are emerging. HnRNP L can either repress or activate splicing (82,244,257,258). It represses splicing through blocking recognition of splice sites. When it binds to the 3' splice site, hnRNP L disrupts binding of U2AF65; otherwise, it interferes with recognition of the 5' splice site by U1 snRNP (257). However, hnRNP L can also promote splicing when binding to the 5' splice site, such as the eNOS mentioned above (114). Moreover, hnRNP L repressed the CD45 E5 through ESSs by antagonizing the effect of a flanking ESE, but promoted this exon when its 5' splice site is weakened and the ESE was removed (258). Therefore, hnRNP L can regulate splicing in a context-dependent way.

On the contrary, limited evidence is available for whether and how hnRNP L itself is regulated in the control of splicing, especially for those responsive to cell signaling. It was reported that hnRNP L autoregulates its protein level by manipulating the usage of a small exon E6A that can lead to NMD. This is likely a mechanism for the homeostasis of hnRNP L in cells (136). A few recent studies provided clues for posttranslational modifications of hnRNP L. Treatment of imatinib-insensitive chronic myeloid leukaemia

cells with imatinib and the deacetylase inhibitor valproic acid, which could partially restore sensitivity of these cancer cells to the BCR/ABL tyrosine kinase inhibitor imatinib, led to increased acetylation of hnRNP L (259). Phosphorylation of hnRNP L during differentiation of embryonic stem cells was also observed (260). Recently, a role of hnRNP L phosphorylation in splicing control has been implicated. For example, increased phosphorylation at serine, threonine and tyrosine sites has been observed in the lung cancer cell lines A549 and H838 in comparison with the nontransformed HBEC-3KT cells (68). Screening by site-directed mutagenesis identified a critical phosphorylation site Ser52. Mutation of the Ser52 to alanine dramatically diminished repression of the exon 3-4-5-6 cassette of caspase 9 in A549 cells, resulting in a significant increase of the proapoptotic caspase 9a (68). However, the upstream kinase(s) that phosphorylates hnRNP L is unknown. Another study suggested that increased phosphorylation of hnRNP L may be partially responsible for repression of CD45 exon 4 upon T cell activation (261). Our recent study showed that L binding to the CaRRE1 element was increased by CaMKIV and decreased by phosphatase treatments (16), which also suggests a phosphorylation-dependent mechanism. However, more intensive studies are needed to verify and better characterize regulation of hnRNP L in depolarization-induced alternative splicing.

In summary, the role of hnRNP L in depolarization-regulated splicing is just emerging. Its target exons upon depolarization and underlying molecular bases remain to be investigated.

3.2.4 Future directions

So far, most depolarization-induced alternative splicing events are only partially characterized and thus underlying mechanisms remain to be uncovered. The CaMKIV signaling is a major pathway downstream of depolarization (4-7,9), while the role of other signaling pathways remains to be defined.

The CaMKIV-targeted RNA elements that have been identified include the original CaRREs and other known regulatory elements with different sequence contents (4-7,9), indicating that the RNA elements responsive to CaMKIV are diversified. Besides, the presence of a CaRRE like element near an exon does not necessarily confer its response to depolarization (7), presumably due to gene- and/or cell-context effects. This complicates prediction of co-regulated genes based on the RNA elements. Therefore, better characterization of the requirement for the regulatory elements will be helpful for identifying co-regulated genes by depolarization.

So far, only a few regulatory splicing factors have been identified as mediators of depolarization-induced splicing changes (6,8,9). How many other factors are also involved needs further investigation. Moreover, answers for how trans-acting factors themselves are regulated in the induced-splicing events are important for understanding splicing control by depolarization.

Mechanisms for depolarization-induced cotranscriptional alternative splicing are just emerging (236). How chromatin remodeling induced by depolarization regulate splicing is not fully understood. Also, the observations that transcription factors can bind RNA and splicing factors can modulate epigenetic modifications of chromatin and transcriptional elongation (145,156) suggest extensive communications between the

transcription and the splicing machineries. How the communications can be unraveled requires further studies.

Since the CaRREs are found in many genes based on database search and can be predictive of splicing in response to depolarization (5,7), it would be interesting to investigate whether functionally related genes can be defined through a specific element. This could help explain how a cellular function may be controlled in response to cell activity changes.

4. Physiological/biological significance of depolarization-controlled alternative splicing and relationship to diseases

4.1 Physiological/biological significance

Excitable cells, such as neurons and endocrine cells, respond to extracellular stimuli by exerting certain functions and/or adapting to changes. An important part of their responses involves regulated alternative splicing of genes targeted by stimuli including membrane depolarization (4-7,9). Through the modulation of alternative splicing of important molecular components, such as ion channels and neurotransmitter receptors (4-7), depolarization induces and regulates many physiological functions. Some examples are discussed in this section.

4.1.1 Fine-tuning of electrical property

Electrical properties of excitable cells are mainly controlled by ion channels such as the L-type VGCCs and the BK potassium channels (262-264). The L-type VGCCs mediate fast Ca^{2+} influx and are critical for Ca^{2+} signaling (265). BK channels activated by Ca^{2+} signaling take part in repolarization of cell membrane, and drive fast afterhyperpolarization (AHP), which affects firing patterns of excitable cells (56).

Therefore, they both are important for electrophysiological properties of cell membranes. Their activities are controlled by multiple mechanisms including regulated alternative splicing of their pore-forming α subunits. For example, the *Slo* gene encoding the BK α subunit contains at least 10 alternative exons and can potentially produce more than 600 splice variants in total (56). The α1 subunit of the L-type VGCC $Ca_v1.2$ contains at least 56 exons with 20 of them are found to be alternatively spliced (266), which could produce even more splice variants. Their alternative exons, such as the STREX of the *Slo1* gene and E9* of the $Ca_v1.2$ α1 gene, can be regulated by depolarization (4,5,7). The STREX-encoded peptide resides in the Ca^{2+}-sensing cytosolic domain of the BK α subunit (267,268). Inclusion of the STREX increases sensitivity of the BK channel to Ca^{2+}/voltage, inhibitory modifications and other stimuli (56). The E9* peptide locates in the cytoplasmic linker region between the repetitive domains I and II of the $Ca_v1.2$ α1 subunit (269), an area responsible for electrophysiological property of the channel subunit.

Differential usage of alternative exons will produce channel isoforms with different properties. When these various isoforms assemble into functional tetramers, a large numbers of channels with differential activities will be produced, providing a critical mechanism for the fine-tuning of channel functions (56). For example, E9* of the $Ca_v1.2$ regulates voltage-dependent activation of its E1b-containing subpopulation in cerebral arteries (270) and is critical for constriction of cerebral arteries (271). More strikingly, the pore-forming tetramers of the BK channel are composed of α subunits, which could potentially generate more than 8 billion types of tetramer channels (56). This could already produce an impressively fine spectrum of BK channel activities, which would

confer a globally fluent response to a large range of stimuli such as a gradient of Ca^{2+} concentration (56). Physiological significance of such elegant modulations of electrical properties is best demonstrated by the frequency tuning of hearing sense organs. In lower vertebrates such as turtles and chicks, frequency analyses in the cochlea mainly depend on the abundance and variable kinetics of BK channels in hair cells positioned along the tonotopic axis of the epithelium (56,272). The presence of numerous splice variants of the *Slo* is suggested as a determinant for the BK dynamics.

As discussed, splicing shifts can be stimulus strength-dependent, such as with varying KCl concentrations (7). Therefore, the effects of alternatively spliced ion channels can be further strengthened by ratio tuning of splice variants through varying intensities of signals regulating alternative splicing (56). This mechanism is particularly important for individual cells expressing limited types of splice variants.

Thus, these dynamic modulations of channel composites and activities, together with posttranslational modulations as well as accessory subunits, provide the fine-tuning of electrical properties of excitable cells.

4.1.2 Control of Ca^{2+} homeostasis and cell functions

Intracellular Ca^{2+} level is important for normal cell functions and response to extracellular stimuli. The homeostasis of intracellular Ca^{2+} is tightly controlled through functional modulations of membrane proteins mediating Ca^{2+} flux such as VGCCs, NMDARs and Ca^{2+} pumps (273). Alternative splicing is possibly a major contributor for their activity control. Alternative exons of these proteins can be regulated by membrane depolarization (5,7,8,274). Regulated usages of these exons by depolarization produce differential functions that affect Ca^{2+} movements across the cell membrane. For example,

inclusion of the above $Ca_V1.2$ α1 E9* shifts activation of the channel to a more negative potential, and thus affects its gating property (275). Interestingly, the $Ca_v1.2$ E33 encoding functions opposite to E9* is concomitantly regulated during development of cortical neurons, but in an opposite direction (266). However, whether this regulation is also applied to depolarization-stimulation needs to be verified. Ca^{2+} influx is also controlled through modulating membrane presentation of the NMDAR1 by alternative usages of its exons responsible for channel transportation from the endoplasmic reticulum (ER) to the plasma membrane (7). Moreover, its pharmacological property, gating and distribution are also regulated by splicing of its E5 (7,276). Removal of Ca^{2+} from cells is also regulated by modulating activity of Ca^{2+} pumps. For example, E21 of the Ca^{2+} pump Atp2b1 encoding the domain for its inhibition by calmodulin is repressed by depolarization and thus expected to promote its Ca^{2+}-removal efficiency (7).

The regulated usage of the differential splice variants of these and possibly many other Ca^{2+}-transportation related proteins is therefore an important mechanism for the maintenance of intracellular Ca^{2+} levels. This is critical for many cell functions including synaptic activities. Moreover, the adjustment of the functional isoforms through alternative splicing also represents a molecular basis for cellular adaptation to environment stimuli.

4.1.3 Regulation of cellular secretion

Pituitary cells can secret growth hormone and prolactin spontaneously, or in response to stimuli such as KCl-depolarization, which is mainly Ca^{2+} signaling-dependent (13). The secretion correlates with sustained plateau bursting of action potentials (13,277). BK channel activities are required for generation of the plateau

bursting (13,278). Consistent with this, BK channels are highly expressed in somatotrophs and lactotrophs that secret growth hormone and prolactin respectively, but are low in ganadotrophs (13). Depolarization shifts splicing patterns of its α subunit *Slo* gene and thus induces production of BK channels with differential activities (56). This could change plateau bursting patterns of pituitary cells and consequently modulate secretion of hormones.

Proteins participating in exocytosis of synaptic vesicles and secretory granules are important for secretion of neurotransmitters and hormones, such as Snap25 (12). Snap25 is a key component of the complex called soluble NSF (Nethylmaleimide-sensitive fusion protein) accessory protein (SNAP) receptor (SNARE), which attaches releasing vesicles to plasma membrane for fusion and secretion (12). It has two SNARE domains linked by a region containing four palmitoylated cycteine residues by which it attaches to the plasma membrane. Snap25 has two splice variants through its mutually exclusive exons 5a and 5b which differ in 9 amino acids located in the N terminal SNARE domains and the linkers (217). The two splice variants are functionally close but different in their abilities to maintain stable vesicles through tuning of the interaction of the SNARE complex with other auxiliary factors (12). They undergo developmental transition from Snap25a to Snap25b, a change that is induced similarly by depolarization as well. The switch of the two isoforms affects short-term synaptic function (279), which may contribute to the fine-tuning of activity-dependent neurotransmitter release through exocytosis.

4.2 Emerging role of depolarization-regulated alternative splicing in disease

As mentioned, depolarization stimulates signaling transduction mainly through Ca^{2+}

(4-9). Maintenance of intracellular Ca^{2+} is critical for normal physiological functions of many biological systems such as the nervous, muscular and endocrine systems (280). Disturbance of intracellular Ca^{2+} level or Ca^{2+} signaling can lead to dysfunctions and diseases, such as neuronal death due to excess intracellular Ca^{2+} or lacking of CaMKIV (56,281), as well as neurodegeneration (273).

Therefore, it is important to maintain normal functions of the components controlling Ca^{2+} flux such as voltage- and ligand-gated Ca^{2+} channels and Ca^{2+} pumps. Deregulation of their functions may disrupt depolarization/Ca^{2+}-regulated molecular events including alternative splicing, which may cause diseases. For example, aberrant alternative splicing of VGCCs has been found to affect channel conductance, expression of subunits and possibly integrity of channels, which associate with many diseases (282-284). Interestingly, the depolarization-regulated $Ca_v1.2$ E9* is important in determine the vascular diameter, and altered splicing of E9* has been observed in disorders of the cardiac system (271). Functional NMDARs are heteromeric complexes consisting of NMDAR1 and NMDAR2 subunits (285). Various variant isoforms of the subunits produced by alternative splicing contribute to the modulation of receptor properties. For example, the NMDAR1 E5 modulates the sensitivity of the receptor to pH. NMDARs incorporated E5-containing isoforms are less sensitive to inhibition by proton and Zn^{2+} (285). Consistently, depolarization induces repression of this exon, which promotes the production of NMDARs more sensitive to inhibition, and thus, prevents (7) excessive Ca^{2+} influx. Therefore, level of the E5 isoform needs to be well controlled to ensure proper functions. Abnormally high level of E5-containing isoform of the NMDAR1 was

observed in the brain of myotonic dystrophy type 1 (DM1) patients, which is possibly associated with damaged memory in DM1 (276).

The disrupted expression of other ion channels is also implicated in neuronal diseases. Enhanced BK potassium channel conductance due to a genetic mutation of the α subunit gene is suggested to induce increased excitability of neurons in human epilepsy and paroxysmal dyskinesia disorder (286). In a rat status epilepticus model, the overall expression of the BK channel decreased; however, the ratio of STREX-containing α subunit increased temporally (287). This shift is thought to possibly compensate for the reduction of the BK channel level because the STREX enhances Ca^{2+} sensitivity and thus activity of the channels, which may be associated with the neuronal hyperexcitability in the model animal brains (287). Therefore, deregulated expression of the STREX due to disrupted neuronal activities is a potential contributor of nervous disorders.

Considering that many genes regulated by depolarization encode proteins with important functions including Ca^{2+} homeostasis, metabolism and growth/death, it can be speculated that deregulation of their alternative splicing may lead to disruptions of normal physiological functions and disease. As an area under study, characterization of splice variants associated with or regulated by depolarization, and their relationship with diseases will provide important insights into the mechanisms underlying the diseases, and benefit development of new therapeutic strategies.

5. Summary

In summary, depolarization controls splicing through the modulation of interactions between regulatory RNA elements and trans-acting splicing factors and the induction of chromatin remodeling. Their molecular bases are only partially answered. Verified and

potential CaMKIV-responsive RNA elements are diverse, suggesting the need of further characterization of CaRREs. Investigations of the involved splicing factors and their regulation by depolarization as well as the cotranscriptional regulation are undergoing and will be a major focus for future studies. Answers to these questions can provide deeper insights into the molecular basis of Ca^{2+} signaling-associated physiological functions and will help understand mechanisms underlying related-diseases.

CHAPTER II

Materials and Methods

Materials

Mammalian cell lines

GH_3	rat pituitary tumor cells
HEK293T	human embryonic kidney cells
PC12	rat pheochromocytoma cells

Materials for cell culture

Gibco® Dulbecco's Modified Eagle Medium (D-MEM) powder	Invitrogen
F-10 Nutrient Mixture (Ham) (1X), liquid	Invitrogen
Fetal Bovine Serum (FBS)	Invitrogen & Sigma
Horse serum	Invitrogen
Iscove's Modified Dulbecco's Medium (IMDM), liquid	Hyclone
Gibco® Newborn Calf Serum (NCS)	Invitrogen
Gibco® Penicillin-Streptomycin, 100X Solution	Invitrogen
L-Glutamine, 200mM Solution	Invitrogen
Trypsin, 2.5%	Cellgro
Cell culture plate	BD FALCON
DMSO	Fisher

***E.coli* strains**

DH5α	A gift from Dr. Peter A Cattini's lab
Rosetta-gami 2 (DE3) pLysS	Novagen

Antibodies

Name	Source
Anti-β-actin (AC-74)	Sigma-Aldrich
Anti-β-actin (C4)	Santa Cruz
Anti-c-Myc	Clontech
ANTI-FLAG® M2 (F1804)	Sigma-Aldrich
Anti-hnRNP L (4D11)	Santa Cruz
Anti-hnRNP LL (#4783)	Cell signaling
Anti-hnRNP K (3C2)	Santa Cruz
Anti-hnRNP F/H (1G11)	Santa Cruz
Anti-C23 (Nucleolin) (H-6)	Santa Cruz
Anti-Ser16-PTB	Xie lab
Anti-phospho-Ser16-PTB	Xie lab
Goat anti-Mouse IgG (Fc specific)-Peroxidase	Sigma-Aldrich
Goat anti-rabbit IgG-HRP	Santa Cruz

Chemicals and reagents

Name	Source
1-Butanol	Fisher
2-mercaptoethanol	Sigma-Aldrich
Acrylamide: Bis-Acrylamide 19:1	Fisher
Acrylamide: Bis-Acrylamide 29:1	Fisher
Agarose-GPL/LE	American Bioanalytical
Ammonium persulfate (APS)	Fisher
Ampicillin	Fisher
Bovine serum albumin, RNase free	Fisher
Brilliant Blue G-250	Fisher

Bromophenol blue	Fisher
Calcium chloride	Fisher
Coomassie brilliant blue R250	Fisher
Dimethyl Sulfoxide (DMSO)	Fisher
Dithioreitol (DTT)	Fisher
Ethanol (95%)	Fisher
Ethidium bromide	Sigma-Aldrich
Ethylenediamine Tetraacetic Acid (EDTA)	Fisher
Gibco®) D-Glucose	Sigma-Aldrich
Glacial acetic acid	Mallinckrodt AR
Glycerol	Fisher
Glycine	Fisher
IGEPAL® CA-630	Sigma-Aldrich
Imidazole	Fisher
Instant skim milk powder	Nestle
Isopropanol	Fisher
Isopropyl 1-thio-β-D-galactoside (IPTG)	Fisher
Kanamycin	MP Biochemicals
Magnesium chloride	Fisher
Methanol	Fisher
N-(2-Hydroxyethyl)piperazine-N'-2-ethanesulfonic Acid (HEPES)	Fisher
N,N,N ′,N ′-tetramethylenediamine (TEMED)	Fisher
phenol/chloroform	Fisher
Phenylmethylsulfonyl fluoride (PMSF)	Fisher
Phosphate Buffered Saline (PBS) Tablet	MP Biomedicals
Polyoxyethylene-20-sorbitan Monolaurate (Tween 20)	Fisher
Poly(ethylene glycol), M.W. 8000 (PEG8000)	Sigma-Aldrich

Ponseau S	Fisher
Potassium chloride (KCl)	Fisher
Sodium chloride (NaCl)	Fisher
Sodium dodecyl sulfate (SDS)	Fisher
Sodium Orthovanadate (Na_3VO_4)	Fisher
Tris base	Fisher (J.T. baker)
Triton X-100	Fisher
tRNA from yeast	Sigma-Aldrich
Urea	Fisher
Xylene Cyanole FF	Fisher

Enzymes and buffers

Product	**Source**
1x dephosphorylation buffer	Invitrogen
5x First strand buffer	Invitrogen
5x Forward buffer	Invitrogen
AgeI	NEB
ApaI	Invitrogen
BamHI	Invitrogen
BbsI	NEB
BglII	Invitrogen
Calf intestinal alkaline phosphatase (CIAP)	Invitrogen
dNTP set	Invitrogen
DTT, 0.1M	Invitrogen
GST-CaMKIV	BioMol
M-MLV Reverse Transcriptase	Invitrogen
Restriction endonucleases and buffers	Invitrogen
RNA guard	GE Health

RNase inhibitors	BIO BASIC INC
RQ1 DNase 10X Reaction Buffer	Promega
RQ1 DNase Stop Solution	Promega
RQ1 RNase free Dnase	Promega
SuperScript™ III Reverse Transcriptase	Invitrogen
T4 DNA ligase	Invitrogen
T7 RNA polymerase	Xie lab
Taq DNA polymerase	Xie lab
TPCK-trypsin	Sigma-Aldrich
TLCK-chymotrypsin	Sigma-Aldrich

Kits

Kit	**Source**
GenElute Mammalian Total RNA Miniprep Kit	Sigma-Aldrich
QIAquick Gel Extraction Kit	QIAGEN
Plasmid Mini Kit	QIAGEN
Plasmid Midi Kit	QIAGEN
QIAEX II Gel Extraction Kit	QIAGEN

Others

Product	**Source**
Filter (0.22 μm)	NALGENE
Lipofectamine 2000	Invitrogen
PolyJet™ DNA In Vitro Transfection Reagent	Signagen®
polyvinylidene difluoride membrane	Millipore
protein G sepharose beads	Pierce
Ni-NTA agarose beads	Invitrogen

Novex® 4–20% Tris-Glycine gel	Invitrogen
TLC plate	EMD Chemicals
Western blotting detection reagent	GE Healthcare

Buffers and solutions

10x Tris-buffered saline (TBS)

Tris ·HCl, pH 7.5	20 mM
NaCl	500 mM

10x PCR buffer

Tris ·HCl, pH9.0	100 mM
KCl	500 mM
Triton X-100	1% (v/v)

PCR mix (for 100 x 12.5µl-reactions)

10x PCR buffer	125 µl
100mM dNTP	2.5 µl each
25mM $MgCl_2$	75 µl
H_2O	872 µl

Use 10.82µl for each 12.5µl PCR reaction

Chromatography solution

1-Butanol	37.5 ml
Glacial acetic acid	7.5 ml
Pyridine	25 ml
H_2O	30 ml

DG buffer

Hepes-KOH (pH 7.9)	20 mM
L-glutamic acid-potassium	80 mM
Glycerol	20% (v/v)
EDTA	0.2 mM

KRG buffer

Hepes-KOH (pH 7.5)	10 mM
$CaCl_2$	2.1 mM
NaCl	128 mM
MgSO4	29 mM
KCl	5.2 mM

NP-40

20% IgePal	0.75 ml
1M Tris.Cl, pH7.6	0.5 ml

4M NaCl	1.875 ml
0.5M EDTA, pH8.0	0.1 ml
H_2O	~46.775 ml

Radio immunoprecipitation assay (RIPA) buffer

Tris HCl, pH 7.6	50 mM
NaCl	150 mM
Triton X-100	1% (v/v)
DOC (Deoxycholic acid)	0.5% (w/v)

TB solution

Pipes	6.05 g
$CaCl_2$ (Without H_2O)	4.41 g
KCl	37.28 g

Methods

Plasmid construction. The plasmids, Flag-CaMKIV-dCT (CaMKIV or IV), its dominant negative mutant -dCTK75E (CaMKIVm or IVm, kinase-dead) and Flag-PKA (A), are as described (4,288). In the Flag-PKA mutant (Am), the Lys73 and Lys169, phosphate-binding residues (289), were mutated to Glu73 and Glu169, respectively (numbered according to NCBI protein accession no. AAA39936, corresponding to Lys72 and Lys168 in the previous report In the Flag-PKA mutant (Am), the Lys73 and Lys169, phosphate-binding residues (289), were mutated to Glu73 and Glu169, respectively (numbered according to NCBI protein accession no. AAA39936, corresponding to Lys72 and Lys168 in the previous report (289)), using primers FlagPKA1 (5′-CGGGATCCGCCATGGACTACAAGGACGACGATGACAAAGCCGCCGCGGGCA A CGCCGCCGCCGCCAAG-3′), PKAK73Es (5′-GGGAACCACTACGCCATGGAGA TCTTAGACAAGCAGAAG-3′), PKAK73Ea (5′-CTCCATGGCGTAGTGGTTCCCACTC-3′), PKAK169Es (5′-CATCTACCGGGACCTGGAGCCCGAGAATCTTCTCATC-3′), PKAK169Ea (5′-

CTCCAGGTCCCGGTAGATGAGGTC-3′), and PKA2 (5′-GCTCTAGACTAA AACTCAGTAAACTCCTTG-3′) (289)), with the mutated codons underlined. Briefly, a fragment A was amplified using primers FlagPKA1 and PKAK73Ea by polymerase chain reaction (PCR), which mutated Lys73 to Glu73. A fragment B was generated using primers PKAK73Es and PKAK169Ea to mutate Lys169 to Glu169. A fragment C was amplified using primers PKAK169Es and PKA2. The full-length FLAG-PKA mutant DNA was obtained by overlap PCR using the fragments A, B and C as templates, and then cloned into BamHI/XbaI sites of pcDNA3.1 (+). The cloned mutant DNA was verified by sequencing. The pPKCγ-EGFP is from the Clontech Laboratories, Inc.

For *in vivo* selection of CaMKIV/PKA-responsive RNA elements, the splicing reporter minigene 33SB, which is a chimera of pDUP4-1 and pL53In, was constructed (78,290). Briefly, the ApaI/BglII fragment of pDUP4-1 was cloned into the ApaI/BamHI sites of pL53In (a gift from Dr. Harold König), and SalI and BamHI restriction sites were introduced into the 33-nucleotide (nt) middle exon (291), resulting in an insert fragment: actgactctctctgcctattggtctattttcccacccttagGTCGACGGTGGTGCCATGGCAGGCCGGAT CCAGgttggtatcaaggttacaagacaggtttaaggagaccaat (with exon in upper and intron in lower cases, SalI and BamHI sites in bold, and the replacement cassette for random sequences underlined). For cTNTβ splicing reporters, the SalI-BamHI fragment of the cTNTβ minigene (a kind gift of Dr. Thomas A. Cooper) (291), was substituted with that of 33SB-derived constructs containing cloned elements. To construct the splicing reporter minigene DUP175-KK7, the first 7 nt of DUP175 middle exon was replaced with CAAAAAA.

The hnRNP L-FLAG expression plasmid was provided by Dr. Stefan Stamm's lab,

as described previously (16). hnRNP LL cDNA was purchased from the OPEN Biosystem (Clone ID:) and then cloned into pCMV-Myc. hnRNP L and LL mutants were made by PCR using Pfu DNA polymerase. The primers carrying hnRNP L serine-to-alanine mutants are L-S326m3R (ATGACCATACTGGGGGCCATAGCGAGCTGGGCCCCGGCGGTGA), L-S513A2 (GGCAAAAGCGAGCGTAGCTCCGCTGGGCTGCTGGAGTGGGAC) and LL-S498A-F (TTCAGCCAAAACACTTGCTGGGCTATTAGAATG), cloned into pcDNA3.1+ and pCMV-Myc respectively, and confirmed by sequencing. To express tagged hnRNP L and LL in GH_3 cells, hnRNP L-FLAG, Myc-hnRNP LL and their mutants were subcloned into the lentiviral vector cppt2E (292). The lentiviral plasmid pFG12-shL (shL), as reported (16), targets the 3' untranslated region of hnRNP L; pFG12-shPTB and -shK are similarly constructed. Their shRNA sequences are listed in table 4; the pLKO.1-shLL against hnRNP LL (shLL, Clone ID: TRCN0000075101; sequence:

ccggCGACAGGCTCTAGTGGAATTTctcgagAAATTCCACTAGAGCCTGTCGtttttg,

with the nucleotides targeting LL in uppercases) (135), was purchased from the OPEN Biosystem's human shRNA library in the Manitoba Proteomic Centre.

Table 4. Lab-designed shRNA sequences for hnRNP L, hnRNP K and PTB

Name	**Sequence**
hnRNPL1906s	tccctcccaagattaaccttcacTTCAAGAGAgtgaaggttaatcttggga
hnRNPL1906a	aaaatcccaagattaaccttcacTCTCTTGAAgtgaaggttaatcttggga
hnRNPK1767s	tccccagcagcagagtgagtgacTTCAAGAGAgtcactcactctgctgctg
hnRNPK1767a	aaaacagcagcagagtgagtgacTCTCTTGAAgtcactcactctgctgctg
PTB2015s	tccctcaagtgacatgattctccTTCAAGAGAggagaatcatgtcacttga
PTB2015a	aaaatcaagtgacatgattctccTCTCTTGAAggagaatcatgtcacttga

Sense sequences of target proteins are underlined in each forward strand.
Each loop region is capitalized.

For RNA binding assay, the 175ST-1 plasmid was made by cloning a PCR fragment from the DUP175ST5, between the AatII and BglII sites of a pGEM-T based vector. The fragment starts from the upstream primer DUP9a (5'-GTTTAGTGAACCGTCAGATC-3') with an AatII site added to the 5' end till the BglII site in the 2nd intron. For 175ST-1m, the CaRRE1 sequence was mutated to the corresponding fugu fish sequence "Fugu_a", as reported (16). The plasmids were linearized with BglII before *in vitro* transcription using T7 RNA polymerase. The 175ST-1S and -1Sm templates were PCR amplified from the plasmids using primers aligned with the 1st and 2nd exons, respectively. The pET28a-hnRNP L was recloned from the hnRNP L-FLAG into pET28a by inserting its open reading frame fragment at the EcoRI site of pET28a.

Cell culture. Rat GH_3 pituitary cells express BK potassium channels. Previous studies have showed that Ca^{2+} signaling upon membrane depolarization regulates splicing of the STREX exon of the BK channel Slo gene in GH_3 cells. Therefore, the same cell line was chosen for splicing assays of endogenous exons including STREX. GH_3 cells were maintained in F10 media containing 10% horse serum, 2.5% fetal bovine serum (FBS) and 1% penicillin/streptomycin/glutamine solution (PSG).

HEK293T cells are derived from human embryonic kidney cells and have been widely used in biomedical research because they are easy to grow and transfect. Therefore, these cells were chosen for splicing assays of splicing reporter minigenes, mammalian overexpression of recombinant proteins, as well as lentivirus preparation. HEK293T cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplied with 10% newborn calf serum (NCS) and 1% PSG. For lentivirus preparation, HEK293T cells were

cultured in Iscove's Modified Dulbecco's Medium (IMDM) containing 10% FBS and 1% PSG.

PC12 cells are derived from rat pheochromocytoma. Because they are responsive to the activation of the PKA pathway, these cells are used for detecting splicing of selected element-containing exons in response to PKA activation. PC12 cells were grown in DMEM supplemented with 10% horse serum and 5% fetal bovine serum.

Western blot analysis. Western blot analysis was based on the procedure as described previously (293).

To test the kinase activity of Flag-PKA and its mutant, overnight HEK293T cultures in 12-well plates were transfected with 0.6 μg of Myc-PTB plasmid, or cotransfected with 0.6 μg of pcDNA3.1(+), Flag-PKA, or Flag-PKA mutant plasmid. Cells were harvested about 1.5 days after transfection and sonication-lysed in Buffer E (20 mM HEPES KOH, pH 7.9, 100 mM KCl, 0.2 mM EDTA, 10% (v/v) glycerol, 1 mM dithiothreitol) (294), containing 1 mM Na_3VO_4, 10 mM $Na_4P_2O_7$, and 10 mM NaF. A fraction of the cell lysates cotransfected with Myc-PTB plus Flag-PKA or PKA mutant plasmid was treated with calf intestinal alkaline phosphatase (CIAP) in 1× dephosphorylation buffer at 37 ℃ for about 30 min. For Western blot analysis, 5-μg cell lysates of each non-PKA cotransfected sample and 2.5 μg of each PKA-cotransfected sample (including those treated with CIAP) were resolved in Novex® 4–20% Tris-Glycine gels and transferred onto polyvinylidene difluoride membranes, followed by blocking in TBS (500 mM NaCl, 20 mM Tris-Cl) containing 5% (w/v)-dried skim milk. Total Myc-PTB and phospho-PTB were then probed by c-Myc monoclonal antibody and anti-phospho-Ser16-PTB antibody (288). After three washes in TBS, membranes were incubated with the appropriate

secondary antibody for about 1 h, followed by three washes in TBS. Poteins were then visualized by applying Western blotting detection reagents and exposing to x-ray films. For detection of PKA and PKA mutant protein, membranes were washed in TBS and reprobed by a monoclonal ANTI-FLAG® M2 antibody.

To detect the phospho-Ser513 of hnRNP L, 1mM Na_3VO_4 was pre-added to the dry milk suspension to block protein phosphatases. Endogenous PTB was detected by anti-Ser16-PTB antibody,

Selection for CaMKIV- or PKA-responsive RNA Elements. To create a library of splicing reporters, a synthesized nominally random sequence of 13 nt in a primer was substituted for the 13-nt replacement cassette in 33SB in PCR reactions. The PCR products were digested with ApaI and BglII, and ligated with ApaI- and BamHI-digested pL53In. The resulting plasmid pool contained $\sim 1.16 \times 10^7$ reporter clones as estimated from transformation of Escherichia coli cells with an aliquot of the plasmids. The complexity of the sequence pool was verified by randomly sequencing 17 clones, with no identical sequences obtained. The pool was cotransfected with 10 μg of Flag-PKA or Flag-CaMKIV-dCT plasmid and 5 μg of an EGFP plasmid using Lipofectamine 2000 into HEK293T cells in a 10-cm dish ($\sim 7 \times 10^6$ cells). The transfection efficiency was estimated 24 h later to be at least 90% by counting green cells under a fluorescence microscope. Total RNA was extracted using the GenElute Mammalian Total RNA Miniprep Kit, followed by digestion with RQ1 RNase-free DNase to remove contaminating plasmid DNA, which was confirmed by the absence of PCR products using an RT sample without reverse transcriptase. RT-PCR was then performed to selectively amplify middle exon-included mRNA using primers targeted to splice

junctions (primer SXenh-J1, 5′-GGAGGACCCACAAGGTCG-3′ and primer SXenh-J2, 5′-CCAGTTGTGCCACTGGAT-3′). The PCR products were cloned as SalI-BamHI fragments back into the 33SB vector and the selection cycle went through multiple rounds, repeating the same procedure in the subsequent rounds as in the first one except that about 1×10^6 plasmid clones (as estimated by transformation in E. coli) were cotransfected with 1.5 μg of Flag-PKA (or CaMKIV-dCT) and 1 μg of the EGFP plasmid into about 1×10^6 HEK293T cells.

Sequence Analyses and Database Searches. The selected 13-nt sequences were analyzed with Mutiple Em Motif Elicitation (MEME) (295), for consensus motifs. The motif-containing human exons were identified from the ASAPII database (296). The constitutive human exons were obtained from the HOLLYWOOD database (297,298).

Splicing assay. For *in vivo* selection of RNA elements, splicing of reporter minigenes and endogenous genes was examined either by regular reverse transcription-polymerase chain reaction (RT-PCR) or primer extension. For primer extension, briefly, RNA samples were heated at 85 ℃ for 5 min, followed by immediate chilling on ice for 2 min, and then applied to reverse transcription by incubation with Superscript III at 50 ℃ for 1.5 h. The resulting products were resolved in 5.3% denaturing polyacrylamide gels, dried and visualized by radioautography. Intensities of bands were quantified by ImageJ (NIH, U.S.A).

Splicing of endogenous genes in GH_3 cells were examined by ^{32}P-labeled PCR modified from a previous procedure (4). Essentially, 25~150ng of cytoplasmic RNA was reverse transcribed in a 10-μl reaction, and 0.5 or 1 ul of each RT product was then

applied to a 12.5-μl PCR reaction, by annealing at 60 ℃ and running for 25 cycles. PCR products were resolved into 6% polyacrylamide gels and visualized by radioautography.

Production of lenti-viral vectors and RNA interference (RNAi)/complementation assay. This was according to our published procedure (16) with slight modifications. Briefly, overnight cultures of HEK293T cells at ~80% confluence were transfected with 5 μg of pHCMVG, 12.5 μg of pCMVDR8.2DVPR, and 12.5 μg of the lentiviral vectors (FG12-shL, pLKO.1-shLL, cppt2E-hnRNP L-FLAG and its S513A mutant, cppt2E-Myc-hnRNP LL and its S498A mutant) using PolyJet™ *in vitro* DNA transfection reagent (SignaGen®) according to the instructions of the manufacturer. After 18 h, the media were refreshed. On days 3 and 4, supernatants were collected, pooled, filtered (0.22 μm, Nalgene), further concentrated ~100 times by ultracentrifugation (16) or by precipitation containing ~8.4% PEG8000 (Sigma-Aldrich) and ~0.3 M NaCl, and centrifuged at 20,000 rpm for 30 min (Beckman Avanti® J-E, rotor JA-25.50). Virus pellets were resuspended in culture media and saved at -80 ℃.

For transduction, rat GH_3 pituitary cells at a density of ~2 x 10^5 cells/well in a 24-well plate were transduced using shRNA-carrying viruses for 3 h, and 24h later using both shRNA and protein-expressing ones and then transferred to a 12-well plate. On day 6, cells were depolarized using 50 mM KCl for ~6 h before harvest for both protein and RNA analyses.

Phosphopeptide mapping. This experiment was performed on the basis of our published procedure (164,288) using anti-FLAG for immunoprecipitating hnRNP L-FLAG/mutants and anti-Myc for Myc-hnRNP LL/mutants. The precipitated proteins were digested by sequencing-grade trypsin and chymotrypsin (Sigma-Aldrich) for peptide mapping in

electrophoresis followed by thin layer chromatography on 10 cm X 10 cm cellulose TLC plates (EMD Chemicals, Inc.).

For peptidemapping of *in vitro* phosphorylated peptides by CaMKIV, 20 ng of GST-CaMKIV (BioMol) was incubated with each of the peptides ERSSSGLLEW (Ser513) and ERSS($_P$-S)GLLEW (p-S: phospho-Ser513) plus purified Myc-CaMKK2(~10 ng) (164), in kinase reaction buffer (1 mM calcium, 20 mM Tris-HCl, 0.1% Tween-20, 8 mM $MgCl_2$, 0.1 mM cold ATP, 83 pM [γ-^{32}P]ATP, 0.05 mM DTT), incubated at 30 ℃ for 30 min, mixed with SDS loading buffer, denatured at 95 ℃ for 5 min and then spotted onto TLC plates for two-dimensional gel electrophoresis and chromatography, respectively.

Phospho-Ser-513-specific antibody. The antibody was made against a synthetic phospho-Ser513 peptide ERSS(pS)GLLEW (Fig. 10B), similarly as previously described (288), in the Alpha Diagnostics Inc. The antiserum showed at least 4 times of immunoreactivity for the phospho-peptide over the nonphosphopeptide ERSSSGLLEW when diluted 100,000 times. The specificity to the phospho-Ser-513 was further confirmed by Western blot using a S513A mutant as shown in Fig. 11A.

Immunodepletion, UV-crosslinking and immunoprecipitation. Immunodepletion was performed according to a published procedure in the presence of 0.5 M NaCl (299). The resulting nuclear extract was used directly for UV crosslinking at a final NaCl concentration of 90-180 mM.

UV crosslinking and immunoprecipitation were performed on the basis of our described procedure (16), with several modifications. For RNA binding assays in Figure 4, DNA templates for RNA probes were amplified from highly PKA or CaMKIV-

responsive clones A3.15, C7, and C42 with a T7-tagged primer. RNA probes were *in vitro* transcribed with T7 RNA polymerase using using [α-^{32}P]CTP. The RNA probe A3.15 sequence (the 13-nt replacement cassette, underlined) is: gcuauuuucccacccuuagGUCGACGGUGCACAGCAAAAAUACCGGAUCCAGguuggua ucaagguuacaagacagguuuaaggagaccaauagaucuucag. RNA probes C7 and C42 are different from A3.15 only at the 13-nt inserts. The control probe CG (CG repeat, underlined) is: GUCCAUGUGUCGCGCGGUUAAAUCUUCUGGAAAGUGUGUACACAAUGUUC CGGAAGGCUGACCUCCCUUCAAAUGUCACUCGCCAG; mCaRRE1 probe from the upstream 3′-splice site of DUP175ST is the same as described previously (16). For RNA binding assays in Figure 12, HeLa (~22 μg) nuclear extract was incubated with 7.5×10^5 cpm of [α-^{32}P]UTP-labeled RNA transcripts in 12.5 or 25 μl of cross-linking reactions at 30 °C for 10 min, irradiated with UV light (254 nm) for 45 min on ice, and digested with 10 units of RNase T1 plus 4.0 μg of RNase A for 30 min at 30 °C**.**

For immunoprecipitation, 2.0 μg of anti-hnRNP L (4D11, Santa Cruz Biotechnology) or 4 μg anti-U2AF65 (MC3, Sigma-Aldrich) antibodies bound to protein G sepharose beads (Pierce) were incubated with 2-4 volumes of UV crosslinking reactions with rotation at 4 °C overnight. The beads were then washed 3 times with 1 ml of RIPA buffer (containing 150 mM NaCl). The resulting proteins were processed as described (16).

Expression and purification of recombinant His-hnRNP L: One liter of overnight culture of pET28a-hnRNP L plasmid-transformed *E.coli* Rosetta-gami 2 (DE3) pLysS (Novagen) was induced with 0.3 mM isopropyl 1-thio-β-D-galactoside for 3 h at 37 °C before harvesting by centrifugation. The bacteria were then resuspended and sonicated in

4 ml of cold PBS (phosphate buffered saline) containing 8 M urea and 20mM imidazole (pH 8.0) and centrifuged at 10,000 rpm for 25 min at 4 °C. The supernatant was applied to nickel-nitrilotriacetic acid-agarose beads (Invitrogen), incubated at 4 °C for 3 h, and then the beads were washed in 30 ml of cold PBS buffer (containing 6 M urea, 50 mM imidazole) followed by sequential wash with decreasing concentrations of urea (6 M, 4 M, 2 M, 1 M, 0 M) in cold PBS. His-hnRNP L was eluted with 1 ml of PBS buffer (containing 1 M imidazole, 1M KCl, 100 mM EDTA, 100 mM DTT) and dialyzed three times against buffer DG (20 mM HEPES-KOH, pH 7.9, 20% glycerol, 80 mM potassium glutamate, 0.2 mM EDTA, 0.2 mM PSMF, 1.0 mM DTT). The concentration of hnRNP L was determined by comparing its band intensities with standard BSA proteins in a SDS-PAGE gel stained with Coomassie Blue using scanned images in ImageJ software. Treatment of His-hnRNP L with decreasing concentrations of urea restored about 21% of the UV-crosslinking activity of the protein as indicated by its ability to interact with RNA and inhibit the binding of U2AF65 to the 3' splice site of STREX (Fig. 12E).

CHAPTER III

Overview of the Thesis Work

The theme of the thesis

The overall goal of this thesis project is to understand the molecular basis for the regulation of alternative pre-mRNA splicing by membrane depolarization. The splicing of the STREX exon of the BK potassium channel *Slo1* gene (4), which is controlled by the depolarization/CaMKIV pathway in pituitary cells and cerebellar neurons (4,5), was used as a major experimental model.

Activity-dependent long term changes of cellular electrical properties are important for development and physiological functions such as neuronal memory, muscle contraction or hormone secretion (13,300-302). However, the molecular mechanisms for the regulation of ion channel expression and function in this process remain largely unknown. Alternative pre-mRNA splicing of ion channels contributes greatly to their functional diversity (56,303,304). The regulation of their alternative splicing by membrane depolarization provides a unique mechanism for the fine-tuning of the activity-dependent changes of electrical properties (56).

The BK potassium channels are widely expressed in many cell types including neurons and endocrine cells. Their activity is modulated by tissue-specific β subunits, posttranslational modifications, as well as alternative splicing of the pore-forming α subunit (303). There are four genes encoding the α subunits in mammals (264), with the *Slo1* being the best studied by far. Transcripts from the *Slo1* gene undergo extensive alternative splicing, capable of producing hundreds of variant subunits and billions of BK

channel tetramers (56,264,305). These voltage/calcium-sensitive channels (306) couple electrical properties with calcium signaling. Loss of the channels causes defects in brain, smooth muscle and endocrine functions such as neurotransmitter release, ethanol sensitivity, blood pressure and stress response (264,307,308).

One of the most intensively studied *Slo1* exons is the STREX (178). STREX encodes a 58-amino acid, cysteine rich-fragment residing in the Ca^{2+}-sensing cytosolic domain of the α subunit (56). Inclusion of STREX induces enhanced sensitivity to Ca^{2+}/voltage, glucocorticoids, hypoxia and stretch, negative regulation by PKA and oxidation (56), and insensitivity to alcohol (309).

The level of STREX is regulated by stimuli altering cell activities, such as hormones (178) and cell membrane depolarization (4,5). In GH_3 pituitary cells and cerebellar neurons, depolarization induces repression of STREX through L-type calcium channels and the CaRRE1 (4,5,7). This regulation represents a way of sending cell signals to alternative splicing of targeted genes. Indeed, other CaRRE-like RNA elements essential for depolarization-induced splicing of a group of endogenous exons have been characterized as well (5,7). Some of them are CaRRE1 variants, however, some show distinct sequence characteristics from CaRRE1, such as the CaRRE2 and the UAA(A) element (7,9), suggesting that the CaMKIV-targeted RNA elements are more diversified than anticipated. Moreover, one of them is possibly targeted by PKA as well (6,188). Therefore, depolarization-responsive RNA elements including CaRREs remain to be analyzed more comprehensively.

On the other hand, the characterization of regulatory splicing factors functioning through CaRREs is very limited. Investigations of what factors are required and how they

mediate the regulated splicing are also important for understanding the control of STREX by depolarization.

Therefore, my thesis project focused on further characterization of CaMKIV/PKA-responsive RNA elements and identification of trans-acting splicing factors required for the depolarization-regulated exons including STREX.

Thesis work

In the first part of the thesis project, we applied *in vivo* selections using SELEX to further characterize RNA elements targeted by the kinases downstream of depolarization. Taking advantage of the context-dependent promotion of exon inclusion in a splicing reporter, this selection enriched novel CaRREs and also PKA-responsive RNA elements. These elements are similar to the STREX CaRRE1 based on their CA repeats, or are A-rich motifs not previously known to respond to these kinases. Consistently, a common binding factor for the CA-rich ones was identified as hnRNP L that is known to bind CA repeats (114), suggesting that hnRNP L is a factor involved in the regulation of STREX. This part of work led to the publication in the JOURNAL OF BIOLOGICAL CHEMISTRY in 2009 (310).

Consistent with the discoveries documented in the first part, my colleagues demonstrated that hnRNP L is an essential mediator of CaMKIV-induced splicing repression through CA repeats and is one of the factors in the depolarization-induced repression of the STREX (16). Moreover, hnRNP L binding to the CaRRE1 is phosphorylation-dependent (16).

Therefore, in the second part of this project, we verified the critical role of hnRNP L in depolarization-induced repression of STREX and investigated its underlying

mechanism. These studies revealed that serine 513 (Ser513) of hnRNP L is critical for the regulated-repression of STREX. Phosphorylation of the Ser513 by CaMKIV enhanced hnRNP L binding to the CaRRE1 and consequently resulted in reduced interaction of the constitutive splicing factor U2AF65 at the 3' splice site. This is expected to interfere with the assembly of spliceosome at this region and thus cause skipping of STREX. This part of work then led to the publication in the JOURNAL OF BIOLOGICAL CHEMISTRY in 2012 (311).

The above studies also indicated that STREX is controlled by other splicing factors as well. Therefore, the last part of the project was aimed to investigate the combinatorial control of STREX by different splicing factors and their specificities. Moreover, we also examined the effects of these factors on other endogenous exons harboring CaRRE1 variants or CA repeats in comparison to STREX. These studies revealed differential regulation of the exons by a group of hnRNPs and verified the effect of the hnRNP L Ser513 on another depolarization-regulated exon.

Overall implications

This study provides a direct functional link between a Ca^{2+} signaling kinase and a splicing regulator, and thus completes the delineation of a pathway regulating alternative splicing of BK potassium channels. This helps explain not only the molecular basis of cell signal-regulated alternative splicing during gene expression but also the physiological functions, such as memory formation and secretion of growth hormones, triggered by cellular activities through the regulation of ion channels.

CHAPTER IV

In vivo selection of kinase-responsive RNA elements controlling alternative splicing

Introduction

A group of endogenous exons are regulated by depolarization through CaMKIV. At least two types of degenerative CaRREs are identified, suggesting that CaMKIV-targeted RNA elements are somehow diversified. However, systematic analysis of CaMKIV-responsive RNA elements has not yet been reported. One possible reason could be the difficulty in enriching for such elements from spliced mRNA in cells when the kinase shows exon-exclusion activity through the CaRREs in the current splicing reporter systems (4,5,7). Recent studies showed the PKA pathway is potentially another signaling pathway involved in depolarization-induced alternative splicing (6,188). However, few studies of the PKA-targeted RNA elements have been reported.

Therefore, we sought to isolate and analyze RNA elements responsive to CaMKIV and PKA using SELEX-derived strategy based on the context-dependent promotion of exon inclusion by the kinases.

This part of work has been published in THE JOURNAL OF BIOLOGICAL CHEMISTRY (310).

Results

A selection strategy based on a context-dependent promotion of exon inclusion by CaMKIV and PKA

CaMKIV inhibited splicing through the CaRRE1 element in the DUP175-based reporters (4,5,7,16); however, in reporters derived from pL53In (290), CaMKIV enhanced splicing through the same element (Fig. 1A).

In this test, CaRRE1 responses to coexpressed constitutively active Flag-CaMKIV-dCT (CaMKIV or IV) or its kinase-dead mutant (IVm) were examined in the pL53In-based splicing reporter 33SB-CaRRE1a or b (Fig. 1A), containing exons/introns and promoters different from the previous DUP175 (4,290,312). In the 33SB vector transcripts, inclusion of the middle exon (63%) was essentially unchanged by CaMKIV (lanes 1 and 2). Replacing part of the middle exon with CaRRE1a or CaRRE1b sequences reduced the level of exon inclusion to 38 and 34% (lanes 3 and 5), respectively, indicating that the CaRRE1 sequences are splicing silencers. However, unlike in DUP175, the exon inclusion was increased by overexpressed CaMKIV (63 and 49% exon inclusion, lanes 4 and 6, respectively).

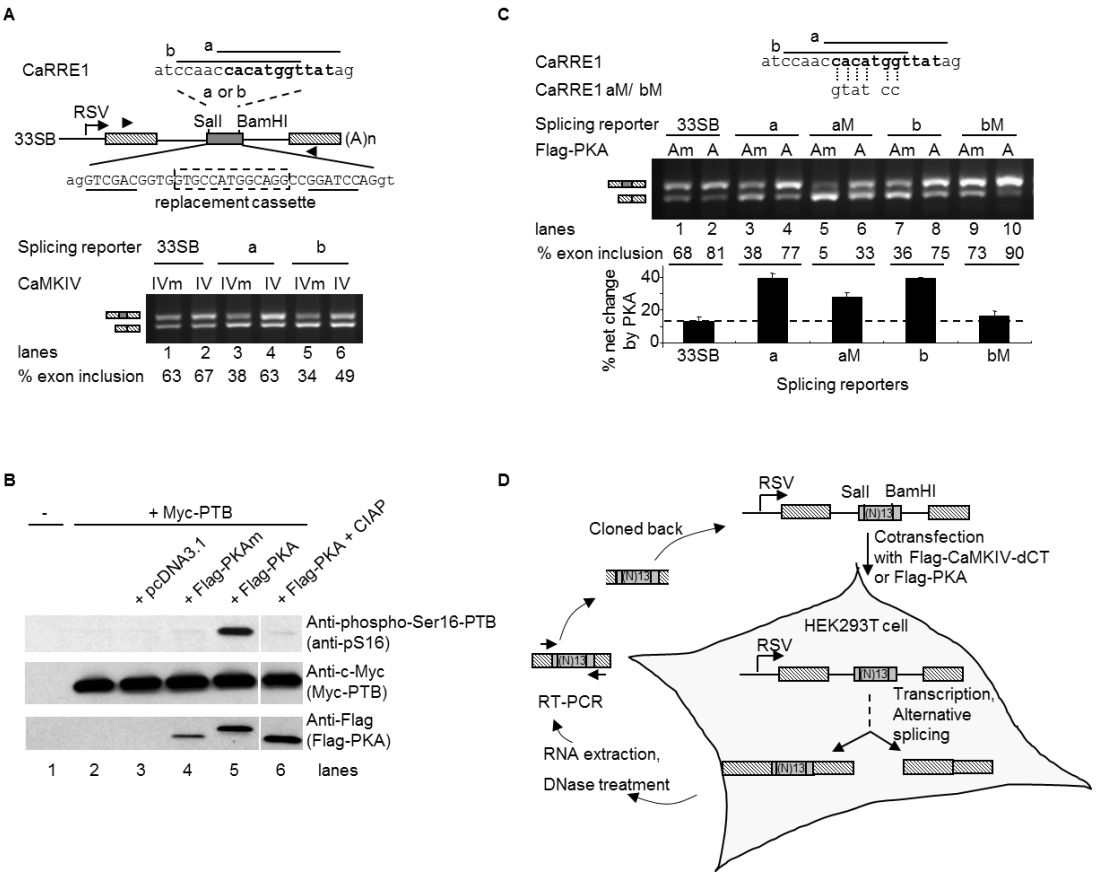

Figure 1. Selection of CaMKIV- or PKA-responsive RNA elements based on a context-dependent promotion of exon inclusion by the kinases. **A**, CaMKIV promotion of exon inclusion through CaRRE1 in 33SB. Upper, diagram of CaRRE1 (consensus motif from the previous reports in bold) in 33SB derived from the vector pL53In based on the insulin gene (exons as black-slashed boxes). Each of the two 13-nt sequences, CaRRE1a (a) and CaRRE1b (b), replaced the 13-nt cassette (boxed in dotted line) between the SalI and BamHI sites (underlined) in the middle exon. RSV, Rous Sarcoma Virus promoter. Exon sequences are in upper case and intron in lower case. Lower, agarose gel of RT-PCR products from HEK293T cells cotransfected with the splicing reporters and Flag-CaMKIVdCTK75E (IVm) or Flag-CaMKIV-dCT (IV) as indicated above the gel. Spliced products are indicated to the left and percentages of exon inclusion below each gel. Lines, introns. Arrowheads, locations of PCR primers. (A)n, poly(A). **B**, phosphorylation activity of the wild type Flag-PKA (PKA) or inactivity of the kinase-dead Flag-PKA mutant (PKAm), respectively. Shown are Western blots indicating the phosphorylation level of the PKA target Ser16 of the Myc-tagged PTB protein (top panel) compared with the total protein (middle panel) expressed in HEK293T cells with vector pcDAN3.1(+), PKAm, or PKA (bottom panel), as indicated above the gels. The sample for the last lane was pretreated with calf intestine alkaline phosphatase (CIAP) before loading. PTB phosphorylation is increased about 7-fold by PKA, about 4-fold of that induced by forskolin on the phosphorylation of the endogenous PTB Ser16 as shown in Xie et al. (288). **C**, PKA effect on the 33SB-CaRRE1 reporters. Shown is an agarose gel of RT-PCR products of CaRRE1 or its mutant splicing reporters

cotransfected with Flag-PKAm (Am) or Flag-PKA (A) into HEK293T cells as indicated above the gel. The mutated CaRRE1 nucleotides are indicated below the CaRRE1 sequences. Below the gel are the percentages of exon inclusion for each lane and a bar graph of the percent net changes by PKA for each reporter. **D**, strategy for *in vivo* SELEX enrichment of CaMKIV or PKA-responsive RNA elements controlling alternative splicing.

A similar promoting effect on exon inclusion by coexpressed Flag-PKA was also observed in the 33SB-CaRRE1 splicing reporters (Fig. 1, B and C). For this assay, we made a double mutant of PKA (PKAm, K73E, K169E) from the wild type Flag-PKA, which phosphorylates the Ser16 of PTB (Fig. 1B) (288,293). In the PKAm, the residues Lys73 and Lys169, essential for phosphate-binding (289), were mutated to Glu73 and Glu169, respectively. The PKAm showed no kinase activity on the PKA target Ser16 of PTB (Fig.1B) and exhibited no effect on the exon inclusion (Fig. 1, A and C, compare lanes 1, 3, 5 in A with 1, 3, 7 in C, respectively, p=0.25, paired Student's *t* test), in comparison with the CaMKIVm that is known to be kinase-dead and have no effect on splicing (4,7,201). In contrast, the wild type Flag-PKA increased the exon inclusion level to 77 and 75%, respectively (Fig. 1C, lanes 4 and 8), similarly to the effect by CaMKIV (Fig. 1A). Importantly, this increase was reduced to that of the 33SB vector level by mutations in CaRRE1 (Fig. 1C, lanes 5, 6, 9, and 10). Because these clones contain the same promoter, flanking exons/introns and poly(A) signals, their differential responses to CaMKIV or PKA are properties of the insert elements themselves rather than those of the transcriptional unit.

The promoting effect of CaMKIV and PKA suggested that these protein kinases would likely enrich exons containing kinase-responsive RNA elements in a pool of random sequences during repeated selections. Moreover, because the vector already

showed about 65% exon inclusion with CaMKIVm or PKAm (Fig. 1, A and C), constitutive enhancers in the random pool were expected to cause 100% exon inclusion soon in early rounds of selection and could not be further increased by the kinases during subsequent rounds of selection. Thereby this system would likely allow enrichment of silencer elements preferably targeted by the kinases through repeated selections.

According to this approach, a pool of 13-nt random sequences was cloned into the middle exon of the 33SB to obtain a library of splicing reporters (containing about 1.16×10^7 clones). The reporters were cotransfected into HEK293T cells with the constitutively active CaMKIV or PKA plasmids. The spliced products were amplified by RT-PCR, cloned back into the vector, and cycled through five selection rounds (Fig. 1D). Because no PKA-responsive splicing element has been reported yet and also for reasons as found out later (more net changes of exon inclusion by PKA and mostly shared elements between these two kinases as shown below), we analyzed the PKA effect in more detail in the following experiments.

The selected PKA- or CaMKIV-responsive RNA elements are mostly splicing silencers

Preliminary analyses with RT-PCR of the spliced mRNA pools from each selection round (of five rounds in total) indicated that the net changes of exon inclusion by PKA increased and peaked at round 3 (about 3-fold, compared with the starting pool).We therefore randomly picked up 48 clones from the 2nd and 3rd rounds of selection plus the 33SB vector, and analyzed their constitutive splicing and response to PKA coexpression (Fig. 2A).

The constitutive level of exon inclusion of these clones ranges from 0 to 93% (Fig. 2A, from bottom to top). A small group of clones (nos. 1–16) showed higher constitutive level of exon inclusion than the 33SB vector. Most of these clones contain motifs that are similar to the purine- or A/C-rich enhancers in the previous selection (291), as expected. For these clones, the PKA effect to increase exon inclusion is hard to assess since they already have almost 100% exon inclusion even without Flag-PKA coexpression. However, a larger group of clones (nos. 18–49) showed lower constitutive level of exon inclusion than the 33SB vector. Of the 32 clones in this group, 29 (nos. 21–49) showed at least 10% less constitutive exon inclusion than the vector, likely containing splicing silencers. PKA coexpression substantially increased the inclusion of exons in 22 clones (with net changes at least 10% more than the vector) except 7 clones (nos. 27, 31, 44–46, and 48–49). Therefore, PKA appears to relieve the splicing repression effect to promote the inclusion of exons containing PKA-responsive silencer elements.

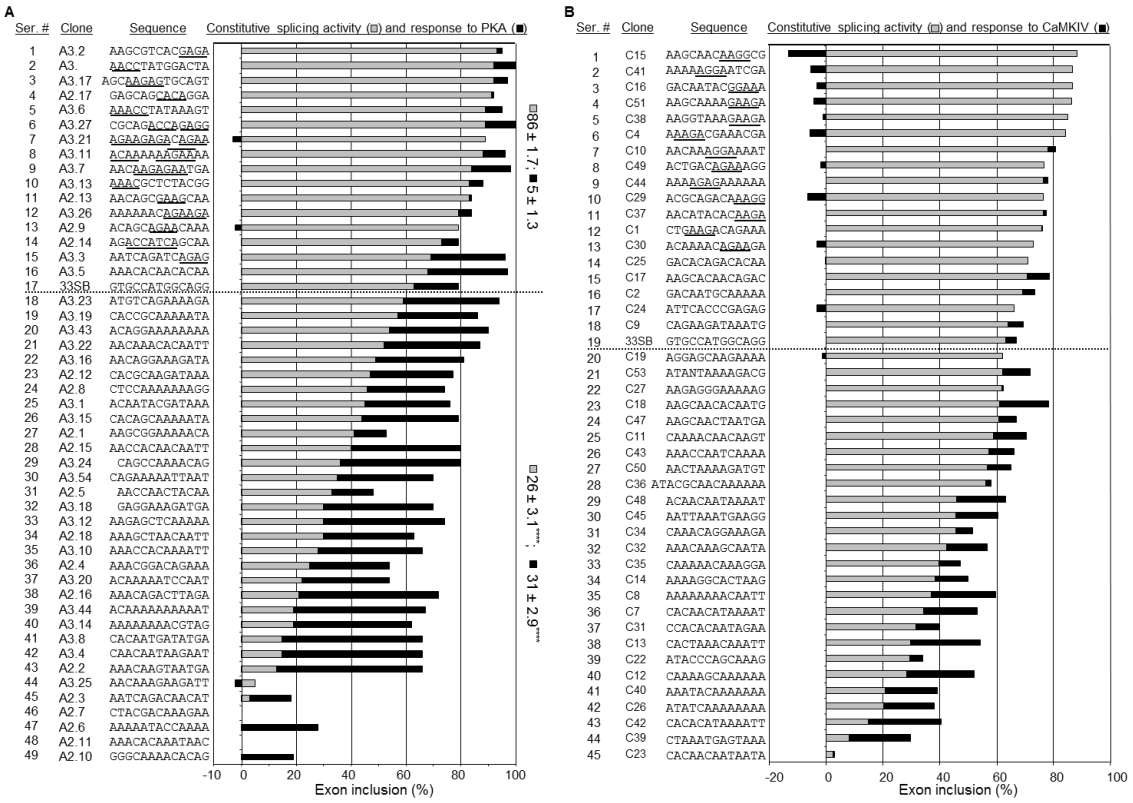

Figure 2. Selected PKA/CaMKIV-responsive elements. A, elements from the selection by PKA, with their serial numbers, clone numbers, sequences, and bar graphs of constitutive levels of exon inclusion (gray bars) and PKA responses (black bars, net changes of percent exon inclusion by PKA) of each clone indicated. The graph is aligned from top to bottom with decreasing constitutive levels of exon inclusion. The average levels of constitutive splicing (±S.E.) or response to PKA of the clones with at least 10% less (silencers) or higher (enhancers) constitutive level than the vector 33SB are indicated to the right of the graph. ****, $p < 0.0001$ compared with the enhancer group, in two tailed Student's t test. **B**, elements from the selection by CaMKIV, indicated similarly in A. In both groups, the vector 33SB is also included, as nos. 17 and 19, respectively, above the dotted lines. Motifs similar to the known purine- or A/C-rich enhancers are underlined in both groups.

Analyses of 44 clones of the CaMKIV group showed similar promotion of exon inclusion through silencer elements though the net change of exon inclusion by CaMKIV is generally less than that by PKA (Fig. 2B, nos. 21–44). Besides, CaMKIV also appeared to generally repress the exon inclusion of the clones containing enhancer elements (nos. 1–13), an effect not seen for the silencers or by PKA.

Overall, the occurrence of clones responsive to PKA or CaMKIV increased from 39% or 38% in the starting pool to 57% (for both kinases) in the 3rd round, supporting that the selection indeed enriched RNA elements responsive to PKA or CaMKIV.

Consensus motifs of PKA- or CaMKIV-responsive elements

To identify consensus motifs of the elements that are PKA-responsive, we first transferred the highly PKA-responsive elements (net changes at least 10% more than the 33SB by PKA, same as in the following texts) from selection rounds 2 and 3 to a heterologous minigene splicing reporter cTNTβ. This experiment identified 14 out of 20 elements that conferred PKA response to this heterologous gene as well (Fig. 3A, b). These transferable elements appeared mostly CA- and A-rich. From these elements, a consensus heptamer motif CARAAHD (R: A or G; H: A, C or T; D: A, G or T, Fig. 3A) was identified using Mutiple Em Motif Elicitation (MEME) (295). In contrast, MEME analysis of the 13nt insert sequences of 17 clones from the starting random pool did not identify any consensus motif that could be found in most clones.

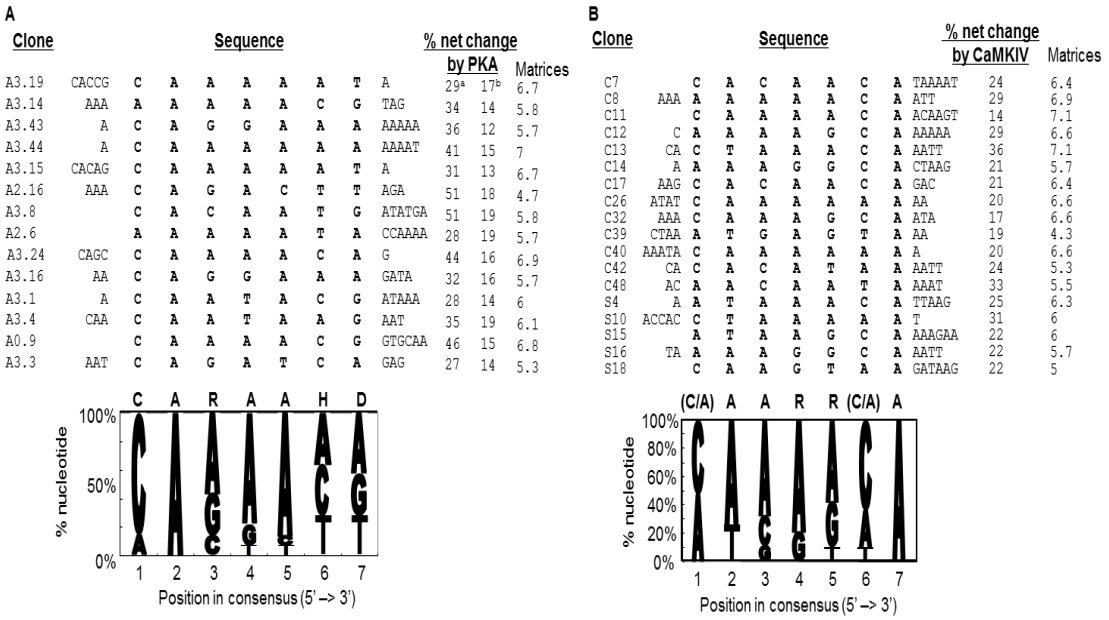

Figure 3. Selected highly PKA/CaMKIV-responsive RNA elements and their consensus motif. A, consensus motif shared by highly PKA-responsive elements. Upper, sequences of fourteen elements, with the nucleotides for the consensus motif aligned in the middle and sequence names to the left and net changes of exon inclusion by PKA in 33SB (a). PKA responses (net changes of percent exon inclusion by PKA) of the elements in another reporter cTNTβ minigene is also listed (b) to the right. Lower, frequency of each nt at each position of the heptamer motif. The height of each nucleotide letter is proportional to its nt frequency, in decreasing order from top to bottom. The consensus sequence is above the graph (R, A or G; H, A, C, or T; D, A, G, or T). The numbers in the clone/element names indicate, from left to right, selection round, number in 33SB, number in cTNTβ. **B**, consensus motif shared by highly CaMKIV-responsive elements. Upper, sequences of the elements from selection round 3 with CaMKIV (C) are listed with the nucleotides for the consensus motif aligned in the middle, followed by the net changes of exon inclusion by CaMKIV. Also included are 5 highly responsive sequences from the starting pool (S). Lower, consensus motif of the highly CaMKIV-responsive elements. The matrices (out of max. 10) of the motifs are indicated to the right of each panel. The CaRRE1 has a matrix score of 5.1 with the "aaccaca" motif.

Similar examination of the 13-nt inserts of a group of highly CaMKIV-responsive clones also identified a consensus motif (C/A)AARR(C/A)A (Fig. 3B, R: purine). This motif is similar to the CA repeats of CaRRE1 (for example, the "CACAT" in clone C42). Moreover, RNA transcripts containing this motif were strongly cross-linked to a protein

similar in size to the hnRNP L (Fig. 4, A and B), which binds the CaRRE1 (16). This protein was immunoprecipitated by 4D11 (Fig. 4B, right), a monoclonal anti-hnRNP L antibody (238). In contrast, barely any PTB signal was detected by the C42 probe compared with mCaRRE1 (compare lanes 2 and 4).

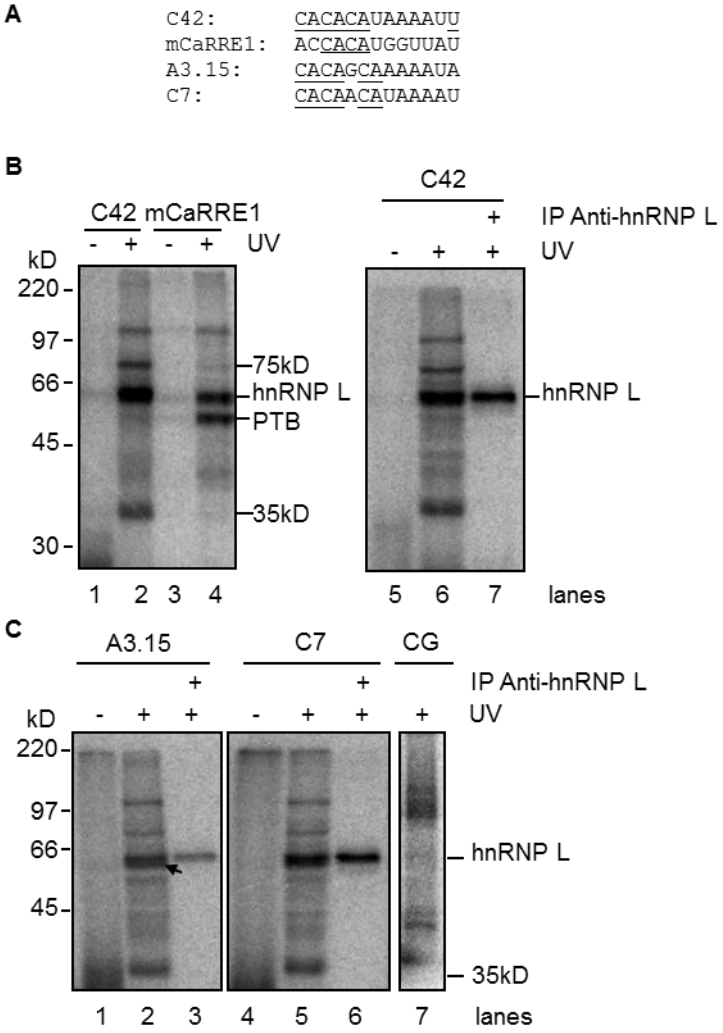

Figure. 4 The CA-rich RNA elements are bound by hnRNP L, a mediator of CaMKIV-regulated splicing through CA repeats. **A**, 13-nt elements in the RNA probes made from the kinase-responsive clones as indicated. Control probe CG is from the gene PRKDC with its CACACA motif mutated to CGCGCG; mCaRRE1 in DUP175 is a positive control for hnRNP L binding. **B**, hnRNP L binds to the RNA probe C42 containing the CACAT motif as in mCaRRE1. HeLa nuclear extracts were subjected to UV-cross-linking with [α-^{32}P]CTP-labeled RNA probes (left), and 6 times of the nuclear

extracts cross-linked to C42 were immunoprecipitated with the anti-hnRNP L antibody 4D11 (right). **C**, hnRNP L binds to the RNA probes containing similar CA repeats but not the CG probe. Similar to those in B, HeLa nuclear extracts were cross-linked to RNA probes A3.15, C7, and CG, and immunoprecipitated with the antibody 4D11. The arrow pointing to the ~62-kDa band below hnRNP L is specific for the A3.15 probe.

Interestingly, similar UV cross-linking immunoprecipitation with the CA-rich A3.15 and C7 RNA probes, but not a control transcript containing CG repeats, also identified hnRNP L as a prominent binding factor (Fig. 4C). In addition, a ~62-kDa protein band just below hnRNP L is clearly visible in the A3.15 sample as well (Fig. 4C, lane 2, arrow). Moreover, hnRNP L binding to C7 and C42 appeared stronger than to the A3.15 probe (compare 4B, lane 7 and 4C, lane 6 with 4C, lane 3).

Of the other two bands (75 and 35 kDa) that are specific for the enriched element probes but not the mCaRRE1 or the CG probe, the 35-kDa band showed no difference in relative intensity among the element probes (Fig. 4C), suggesting that it is not an element-specific band; however, the 75-kDa band showed a much higher relative intensity in the C42 sample.

Thus, the CA-rich motifs enriched in the PKA- and CaMKIV-responsive elements likely bind proteins differentially; one of the common protein factor is the hnRNP L. Taken the random enrichment of these elements together with the essential role of hnRNP L in CaMKIV-regulated splicing through CA repeats (16), hnRNP L is likely a preferred target of PKA and CaMKIV in regulating splicing. Together, the CA-rich content and the identification of hnRNP L as a binding factor suggest that the selection indeed identified consensus motifs that are known to be CaMKIV-responsive.

PKA and CaMKIV specifically share their responsive elements

As shown above, both CaMKIV and PKA promote inclusion of exons containing CaRRE1 (Fig. 1, A and C), and their consensus motifs appear similar in the CA- and A-rich content. Moreover, hnRNP L was identified as one binding factor for both kinase-selected elements (Fig. 4). These together suggest that the two kinases may share similar elements.

To examine the kinase specificity of these elements, four highly PKA-responsive clones were tested with CaMKIV and PKCγ (Fig. 5, A and B). These clones showed clear responses to CaMKIV (Fig. 5A), with CaMKIV-induced net changes ranging from 15 to 31%, well above that of the 33SB vector alone (4%). In contrast, three clones had no response to PKCγ above the 33SB level (Fig. 5B, lanes 1–8), and one clone (A3.44) showed only 9% increase of exon inclusion over the vector (lanes 9 and 10). Thus, the PKA-responsive elements are preferably responsive to both PKA and CaMKIV but not PKCγ.

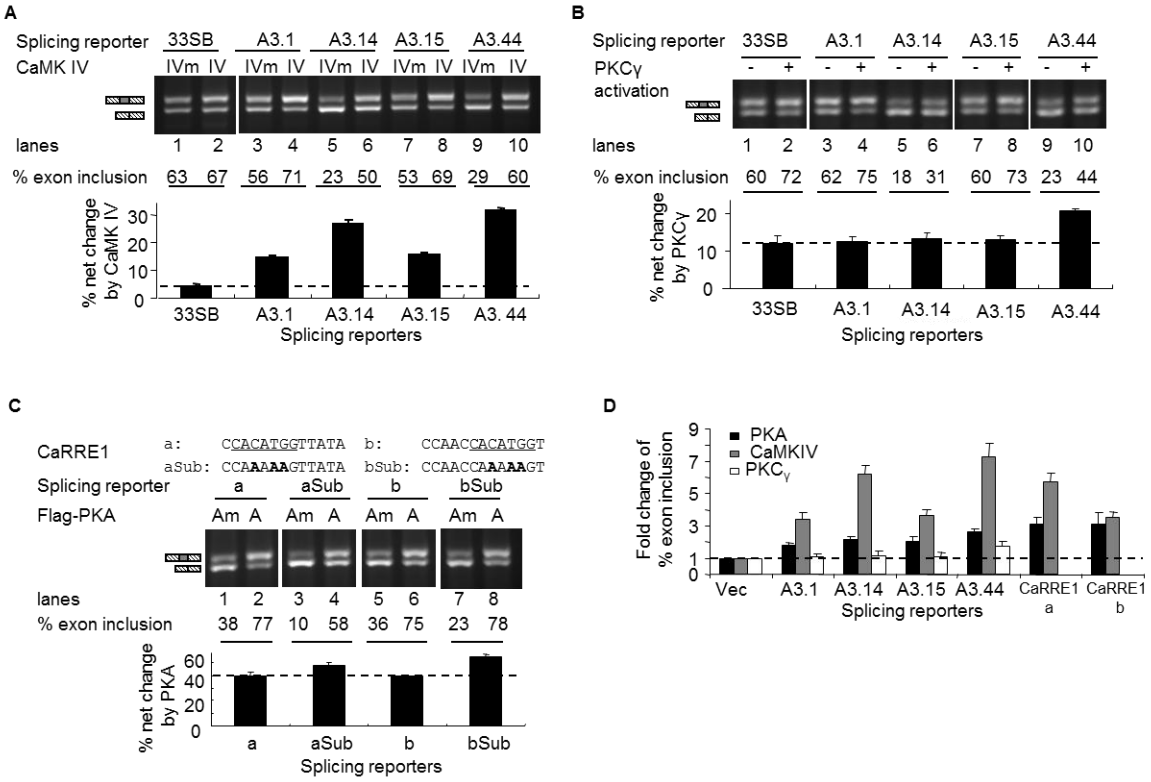

Figure. 5 Kinase specificity of the PKA- or CaMKIV-responsive elements. A and B, CaMKIV and PKCγ effects on the splicing of the individual PKA-responsive clones. Shown are agarose gels of RT-PCR products of the splicing reporters contransfected into HEK293T cells with CaMKIV-dCTK75E (IVm), CaMKIV-dCT (IV) as indicated above the gels. In B, PKCγ-EGFP was cotransfected with all the reporters but was either not activated (−) or activated with TPA (+) as indicated. Below the gels are the percentages of exon inclusion for each lane and a bar graph of the percent net changes by CaMKIV or PKCγ for each reporter. **C**, CaRRE1 motif is replaceable by a consensus motif of the PKA-responsive elements. Shown on top are the 13-nt insert sequences of CaRRE1 (a and b) and its core motif substituted by CAAAAAG (aSub or bSub). Below are agarose gels of the splicing reporter assay for PKA response indicated as in A and B. **D**, comparison between the effects of the kinases on splicing through the RNA elements. Graphed are the fold changes of the percentages of exon inclusion by PKA, CaMKIV, or PKCγ, each normalized to that of vector 33SB (taken as 1.0). Data are from the splicing reporter assays in Figs. 1 and 5, A–C.

The sharing of motifs between PKA and CaMKIV is also supported by that substituting the CaRRE1 core sequence with CAAAAAG, from the consensus motif of

PKA-responsive elements (Fig. 3A), retained the PKA effect (Fig. 5C). Therefore, the CaRRE1 core motif is replaceable by a PKA consensus motif.

Comparisons of these kinase effects through the elements relative to that through the vector sequence indicate that the PKA and CaMKIV effects on splicing are significantly stronger than PKCγ (Fig. 5D). Therefore PKA and CaMKIV specifically share most of these tested RNA elements in controlling alternative splicing.

The consensus motifs of the selected RNA elements are critical for kinase-dependent splicing and are significantly enriched in alternative exons

To verify that the consensus motifs are critical for kinase effects on splicing, two strongly PKA-responsive clones containing CA dinucleotides or A-rich motifs were mutated and tested in splicing reporter assays (Fig. 6A). The wild type clones showed significantly increased exon inclusion by PKA as expected (compare lanes 3 and 4, 7 and 8). Importantly, their responses to PKA were abolished or reduced by mutations in the consensus motifs (compare lanes 5 and 6, 9 and 10). Therefore, these selected motifs are essential for the PKA-dependent splicing changes.

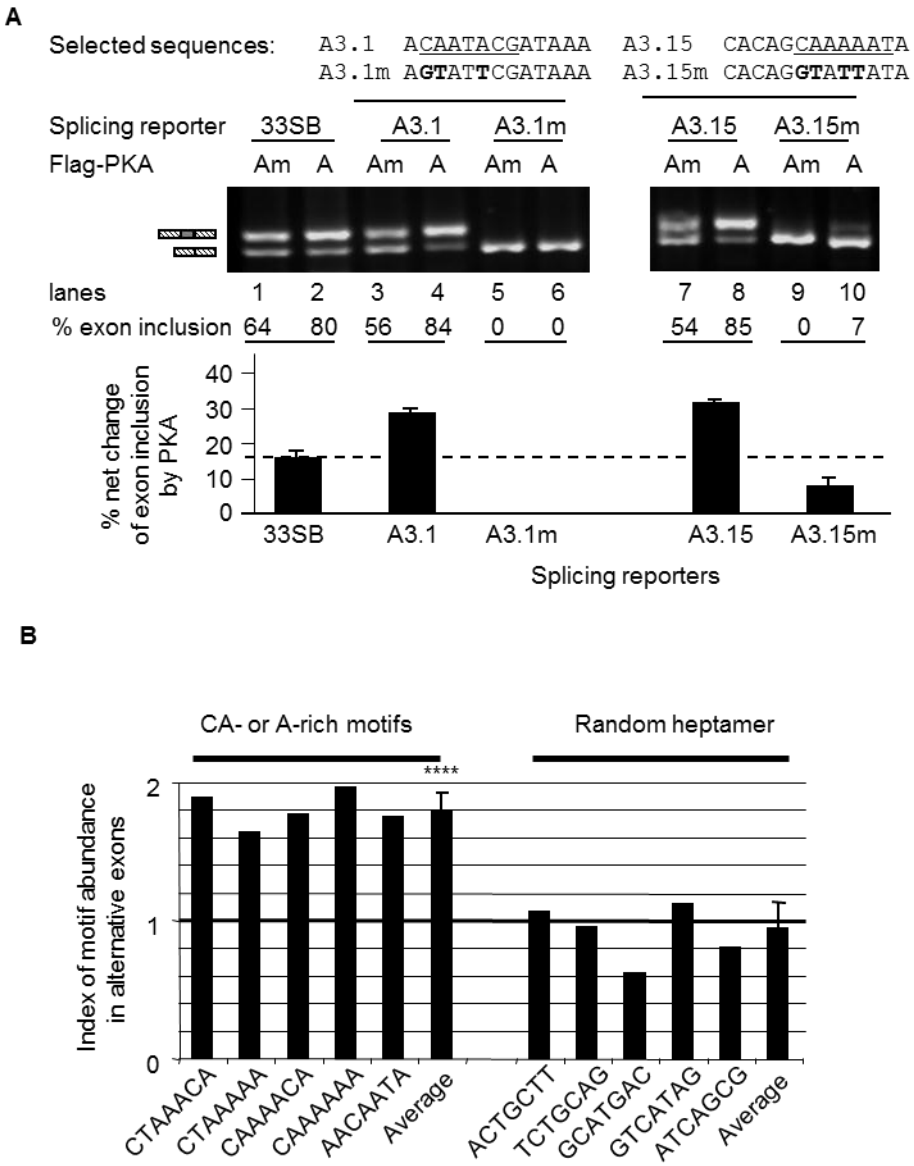

Figure. 6 The kinase-responsive heptamer motifs are critical for the kinase effect on splicing and are significantly enriched in alternative exons. **A**, splicing reporter assay with highly PKA-responsive clones and their mutants. Above the gel are the 13-nt sequences of each clone and mutated nucleotides (bold). The dotted horizontal lines in the graphs mark the baseline percentages of net changes of the vector 33SB by PKA. Below each lane are the percentages of exon inclusion and a bar graph of net changes (aligned between every two lanes) by PKA for each reporter. **B**, bar graph of motif abundance demonstrating the enrichment of the kinase-responsive CA-containing or A-rich motifs in alternative exons in comparison to the random sequences in the control group. Each motif (as indicated) was searched against 6125 alternative or 3775 constitutive exons of 7 to 500 nt in length, and the abundance index was taken as the result of the element occurrences per kilobases of alternative exons divided by the corresponding occurrences of constitutive exons. Therefore, an index number of 1 equals

the abundance of the elements in constitutive exons. The averages of the index numbers of the selected and non-selected motif groups are shown to the right of each group. ****, $p < 0.0001$, two tailed Student's t test between the two groups.

As would be expected from the above results, these motifs should be enriched in alternative exons compared with constitutive exons. We thus searched five CA-containing or A-rich heptamers from the consensus region of the PKA- or CaMKIV-responsive elements against subsets of alternative (6125 exons total) as well as constitutive (3775 exons total) exons from the published splicing databases (296-298). In total, 276 heptamers were found in the alternative exons while as only 108 found in the constitutive exons. On average, there are 45 heptamers in every 1000 alternative exons versus 29 in the same number of constitutive exons; or 38 versus 21 heptamers per 100 kilobases of alternative versus constitutive exon sequences. Relatively, all the five heptamers are present between 1.5 and 2-fold (1.8 ± 0.12, average $\pm$ S.D.) in the alternative exons over the constitutive exons (Fig. 6B). In contrast, five random heptamers all show about 1-fold (0.91 ± 0.2, average $\pm$ S.D.) in the alternative exons. Therefore, these CA-containing or A-rich motifs are significantly ($p < 0.0001$) enriched in alternative exons.

Motif-containing endogenous exons are regulated by cAMP and/or depolarization in cell culture

Of these motifs, the A-rich ones in endogenous exons have not been shown to respond to cAMP and depolarization. In examination of seven A-rich consensus motif-containing exons (from the ASAPII database), we found three exons/genes with detectable expression and two of the three were alternatively spliced (with both exon-included and -excluded products detectable) in the PC12 and GH_3 cells used.

Interestingly, both of the alternatively spliced exons were regulated by cAMP and/or depolarization (Fig. 7).

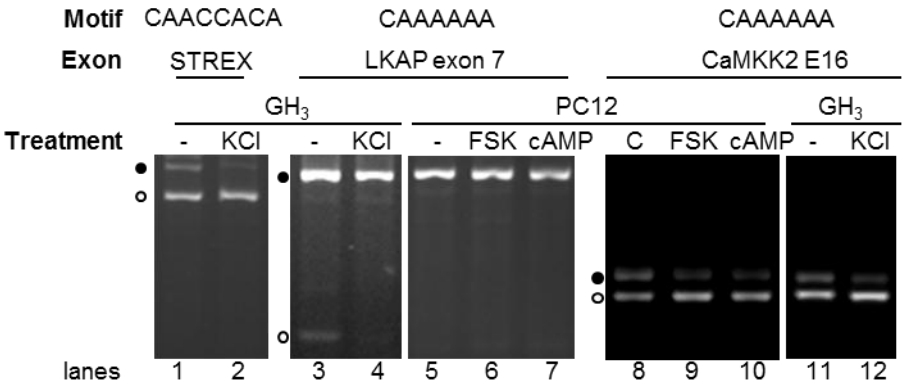

Figure. 7 Regulation of the motif-containing endogeneous exons by cAMP or depolarization in cell cultures. Inclusion levels of endogenous exons STREX, LKAP exon 8, and CaMKK2 exon 16 were analyzed by RT-PCR. Motifs and exon names are above the gels. −, nontreated. C, ethanol, as the vehicle control for forskolin; FSK, forskolin (10 μM), an activator of adenylyl cyclase; cAMP, 8-cpt-cAMP (100 μg/ml), a membrane-permeable cAMP analogue. Exon-included products are indicated by black dots, and exon-excluded products by open circles. STREX-included and -excluded products are 597 and 423 nt, respectively; LKAP, 554 nt and 125 nt; and CaMKK2, 225 nt and 182 nt. The gels are representative of three experiments

One of them, the exon 8 of the Limkain b1 (Lkap, GenBankTM accession no.: AB012133) gene encoding an autoantigen with RNA binding domains (313), contains 2 copies of CAAAAAA. This exon is differentially included in PC12 and GH_3 cells (Fig. 7, lanes 3 and 5), suggesting that its inclusion is regulated. Depolarization of GH_3 cells with KCl led to its complete inclusion (Fig. 7, lanes 3 and 4). In PC12 cells, this exon is already 100%-included before treatment and the forskolin or cAMP effect on its inclusion, if similarly promoting as the depolarization effect, cannot be assessed (lanes 5–7).

Another A-rich motif-containing exon is the CaMK kinase β1 (CaMKK2) exon 16 (314). This exon was regulated by both forskolin and cAMP, as well as by depolarization (Fig. 7, lanes 8–12), and was examined in more detail with hot-PCR and primer extension.

This exon, containing a conserved CAAAAAA motif at its 5′-end in mammalian genes (Fig. 8A), showed an inclusion level of about 37 and 36% in vehicle- and non-treated PC12 cells, respectively in hot-PCR (Fig. 8B, lanes 1 and 2). Upon treatment with PKA stimulus forskolin or cpt-cAMP, the exon inclusion level was reduced to about 21% (Fig. 8B, lanes 3 and 4). Therefore, the CaMKK2 exon 16 is likely controlled by the PKA pathway in PC12 cells. A similar effect was observed by treatment of GH_3 cells with a depolarizing concentration of KCl (25 mM, lane 6), which controls splicing through CaMKIV [4].

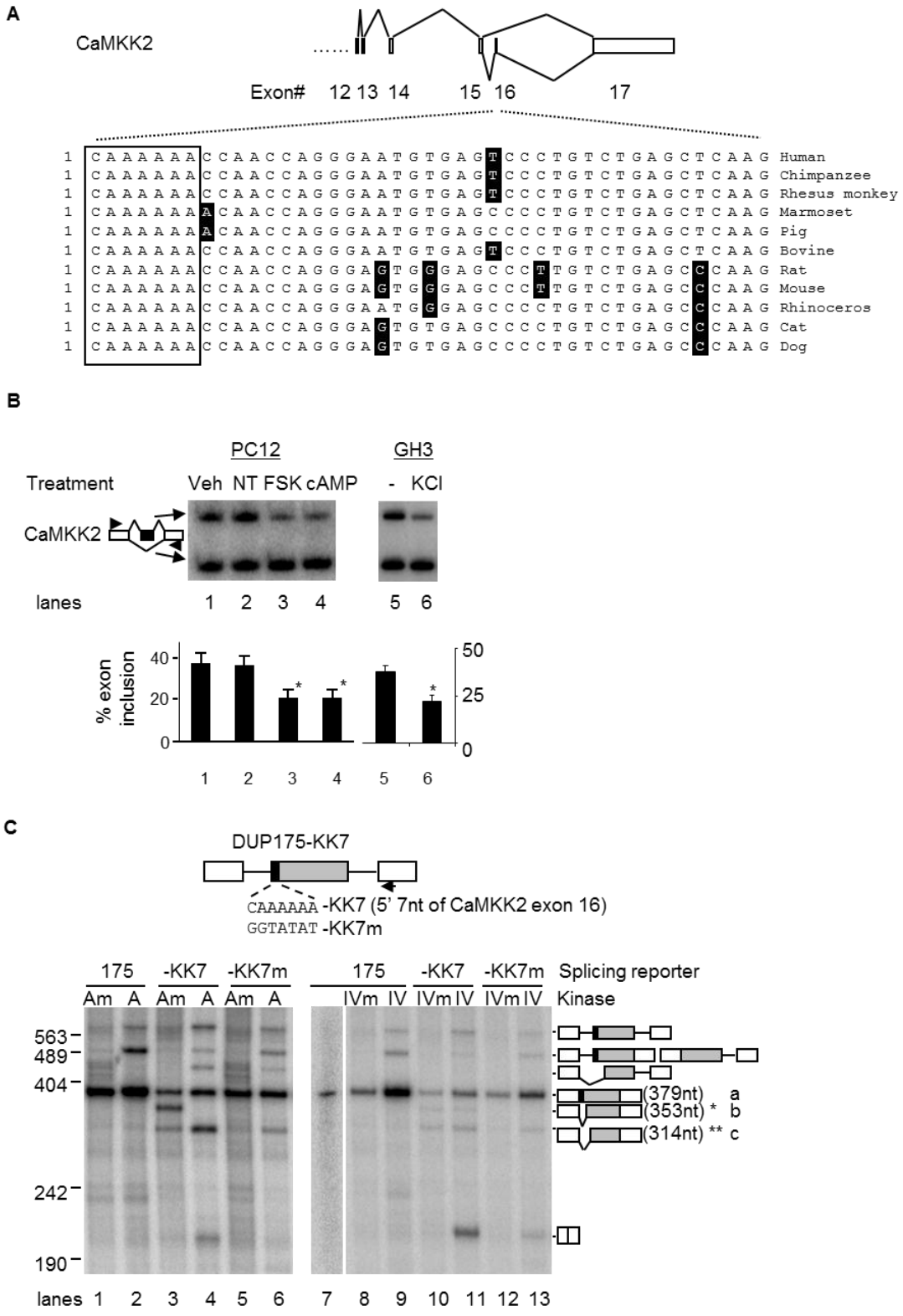

Figure. 8 An A-rich motif mediates splicing regulation of CaMKK2 by PKA and CaMKIV. **A**, diagram of splicing pattern and sequences of CaMKK2 exon 16 with the

conserved motif in mammals boxed. **B**, changes in the inclusion level of the endogenous CaMKK2 exon 16 in PC12 cells upon treatment with PKA stimuli, or in GH_3 cells upon depolarization. Shown is the phosphorimages of denaturing PAGE gels of RT-PCR products of the CaMKK2 gene expressed in PC12 cells not-treated (−) or treated with various chemicals for 7 h as indicated above the gel, or in GH_3 cells untreated (−) or treated with KCl (25 mM). Exon 16-included or -excluded product is diagrammed to the left, and a bar graph of the percent net changes is below the gel. Treatments are indicated as in the legend to Fig. 7. **C**, primer extension assay of the splicing reporters containing the CAAAAAA element from the CaMKK2 exon 16 at the 5′-end of the middle exon in the presence of PKAm (Am), PKA (A), CaMKIVm (IVm), CaMKIV (IV). Shown are phosphorimages of denaturing PAGE gels of primer extension products from mRNA of HEK293T cells transfected with the reporters and kinases as indicated above the gel. Products resulted from the pre-mRNA containing one or both unspliced introns with the deduced structures are diagrammed, based on sequencing similar bands of DUP175CaRRE1 products in other experiments; Asterisks, cryptically spliced products truncating 26 nt (*, band b) and 65 nt (**, band c) of the 5′-end of the 175-nt middle exon. Arrow, location of the primer for primer extension. The gels are representative of three experiments.

To verify the role of the CAAAAAA motif in response to the kinases, we replaced the 5′-end 7 nt of the DUP175 middle exon with the motif and tested its responses to PKA or CaMKIV in HEK293T cells (Fig. 8C). The PKA cotransfection did not induce exon skipping of the DUP175 transcripts (lanes 1 and 2). Transferring the CAAAAAA motif to DUP175 promoted two cryptically spliced products in the presence of PKA mutant (lane 3), but with barely visible complete exon-skipping products (about 7%, without counting the cryptic products, same as below). Coexpressed wild type PKA led to substantial amount of exon skipping (lane 4, about 31% exon-skipping product). Importantly, mutation of the CAAAAAA completely abolished the PKA effect (lane 6). Thus, the CAAAAAA motif is sufficient for the PKA effect on splicing. Coexpressed wild type CaMKIV induced exon skipping as well, with an even stronger effect (lane 11, about 60% exon skipping product), and also in a CAAAAAA-dependent way (lanes 12 and 13).

Taken together, these data indicate that the CAAAAAA motif mediates the control of alternative splicing of the CaMKK2 gene by PKA and CaMKIV. Therefore, the motif provides a converging point for PKA and CaMKIV to cross-talk or feedback regulate CaMKK2 through alternative splicing.

Discussion

Several Considerations about the *in Vivo* Selection System and Identified RNA Elements

Previous reports selected constitutive enhancer or silencer elements in alternative splicing (80,291). There has been no reported selection system to enrich and refine pre-mRNA elements responsive to activated protein kinases. We thus attempted to select kinase-responsive elements by modifying the method used for the constitutive enhancers (291).

The rationale of selection is based on the kinase promotion of exons containing kinase-responsive silencers in a splicing reporter already having about 65% exon inclusion (Fig. 1). As expected, most of the selected clones contain constitutive splicing silencers. The exon inclusion levels of a smaller group of enhancer-containing clones mostly either reached about 100% and not to be increased further by the protein kinase PKA or even reduced by CaMKIV (Fig. 2). Based on this observation, similar selection could be applied to enrich RNA elements responsive to other signaling protein kinases.

The selected elements appear CA- and A-rich. The consensus motif heptamers are critical for the PKA effect and are significantly enriched in alternative exons (Figs. 5 and 6), supporting their role in regulated splicing. Interestingly, CA repeats have been recently found to mediate CaMKIV-regulated splicing, with the hnRNP L as an essential

component (16). Consistently, hnRNP L is identified as a factor binding to the enriched CA-rich elements as well (Fig. 4). Therefore, CA repeats or the CA-rich elements and hnRNP L are likely preferred targets of CaMKIV in controlling splicing. The consensus sequence and the matrices should be helpful for recognizing these regulatory elements in the "sea" of the genomic sequences.

The CA-rich content also bears some similarity to the A/C-rich elements but the latter are splicing enhancers, as tested in a similar 33-nt exon derived from the β-globin gene as well (291). Moreover, the A/C-rich enhancer-binding protein YB-1 (~50 kDa) is not observed as a prominent band in UV-cross-linking (Fig. 4). The A-rich contents of these elements have not been observed in PKA- or CaMKIV-regulated splicing. A known A-rich element is the AAAAUU in the exon v5 of the CD44 gene (161), but it is an enhancer. Therefore, the similarities and differences between the selected and these known elements need further investigation.

The number of clones contained in the starting pool is about one-sixth of a full coverage (6.7×10^7 clones) of 13-nt random sequences. The CaMKIV selection did not enrich elements similar to the G-rich CaRRE2 (7). Moreover, this system does not allow the selection and assessment of constitutive splicing enhancers in kinase-promoted exons. Therefore, besides the selected elements, the existence of other sequences that are also responsive to these kinases is not ruled out. Despite these considerations, this selection does provide the first example to enrich RNA elements that are responsive to protein kinases.

Context Dependence of Cell Signal-regulated Alternative Splicing

Context dependence of pre-mRNA elements on splicing has been observed in many

cases (56,225,315,316), but opposite effects of a kinase in splicing through the same element in different minigenes have not been observed. We have shown here that, through the CaRRE1, CaMKIV inhibits exon inclusion in one minigene, DUP175ST (4), but promotes exon inclusion in another minigene, 33SB-CaRRE1 (Fig. 1). The promoting effect of CaMKIV, as well as PKA, is also seen through the SELEX-enriched elements (Figs. 2, 5, and 6). Consistent with the context-dependent effect through these elements, PKA and CaMKIV also inhibit the inclusion of the motif-containing exon in DUP175 (Fig. 8).

The molecular mechanisms underlying the context-dependence of a protein kinase through an element is unknown. In the case of SRp38, the splicing enhancing or repressing effects can be determined by the phosphorylation/dephosphorylation status of the protein (109). In our splicing reporter assays, the protein kinases used for the reporters DUP175ST and 33SB-CaRRE1 are the same (PKA or CaMKIV). Therefore, any changes of the phosphorylation status of a splicing factor, for example hnRNP L, are likely to be the same for both reporter systems. However, these reporters differ in their promoters, lengths/sequences of the middle exons, flanking exons/introns, and the locations of the CaRRE1 element relative to the splice sites (Fig. 1). Interestingly, the mCaRRE1 in DUP175 and the selected elements in 33SB have several different bound factors: PTB specific for mCaRRE1 while as the 75-kDa protein specific for the enriched element probes (Fig. 4). Moreover, a ~62-kDa protein smaller than hnRNP L is specific for the A3.15 element (Fig. 4C, lane 2). Taking these differences together with the known combinatorial control of mammalian splicing by multiple elements and influence by promoters (315,317), one possibility may involve differential recruitment of trans-acting

factors in addition to hnRNP L to the target and/or its surrounding elements/promoters by the kinases, resulting in either inhibition or promotion of splicing depending on the balance between the positive and negative regulators.

Effect of the Enriched Elements in Cells and Shared Target Elements/Exons in PKA- and CaMKIV-regulated Alternative Splicing

Similar to the previously identified depolarization-responsive elements that respond to depolarization only with multiple copies of the elements or in combination with other elements (4,6,7), a splicing reporter containing only one copy of an hnRNP L high affinity sequence, as used previously (16), was not sufficient to respond to depolarization (data not shown). It is thus likely that the CA-rich elements also require multiple copies (such as 2 copies of CAAAAAA in the LKAP exon 8, Fig. 7) or with the help of other elements in alternative exons to confer response to endogenous signals.

The ASAPII exons containing these enriched motifs have average MaxEntScan scores of 7.5 (±2.98) for the 3′-splice sites and 7.9 (±2.8) for the 5′-splice sites (n = 263) as measured according to the maximum entropy model (318). These scores are not significantly lower than that of the constitutive exons (7.9 ± 3.6, 8.3 ± 3.1, for the 3′- and 5′-splice sites, respectively) (319). The relatively strong splice sites may make the exons mostly included as the LKAP exon but with the presence of the CA- or A-rich elements can still be regulated by the activation of protein kinases.

The cAMP and Ca^{2+} signaling pathways are known to converge at the Ser133 of the CREB protein in transcriptional control (320). Their effects on alternative splicing through common RNA elements have not been observed. Interestingly, the 33SB-derived reporters used here demonstrate preferred responses of the CA- and A-rich elements to

both kinases, but not PKCγ. The control of alternative splicing by PKA and CaMKIV through the same RNA elements adds another possible point of convergence between these two important signaling pathways.

Among the common target elements shown here however, their responses to PKA and CaMKIV overexpression are not always the same (Fig. 5D). Specific elements with variations in nucleotide sequences are preferred by each of the two kinases (Fig. 5D). Related to this variation, in the exon of a DUP175-derived splicing reporter, a CA repeat element strongly responded to CaMKIV but barely to PKA (16), unlike in the 33SB-derived reporters (Figs. 1, 5, and 6). In this case, the reporter context appears to play an important role on the PKA effect. The shared, but differential, splicing regulation through these RNA elements by PKA and CaMKIV likely helps to refine gene expression profiles tailored for precise functions of genes and cells.

PKA is known to control the CaMKK1 (CaMKKα) through direct protein phosphorylation (321-326). The control of CaMKK2 splicing by PKA and CaMKIV through a common RNA element adds a novel way of signal cross-talk/feedback between the PKA and CaM kinase pathways. Compared with direct phosphorylation, one likely advantage of this splicing regulation is that it can be temporally longer and involved in cell functions requiring gene expression to take effect. Therefore, this regulation provides an example where the selected RNA element could play a role in regulating the important signaling networks in cells.

Summary

CaMKIV- and PKA-responsive RNA elements were isolated through our in vivo selection system. Identified responsive elements are mostly splicing silencers. Highly responsive elements appear as CA- or A-rich. The CA-rich elements are similar to the known CaRRE1 by their interactions with hnRNP L. Moreover, alternative exons carrying an A-rich element are regulated by depolarization and cAMP.

Contributions

I am the co-first author of this paper. To screen for kinase-responsive elements from a pool of 13-nt random sequences, I prepared and sent at least 54 plasmids for sequencing, which were then used for testing kinase response by my colleague, Mr. Li (Fig.s 2B and 3B). To examine kinase responses in splicing reporter minigenes, I made the kinase-dead mutant of PKA and tested its effect on phosphorylation of PTB (Figure 1B). To test cell signaling responses of endogenous exons containing the consensus motifs, I did splicing assays by semi-quantitative RT-PCR (Fig.s 7 and 8B). I cloned the CAAAAAA motif and its mutant into a splicing reporter minigene and then performed splicing assays by primer extension (Fig. 8C). I also revised the manuscript with my advisor, Dr. Jiuyong Xie, before its submission.

CHAPTER V

A Conserved Serine of Heterogeneous Nuclear Ribonucleoprotein L (hnRNP L) Mediates Depolarization-regulated Alternative Splicing of Potassium Channels

Introduction

Following the finding detailed in Chapter IV that the CA-repeat motif is a characteristic of CaRREs and hnRNP L is a binding factor, our lab further demonstrated that hnRNP L is an essential mediator for CaMKIV-regulated alternative splicing through CA repeats (16). Moreover, hnRNP L is not only a constitutive repressor of the STREX, but also one factor responsible for partial repression of the exon by depolarization. The involvement of hnRNP L in the depolarization-induced splicing change suggests that L itself is regulated. However, it is not clear, as yet, how hnRNP L modulates the cell signal-responsive repression of STREX.

Therefore, we decided to verify the critical role of hnNRP L and study the underlying mechanism by focusing mainly on whether and how hnRNP L is regulated by the depolarization/CaMKIV signaling pathway, as well as its effect on the regulated-splicing of the STREX.

This part of work has been recently published in THE JOURNAL OF BIOLOGICAL CHEMISTRY (311).

Results

HnRNP L and L-like proteins are required for depolarization-induced repression of the STREX exon

Our previous studies indicate that hnRNP L binds the CaRRE1 to repress STREX splicing (Fig. 9) but that knockdown of hnRNP L alone did not abolish the depolarization effect (16), suggesting the involvement of other factors such as hnRNP LL (135,254). We thus carried out RNA interference against hnRNP L as well as LL proteins by expressing short hairpin RNAs against either hnRNP L (shL), LL (shLL) or both (shL + shLL) in GH_3 pituitary cells (Fig. 9D). Each shRNA specifically knocked down its expected target but not the other proteins examined. Knocking down either hnRNP L or LL protein alone promoted (shL) or inhibited (shLL) the STREX exon inclusion in non-treated cells (NT), suggesting that hnRNP L and LL may have differential effect on STREX inclusion in un-depolarized conditions. But neither knockdown abolished the repression effect by depolarization (Fig. 9E). In contrast, knocking down both hnRNP L and LL nearly abolished the depolarization effect (shL+shLL, $p = 0.2$). Thus, hnRNP L and LL are required for the repression of STREX by depolarization, but neither one by itself is sufficient to mediate this effect in GH_3 cells. Because our previous work has demonstrated a critical role for the CaMKIV phosphorylation of hnRNP L in its interaction with the CaRRE1 element (16), hereafter we will focus on hnRNP L as an example to delineate the pathway from CaMKIV to the spliceosome.

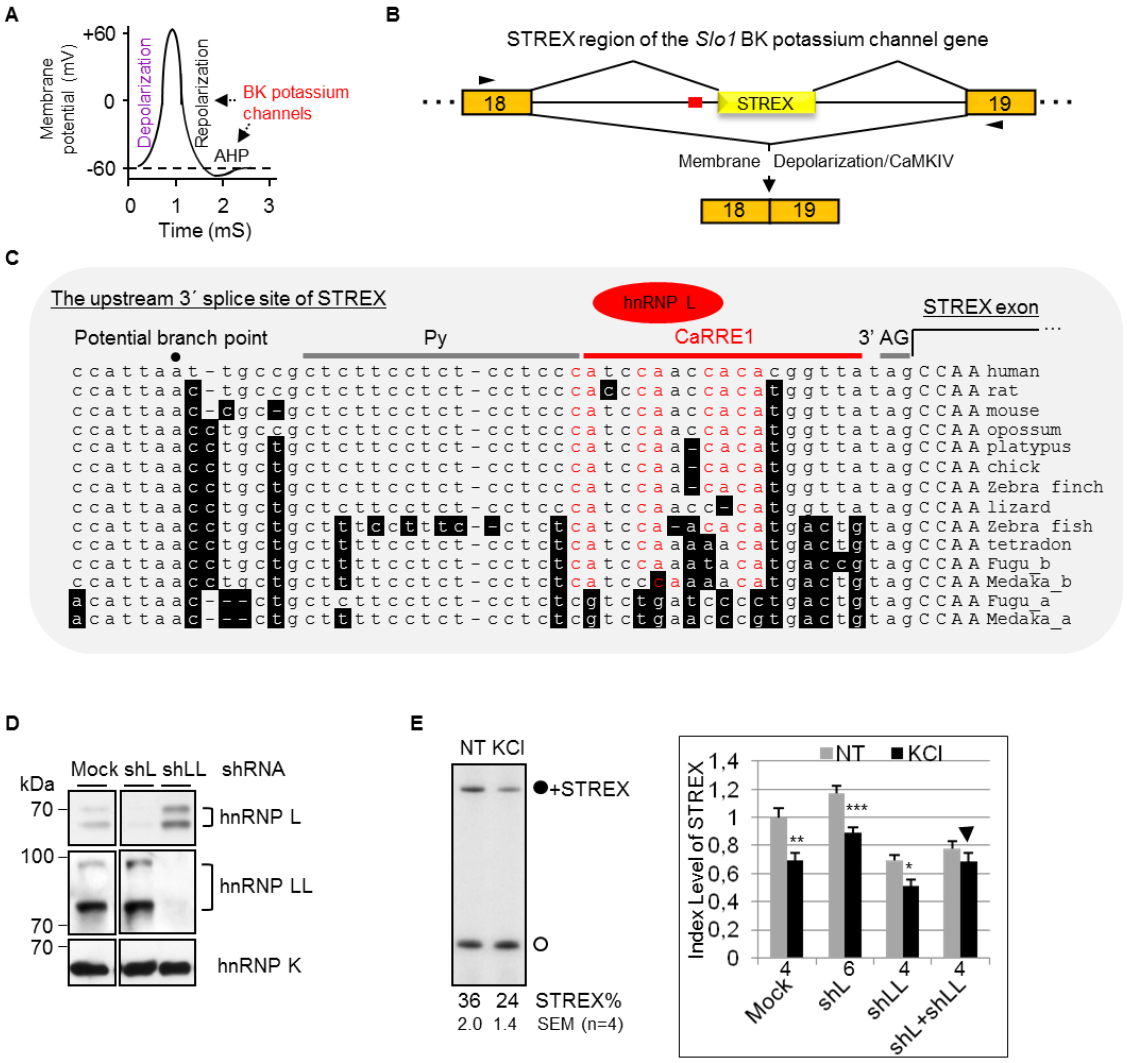

Figure 9. Essential role of hnRNP L and L-like proteins in depolarization-induced repression of the STREX exon of Slo1 BK potassium channel transcripts. A, diagram of the physiological context of the splicing regulation in neurons or endocrine cells including GH_3 cells. Shown is the relationship between membrane depolarization and BK channel function in an action potential. The two arrows point to the critical effect contributed by the opening of BK channels upon depolarization. In these cells, both STREX-included and excluded variants have been observed and regulated by depolarization. The regulation is expected to change the BK channel properties and thus the action potentials/firing properties of the cells. AHP: afterhyperpolarization. **B,** diagram of the pre-mRNA region around the STREX exon (not to scale). In GH_3 cells and neurons, inclusion of the STREX exon is repressed upon membrane depolarization through the CaMKIV pathway, resulting in more *Slo1* transcripts with exons 18 and 19 joined directly. Red bar: CaRRE1 element. Arrow heads: location of PCR primers rSlo1 and rSlo2. **C**, hnRNP L (red oval) binding region within the CaRRE1 (red bar), and the element's unusual "insertion" between the otherwise adjacent polypyrimidine tract (Py) and 3′ AG (gray bars), within the upstream 3′ splice site of STREX among species from

fish to human. The hnRNP L-preferred "ca" dinucleotides are in red. Nucleotides different from human sequence are shaded. Fugu and medaka fish each has two paralogous *Slo1*/STREX (305), one of which (Fugu_a) does not contain the CaRRE1 element (5). **D**, efficiency and specificity of the lentivirus-mediated RNA interference against hnRNP L (shL) or LL (shLL). Shown are Western blots using antibodies against either hnRNP L, LL or K (protein loading control). Note that two bands corresponding to hnRNP L and two bands corresponding to hnRNP LL are specifically detected by anti-hnRNP L and -LL antibodies and knocked down by the specific shRNA, respectively. The sizes of the hnRNP LL bands, bigger than the ~60kDa human hnRNP LL in the supplier's datasheet, are likely from alternative splicing in the rat GH_3 cells. Molecular size marker (kDa) is shown to the left. **E**, effect of RNA interference against the hnRNP L/LL proteins on depolarization-induced repression of STREX. On the left is a representative PAGE gel of semiquantitative RT-PCR products with STREX inclusion (●)/exclusion (○) in non-treated (NT) or depolarized (KCl, 50mM) GH_3 cells. On the right is a bar graph (mean ± SEM, n = 4 or 6, as indicated below each pair of columns) of STREX inclusion levels of various groups normalized to the NT sample of the mock-transduced group. Black arrowhead points to the loss of significant changes in the KCl treated samples compared to its paired NT samples. p-values are: ***: $p<0.001$, **: $p<0.01$, *: $p<0.05$, in two tailed Student's t-test.

The highly conserved Ser513 of hnRNP L is a phosphorylation target of CaMKIV

To determine how hnRNP L is regulated, we examined its changes upon depolarization. Western blot analysis did not detect changes in its protein level in KCl-treated GH_3 cells (data not shown). We previously found that hnRNP L was phosphorylated when coexpressed with a constitutively active CaMKIV (16). The phosphorylation resulted in increased binding to CaRRE1 and is phosphatase-sensitive (16). We thus mutated hnRNP L at several conserved amino acid residues within CaMKIV consensus sequences (Arg-X-X-Ser/Thr) (327,328), including Ser326 and Ser513 (Fig. 10, A and B). The effect of the mutations on phosphorylation by CaMKIV in HEK293T cells was analyzed by phosphopeptide mapping. Mutation of Ser326 did not result in different patterns of phosphopeptides from that of the wild type hnRNP L (Fig. 10C). However, mutation of Ser513, whose consensus peptide is highly conserved among

chordates and arthropods (Fig. 10B), completely abolished the major phosphopeptides (Fig. 10C). Therefore, Ser513 is likely a target of CaMKIV.

To verify that Ser513 is a direct target of CaMKIV, we carried out in vitro phosphorylation followed by phosphopeptide mapping with synthetic peptides containing P-Ser513 (ERSS(p-S)GLLEW, p-S: phosphorylated Ser513) and unphosphorylated Ser513 (ERSSSGLLEW). The CaM kinases in the reaction were phosphorylated in the absence of peptide substrates as expected (Fig. 10D, left panel). Addition of the peptide P-Ser513 as a substrate resulted in a similar pattern (Fig. 10D, center panel). In contrast, addition of the unphosphorylated Ser513 peptide resulted in a strong ^{32}P-labelled spot not seen in the background (Fig. 10D, right panel, compared with the left panel). Therefore, Ser513 is a direct target of CaMKIV.

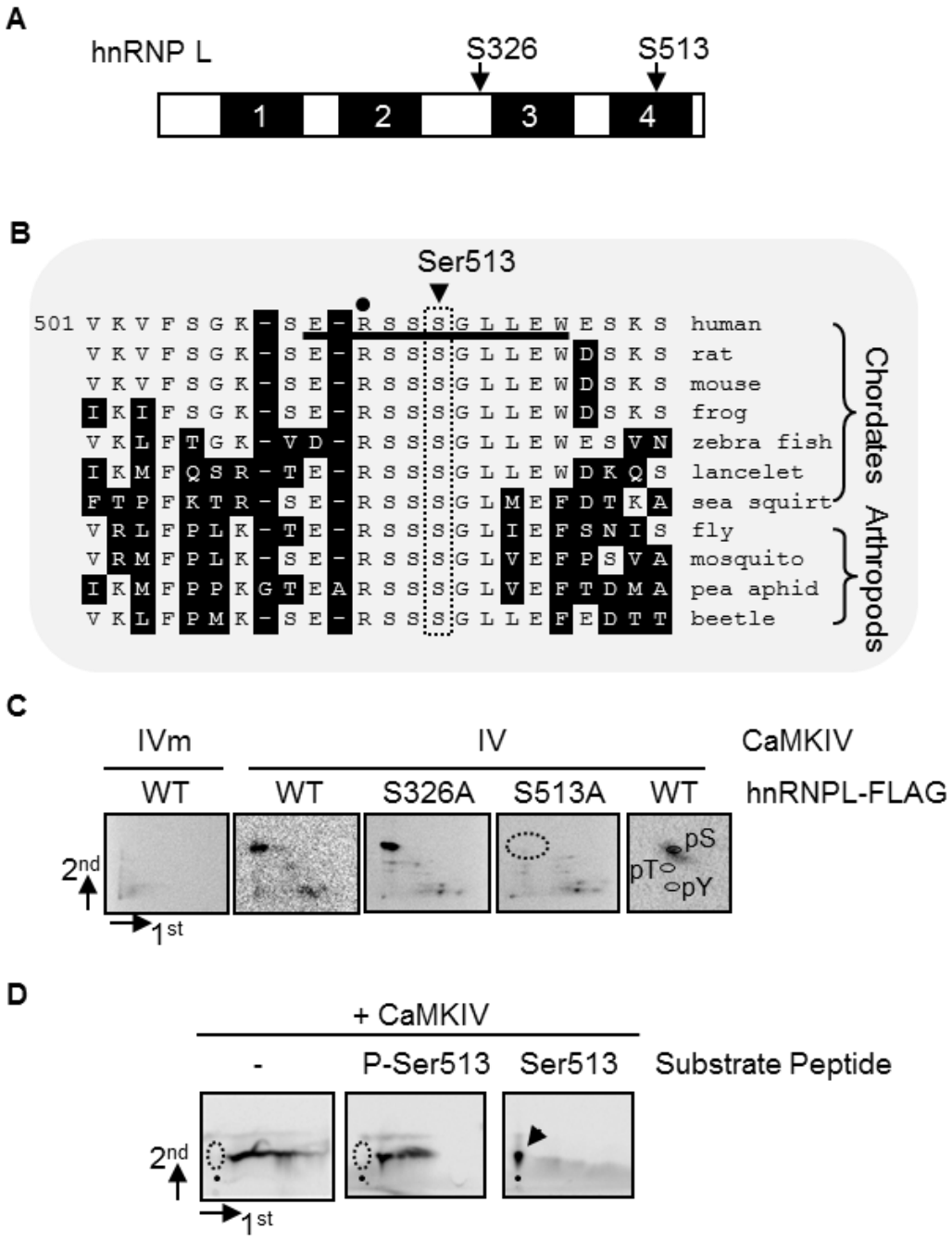

Figure 10. The highly conserved serine 513 of hnRNP L is a major phosphorylation target of CaMKIV. **A,** diagram of the RNA recognition motif (RRM) domains (dark) of hnRNP L protein and locations of two potential CaMKIV target serines (not to scale). **B**, serine 513 (S513 or Ser513) is highly conserved between phyla chordata and arthropoda. Shown are amino acid sequences around Ser513 in an alignment of hnRNP L proteins from different species. Amino acids different from human hnRNP L are shaded. The black dot indicates the critical arginine residue of the CaMKIV target consensus. The heavy line indicates the peptide used for making the anti-pSer513 antibody. The common

names of the species are indicated to the right. Their binomial nomenclatures are: human*: Homo sapiens;* rat*: Rattus norvegicus;* mouse*: Mus musculus;* frog*: Xenopus tropicalis;* zebrafish*: Danio rerio;* lancelet*: Branchiostoma floridae;* sea squirt*: Ciona intestinalis;* fly*: Drosophila melanogaster;* mosquito*: Anopheles gambiae;* pea aphid*: Acyrthosiphon pisum;* beetle*:Tribolium castaneum.* **C**, two dimensional phosphopeptide mapping of [^{32}P] orhtophosphate-labelled hnRNP L-FLAG (WT) or its mutant proteins coexpressed with inactive (IVm) or constitutively active CaMKIV (IV) in HEK293T cells. The position of the phosphopeptide(s) abolished in the S513A mutant sample is circled in dotted line. The positions of standard phospho-serine (pS), -threonine (pT) and -tyrosine (pY) are shown in smaller circles. The sequence and direction of the two dimensions are as marked beside the first panel. **D**, phosphopeptide mapping of [γ-^{32}P]ATP-labeled synthetic peptides by CaMKIV *in vitro*. See text or Methods for peptide sequences. The background signals with substrate peptides are apparently from the phosphorylation/autophosphorylation of CaM kinases used. The dotted circles in the left two panels indicate the absence of the ^{32}P-labelled-phospho-Ser513 peptide (arrowhead) seen in the right panel. The locations of the sample loading spots used as references to pinpoint the expected location of the phospho-Ser513 peptide are indicated with black dots.

Ser513 is essential for depolarization-regulated splicing of endogenous STREX in GH_3 cells

To verify the phosphorylation of endogenous Ser513 and its regulation in cells, we made phospho-Ser513-specific antibody and validated its specificity by Western blot analyses (Fig. 11A). The antibody detected a strong signal from the hnRNP L-FLAG protein coexpressed with CaMKIV in contrast to that with CaMKIVm. Importantly, the signal was abolished by the S513A mutation. Therefore, the antibody is specific for the phospho-Ser513 epitope.

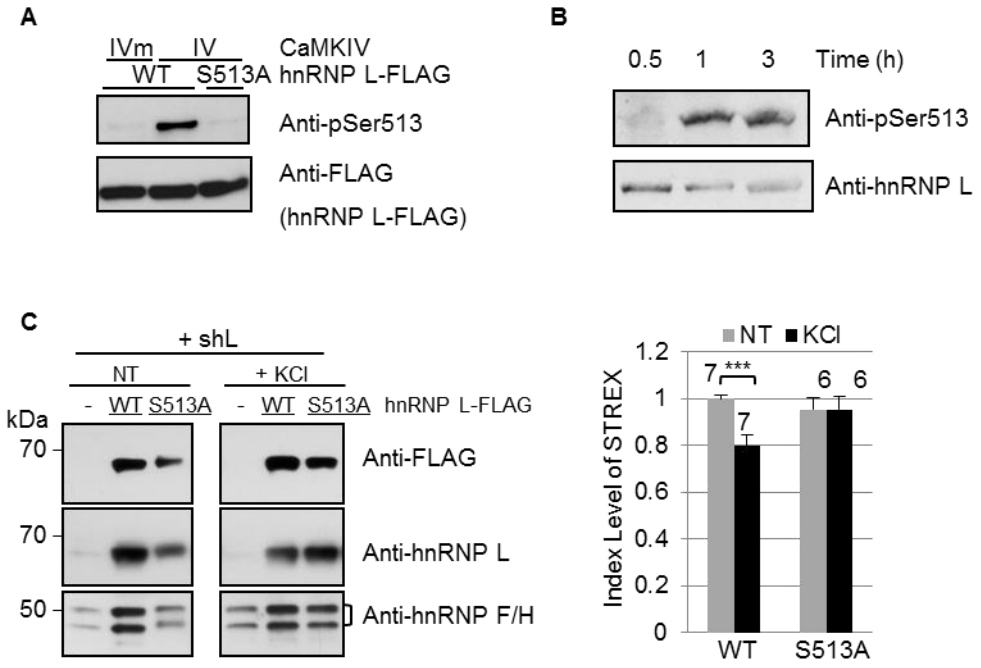

Figure 11. Essential role of Ser513 in depolarization-induced repression of the STREX exon in GH_3 cells. **A**, specificity of the anti-phospho-Ser513 antibody. Shown is a Western blot of protein samples of HEK293T cells co-expressing hnRNP L-FLAG (WT) or its S513A mutant (S513A) with CaMKIVm or CaMKIV, using antibodies against phospho-Ser513 or FLAG. **B**, effect of depolarization on phospho-Ser513 in GH_3 cells. Shown are Western blot analyses of depolarized (KCl, 50mM) GH_3 samples at different time points after KCl addition, using antibodies against phospho-Ser513 (anti-pSer513) or hnRNP L. **C**, effect of S513A mutation on depolarization-induced repression of STREX exon in GH_3 cells. Left panel, Western blot analyses demonstrating the expression of the hnRNP L-FLAG proteins in shL-expressing cells with hnRNP F/H as loading controls. Right panel, a bar graph of the normalized STREX inclusion levels (mean ± SEM, n = 7 or 6 for each pair of samples as indicated) in the hnRNP L-FLAG WT or S513A mutant-complemented GH_3 cells knocked down of hnRNP L. For RT-PCR, primers as in Fig. 9E were used.

We next examined whether the phospho-Ser513 was induced by depolarization with the phospho-Ser513-specific antibody. The phospho-signal was strongly detected at 1 and 3 hours after depolarization by KCl treatment of GH_3 cells (Fig. 11B) in comparison to the loaded hnRNP L protein level in the same blots. Therefore, increased Ser513 phosphorylation can be induced by membrane depolarization.

To determine whether increases in Ser513 phosphorylation play a role in splicing, lentiviral systems were used to express either hnRNP L-FLAG or its S513A mutant together with shL (to deplete endogenous L proteins) in GH_3 cells (Fig. 11C, left panel). Without treatment (NT), the STREX levels in cells expressing hnRNP L-FLAG or its mutant are similar (Fig. 11C, right panel). Upon depolarization by KCl, the STREX level in cells expressing the wild type hnRNP L-FLAG was significantly reduced ($n = 7$ pairs, $p < 0.001$). However, this reduction was abolished in cells expressing the S513A mutant ($n = 6$ pairs, $p = 0.98$). Therefore, Ser513 is essential for depolarization to repress the endogenous STREX exon in GH_3 cells.

Ser513 phosphorylation enhances hnRNP L binding to CaRRE1 and interferes with the binding of U2AF65

To understand the role of Ser513 phosphorylation in splicing repression, the effect of phosphorylation on the interaction of hnRNP L with the CaRRE1 RNA was examined on the basis of the location of Ser513 within the RNA recognition motif 4 (RRM4, Fig. 10A). For this experiment, unphosphorylated, phosphorylated or S513A mutant hnRNP L-FLAG were purified from HEK293T cells by immunoprecipitation, verified them by Western blot analysis (Fig. 11A), and examined their cross-linking efficiency with the CaRRE1 RNA probe in solution (Fig. 12, A and B). The unphosphorylated hnRNP L cross-linked to the probe, as expected. However, the phospho-hnRNP L cross-linked much more strongly. In contrast, the mutation S513A abolished this stronger effect and resulted in even less binding than the unphosphorylated wild type. Taken together, the S513 phosphorylation within the RRM4 of hnRNP L by CaMKIV is essential for

enhanced interaction with the CaRRE1 element within the upstream 3′ splice site of STREX.

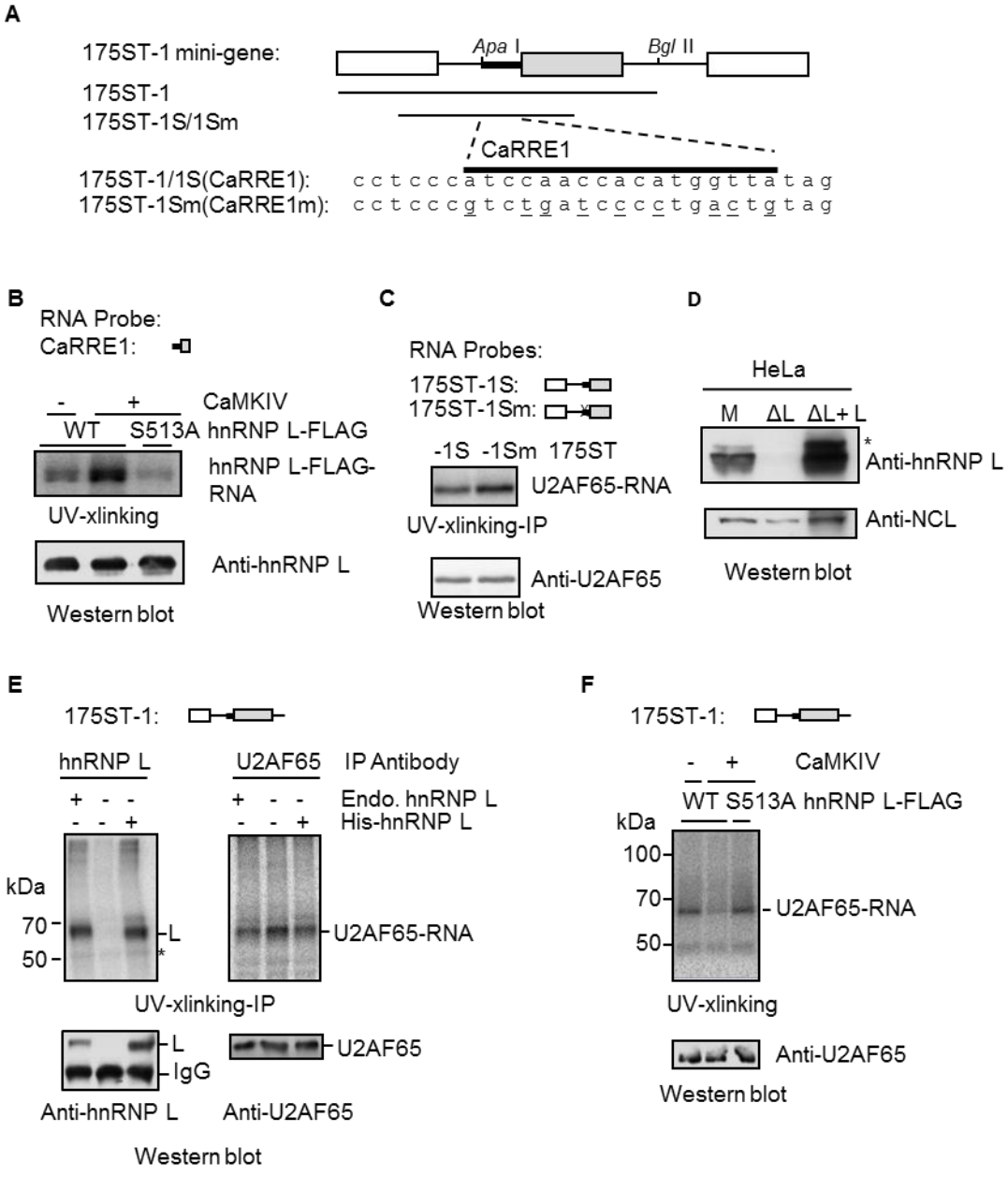

Figure. 12. Effect of Ser513 phosphorylation on hnRNP L interaction with CaRRE1 and U2AF65 binding to the upstream 3' splice site of STREX. **A**, diagram of the 175ST-1 plasmid. Thin lines represent introns, and the boxes represent exons.The thick line corresponds to the 3' splice site of STREX carrying CaRRE1. 175ST-1/1S/1Sm and CaRRE1 RNA probes (dashed lines) were *in vitro* transcribed using plasmids 175ST -

1/1m or PCR fragments derived from them as templates (see methods for details). In 175ST-1Sm, CaRRE1 element was changed to the corresponding "Fugu_a" sequence. The mutated nucleotides are underlined. Heavy bar: CaRRE1 element as in Fig. 9C. **B**, Ser513 phosphorylation enhances hnRNP L binding to the RNA probe containing CaRRE1 (top panel, heavy black bar). In the center panel are phosphorimage signals of ^{32}P-labelled mouse CaRRE1 RNA probe UV-cross-linked to 100 ng of the hnRNP L-FLAG WT or mutant S513A, which were purified by immunoprecipitation from HEK293T cells coexpressed with (+) or without (-) constitutively active CaMKIV. The bottom panel shows a Western blot analysis of input hnRNP L-FLAG. **C**, effect of CaRRE1 mutations in the 175ST-1S transcript (top panel) on U2AF65 binding to the upstream 3' splice site of STREX. Shown in the center panel are representative phosphorimage signals of SDS-PAGE gels of immunoprecipitated U2AF65 from HeLa nuclear extracts cross-linked to 175ST-1S or its mutant 175ST-1Sm. The CaRRE1 (heavy black bar) in the mutant was replaced by the corresponding Fugu_a sequence. The protein level of U2AF65 in the same gel is shown below. **D**, depletion of hnRNP L. Mock-depleted (M), hnRNP L-depleted (ΔL), and depleted/His-hnRNP L complemented (ΔL+L) nuclear extracts were analyzed by Western blot probing with anti-hnRNP L (upper panel). Nucleolin (NCL) was used as a loading control (lower panel). Asterisk, the purified His-hnRNP L as two bands: the upper one is the full length protein, and the lower, main one is cleaved of the His-tag, as confirmed by separate Western blot analyses. **E**, effect of hnRNP L depletion/add-back on U2AF65 binding to the upstream 3' splice site of STREX. The left panel shows the efficient interaction of endogenous and His-hnRNP L with CaRRE1 WT probe 175ST-1 RNA probe. The upper image represents the phosphorimage signals of a PAGE gel of immunoprecipitated hnRNP L cross-linked to the 175ST-1 probe (top panel) in HeLa nuclear extracts mocked-depleted, hnRNP L-depleted or depleted/His-hnRNP L complemented. Asterisk, a nonspecific band. The lower image is a Western blot analysis of the same gel showing efficient immunodepletion of hnRNPL. The right panel shows the representative hnRNP L depletion/add-back effect on U2AF65 binding to the probe in similar experiments. Endo. hnRNP L: endogenous hnRNP L. The lower image is a Western blot analysis of the same gel showing the total U2AF65 protein level in each lane. **F**, effect of Ser513 phosphorylation on U2AF65 binding to the CaRRE1. Shown is a representative gel of phosphorimage signals of U2AF65 in the presence of purified different phosphorylation states of hnRNP L coexpressed with (+) or without (-) CaMKIV. U2AF65 used here was purified by immunoprecipitation from HeLa cell nuclear extract. Below the image is a Western blot analysis of the same gel showing similar U2AF65 protein levels. Gels representative of at least three experiments.

Because the CaRRE1 element is "inserted" between the Py tract and 3' AG (Fig. 9C) (5,16), binding sites for the constitutive heterodimeric splicing factors U2AF65 and U2AF35 (329), respectively, it is reasonable to speculate that regulated hnRNP L binding to CaRRE1 inhibits interaction of U2AF65 with the upstream Py tract of

STREX. To test this hypothesis, we first mutated the CaRRE1 to the corresponding non-CA containing Fugu_a sequence (CaRRE1m, see also Fig. 9C) and examined U2AF65 interaction with longer CaRRE1-containing RNA probes 175ST-1S and its mutant 175ST-1Sm (Fig. 12C). Compared with the wild type, the mutant probe cross-linked more strongly to U2AF65 in HeLa nuclear extracts. This difference thus suggests that the presence of CaRRE1, and likely hnRNP L as well, is inhibitory to U2AF65 binding to the upstream Py tract.

To determine the effect of hnRNP L in the control of U2AF65 binding, we carried out immunodepletion/add-back experiments using HeLa nuclear extracts either mock-depleted, hnRNP L-depleted, or depleted/His-hnRNP L complemented (Fig. 12D). Upon UV cross-linking of these extracts with an RNA probe 175ST-1, similar to 175ST-1S, endogenous hnRNP L and add-back His-hnRNP L efficiently bound to the RNA with almost complete loss of interaction in the depleted extract (Fig. 12E, left panel). In contrast, U2AF65 binding was enhanced upon depletion of endogenous hnRNP L (Fig 12E, right panel). The enhancement was inhibited by His-hnRNP L addition. Therefore, hnRNP L inhibits U2AF65 binding to the upstream 3′ splice site.

To determine whether Ser513 phosphorylation played a role in U2AF65 binding to the 3′ splice site, we used the hnRNP L-FLAG proteins to carry out similar UV cross-linking with purified U2AF65 from HeLa nuclear extracts. The phosphorylated hnRNP L-FLAG strongly reduced U2AF65 binding compared to the unphosphorylated one (Fig. 12F). More importantly, the S513A mutation abolished this effect. Therefore, Ser513 phosphorylation of hnRNP L enhances its ability to inhibit U2AF65 binding to the 3’ splice site.

Taken together, the UV cross-linking experiments (Fig. 12) indicate that Ser513 phosphorylation enhances hnRNP L binding to the CaRRE1 element and interferes with U2AF65 binding to the upstream Py tract.

Discussion

In summary, these data demonstrate that a highly conserved CaM kinase target Ser513 of hnRNP L is essential for membrane depolarization to regulate the alternative splicing of BK potassium channels. The Ser513 phosphorylation controls the interaction of a critical component at the early steps of spliceosome assembly with the pre-mRNA (Fig. 13). This not only provides a direct functional link between a Ca^{2+} signaling kinase and a splicing regulator but also a defined pathway from membrane depolarization to the constitutive splicing machinery when considered with previous studies (4,5,16,56). This regulation forms a unique molecular basis for how depolarization could modulate the splice variant compositions and functions of potassium channels.

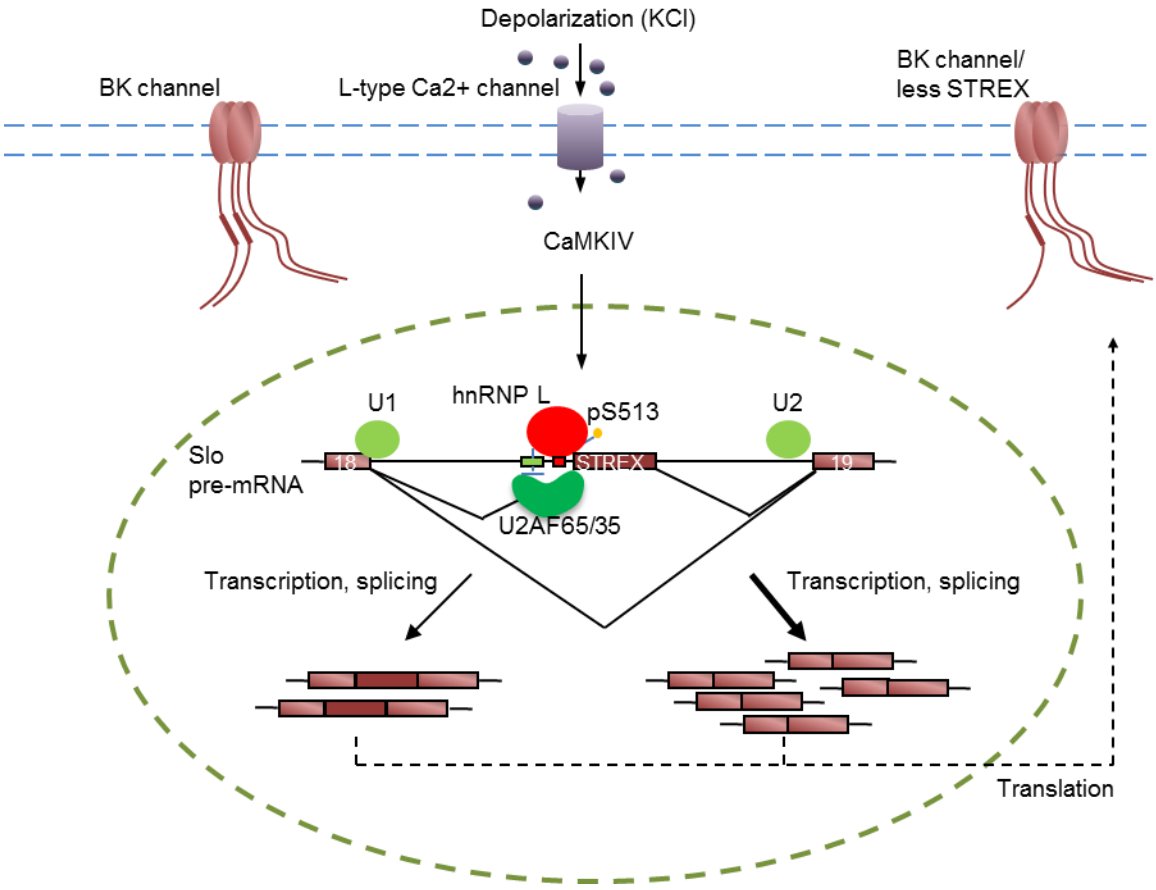

Figure 13. Summary for the role of Ser513 phosphorylation of hnRNP L in mediating depolarization-regulated splicing of the Slo1 BK channels. Membrane depolarization of GH_3 or neuronal cells by high concentrations of KCl (25~50mM) activates the L-type calcium channels (5) and the Ca^{2+}/CaMKIV pathway (4), which leads to Ser513 phosphorylation (Fig. 10). This modification enhances hnRNP L binding to the CaRRE1 element between the Py tract and AG of the upstream 3' splice site of the STREX exon, interfering with the binding of U2AF65 to the Py and thus recognition of the 3' splice site, leading to STREX skipping and joining of the flanking exons. More importantly, the STREX skipping produces BK channels with altered properties, including lower Ca^{2+}/voltage sensitivity as well as lower sensitivities to other factors such as PKA, and thus different electrical properties that are expected to change the cellular excitability upon following waves of electrical stimulation. Darker brown bar: the STREX; green bar: the Py tract; red bar: CaRRE1; solid purple circle: Ca^{2+}.

Through this modulation, depolarization of excitable cells is expected to induce a different combination of variant subunits to form tetramer channels that can alter the repolarization/afterhyperpolarization and thus firing properties of following waves of

action potentials (Fig. 13). As proposed previously (4,56), this regulation likely contributes to the fine-tuning of the long term changes such as electrophysiological memory in neurons (330).

With this critical site identified, how the Ser513 phosphorylation within an RNA recognition motif enhances RNA binding by hnRNP L would be an interesting question for future investigations. Skrisovska *et al.* (331) demonstrated that RNA recognition motifs of hnRNP L interact with each other. Thus, one possibility would be that RRM4 acts as a regulatory domain which upon Ser513 phosphorylation promotes the binding of the RRMs to their cognate sequence elements.

The highly conserved nature of the target Ser513 site (Fig. 10B), the extensive alternative splicing of BK and other ion channels (56), and the many other roles of hnRNP L in RNA metabolism (82,114,124,243,257,332-335), together make it worthwhile to explore a wider role of phospho-Ser513 in excitable cells.

HnRNP L, as a global alternative splicing regulator (82), appears to act in different ways to control alternative splicing. Besides its role as a splicing enhancer when it binds close to the 5' splice site (82), it also acts as a splicing repressor. For the repression of the variable exons 4, 5, and 6 of the CD45 transcripts, hnRNP L binds to the exonic splicing silencer element to repress splicing after the recruitment of U2AF65 (335). For the exon 20 of the TJP1 transcripts, hnRNP L acts through a CA-rich element within the Py tract of the 3' splice site to compete with U2AF65 (257). For STREX, hnRNP L inhibits the binding of U2AF65 (Fig. 12E). However, because the CaRRE1 element bound by hnRNP L is mainly downstream of the Py tract (Fig. 9C), the mechanism of this inhibition remains to be investigated.

Besides hnRNP L, its paralogue hnRNP LL is also required for the regulation of STREX splicing by depolarization (Fig. 9E). Moreover, PTB binds to the upstream 3' splice site of STREX (16). Thus, in response to membrane depolarization, the level of STREX inclusion is likely determined by the interplay among at least three regulatory proteins hnRNP L, LL, PTB, and the constitutive splicing factor U2AF65. Further characterization of these additional factors and their modifications in the regulation is necessary to uncover the underlying molecular mechanisms.

Summary

Phosphorylation of hnRNP L Ser513 is regulated by the depolarization-induced CaMKIV pathway. This depolarization-mediated phosphorylation enhances RNA binding of L and strengthens its splicing-inhibiting effect through interfering with 3' splice site recognition by the constitutive factor U2AF65. Taken together, it is likely that hnRNP L exerts its repressive effects on depolarization-induced splicing of specific target exons through, at least partly, regulated phosphorylation at its Ser513. These thus provide the first link between a specific phospho-serine site of a splicing regulator and its function in Ca^{2+} signaling-triggered alternative splicing.

Contributions

I co-first authored this paper. I constructed the pFG12-shL plasmids and prepared lentiviruses from them and the pLKO.1-shLL plasmids. I then infected GH_3 cells with either lentivirus or both to knock down expression of endogenous hnRNP L and/or LL (Fig, 9D). I mutated the serines 326 and 513 of the hnRNP L-FLAG into alanines and carried out *in vitro* phosphorylation followed by phosphopeptide mapping with synthetic peptides containing phospho-Ser513 or unphosphorylated Ser513 (Fig. 10D). I prepared

lentiviruses expressing either wild-type or the S513A mutant hnRNP L-FLAG, and applied them to knockdown and complementation assays, followed by RT-PCR (Fig. 11C). I expressed hnRNP L-FLAG and the S513A mutant with or without CaMKIV in HEK293T cells and immunoprecipitated the tagged proteins (Figs. 12B and F). I also contributed to the writing of the paper (Related materials and methods from the experiments that I did, and initial revisions of the manuscript).

CHAPTER VI

Other exons differentially regulated by depolarization, hnRNP proteins and Ser513 of hnRNP L

Introduction

We have described in the last chapter that both hnRNP L and LL are involved in depolarization-induced repression of the STREX of the potassium channel *Slo1* gene. We also demonstrated that PTB binds to the RNA probe containing the Py tract upstream of CaRRE1 (16). Besides, a potential binding site, UCCCA (336), for another factor hnRNP K is also found in the same Py tract. Therefore, the STREX is likely controlled by multiple regulatory splicing factors.

To investigate roles of the other factors in the control of the STREX splicing and the specificity of their impacts, including that of the hnRNP L Ser513, on depolarization-induced splicing, we examined the effects of the hnRNPs on a group of endogenous exons including the STREX by RNAi as well as the Ser513 effect on one of the other exons.

Results

Differential regulation of a group of alternative exons by depolarization in GH_3 cells

To identify other cofactors and to evaluate their exon specificities, firstly the alternative splicing of another 11 exons in GH_3 cells was examined. Among them, 10 alternative exons are known to be regulated by depolarization in mouse embryonic

carcinoma P19 cells (7). The transcripts harbor putative CaRRE1 variants either in flanking introns or in exons with various numbers of CA dinucleotides (Table 5).

Table 5. Tested gene/exons harboring putative CaRRE1 type elements or CA repeats

Gene/exon	GenBank No.	Predicted CaRRE/CA	Location of CaRRE/CA	Expression Detected	Alternatively spliced	Change by depolarization
Adcyap1r1/E14	NM_001025372.1	cacaccca	Upstream intron			
D4Ertd22e/E2	NM_001114599	cacuuuua	Upstream intron	*	**	Repressed
Nf2/E16	NM_010898.2	cucauuua	Upstream intron	*		
Pitpnb/E11	NM_053742	uacauuua	Upstream intron	*	**	Repressed
Shc1/E6	NM_011368.3	gacauuua	Upstream intron	*		
Atp2b1/E21	NM_026482.1	CACAUGUA	Exon	*		
Rnf14/E4	NM_020012.1	CACACUUA	Exon	*	**	
Ktn1/E41	NM_008477.1	CACAGUUA	Exon	*	**	
Spna2/E36	DY313870	cacuuuua	Downstream intron	*	**	Repressed
U26/E2	NM_173765.1	cacacuua	Downstream intron	*		
Nrg1/E18	NM_013960	cacaca	introns	*	**	Repressed

Note: the first 10 exons listed in the table carrying putative CaRRE1s were chosen based on the report by Ji-Ann Lee, *et al.*, 2007 (7).

Another exon is in the neuregulin 1 gene (Nrg1), which was identified from our search for CA repeats in the human genome database at the similar position to the STREX CaRRE1 (data not shown). The *Nrg1* gene encodes a growth factor signaling through the ErbB receptor tyrosine kinases (337), and it has been implicated in neuropsychiatric disorders (337) and prolactin secretion by GH_3 cells (338). A $(CA)_3$ motif was found just prior to its last exon E19. Examination of the Nrg1 splicing around this region in the UCSC genome browser (http://www.genome.ucsc.edu/index.html?org=Human&db=hg19&hgsid=273426893) revealed complex patterns (Fig. 14). At least 3 splice variants with different 3' ends are produced through alternative Poly(A) sites following its E17 and E19 respectively, as

well as the cassette exon E18. Another $(CA)_3$ motif is found about 60 nts upstream of E18 (Fig. 15A). Preliminary experiments showed that E18 was repressed by depolarization. Therefore, it was further verified here. Moreover, it would be interesting to examine whether the hnRNPs, particular hnRNP L, regulate this exon as well.

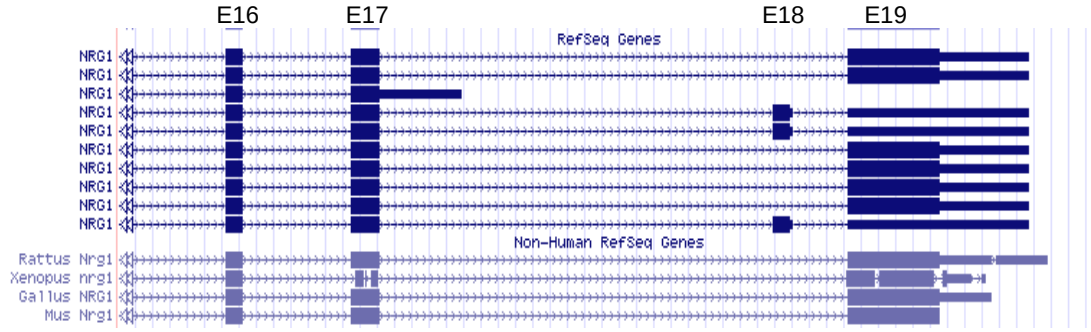

Figure 14. A cut-off alignment of Nrg1 transcripts of human and other species at the region spanning E16 to E19 from the UCSC Genome Web browser. Human sequences are on top. Solid blocks are exons, thinner blocks 3' UTRs, and lines introns. Arrowheads on the intron lines indicate the direction of transcription.

Among the 11 exons, 10 were detectable by RT-PCR (Table 5); 6 of them were alternatively spliced with different levels of inclusion, suggesting that their inclusions are differentially controlled in GH_3 cells. Similar to the STREX, 4 out of the 6 exons were repressed by depolarization (Table 5 and Fig. 15). The exon inclusion levels of these exons were reduced by 10, 24, 10, 9 and 17%, respectively for exons STREX, E11 of the phosphatidylinositol transfer protein, beta gene (Pitpnb), E2 of the RGD1560286 gene (D4Ertd22), E36 of the spectrin alpha 2 gene (Spna2) and E18 of the Nrg1, suggesting that depolarization differentially regulates these exons.

A

Slo-STREX	-ctctcctcccacccaaccacatggttatagCCAAGATGTC-
Pitpnb-E11	-tagcagctcatgattttatttacatttagATGCGTAAGA-
D4Ertd-E2	-tccacttttatatacatttttttcctctagGAAATAGACA-
Spna2a-E36	-CAAGCTGCTGgtaagttctttacacttttaagagttctgg-
Nrg1-E18	-agcacacaaa….ctctttttttttcataagACATAACCTT-

B

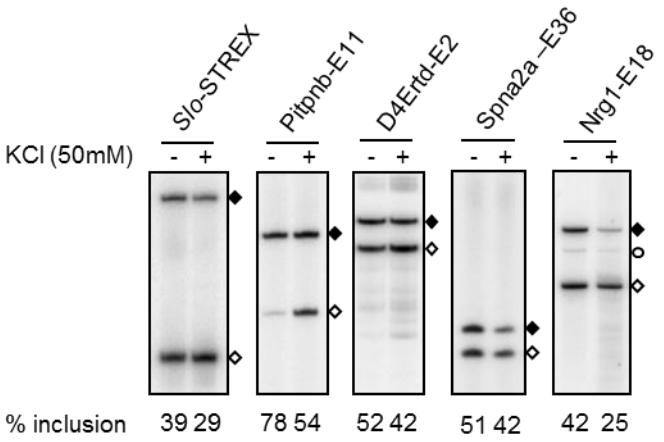

Figure 15. Alternative exons regulated by depolarization in GH_3 cells. **A**, rat genomic sequences nearby the 3' splice sites or the 5' splice site of the 5 alternative exons responsive to depolarization. Gene names and exons are indicated on the left. Intronic sequences are in lower cases, and exonic sequences in upper cases. CaRRE1 type elements or CA-repeat motifs (Nrg1) are underlined. **B**, splicing repression of the selected exons by depolarization. Shown are urea-PAGE gels of [γ-^{32}P] labeled RT-PCR products. Each is a representative of at least two gels. Splice variants are indicated by solid diamonds (exon included) and open diamonds (exon skipped). The Nrg1 variant produced through an alternative 3' end upstream of the E18 is indicated by an open circle.

Differential effect of hnRNP factors on the depolarization-regulated alternative splicing

Next, to examine the effects of the 4 hnRNPs on splicing of the responsive exons, expression of hnRNP L, LL, K and PTB was knocked down using gene-specific shRNA-expressing lentiviruses (Fig. 16A, also see Fig. 9D). Similar to hnRNP L and LL (Fig. 9E), downregulation of PTB and hnRNP K respectively produced different effects on the

STREX, though neither of them abolished depolarization-induced repression of the STREX (Fig. 16B). PTB appears to be a stronger repressor of STREX compared to hnRNP L because its downregulation led to increased level of constitutively expressed STREX. PTB downregulation also led to less repression by depolarization (relative reduction: 31% by mock vs. 17% by shPTB). This indicates that PTB is a regulator of both constitutive and induced repression of the STREX as well. As seen for double knockdown of hnRNP L and LL in the last chapter (Fig. 9E), the depolarization-induced STREX reduction was dramatically diminished in cells knocked down of both hnRNP L and PTB (sh(L+PTB), $n = 3$ pairs, $p = 0.16$) as well. Therefore, it is likely that multiple factors are involved in the regulation of STREX.

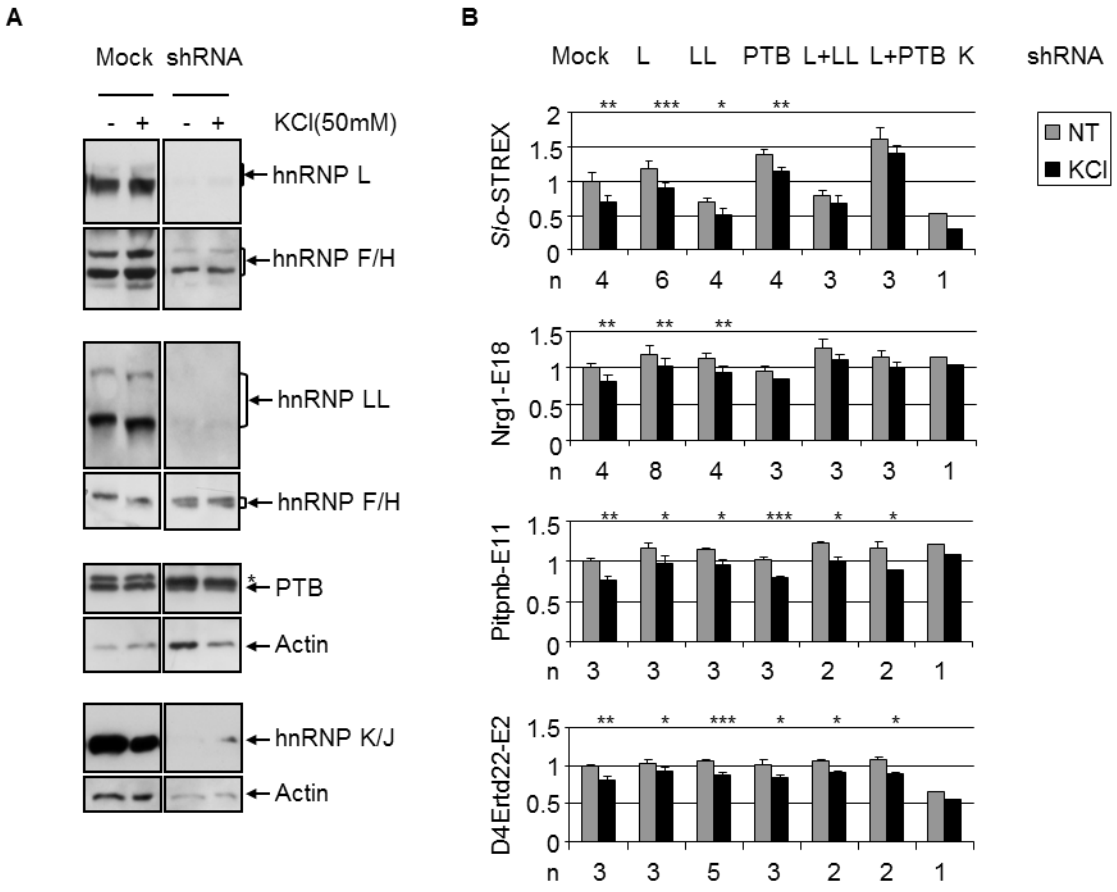

Figure 16. Differential regulation of alternative exons by a group of splicing factors. **A**, Western blot analyses showing knockdown of selected splicing factor by lentiviral vector-expressed gene-specific shRNAs. The band indicated by the asterisk is supposed to be nPTB that is also recognized by the same antibody. **B**, bar graphs summarizing relative inclusion levels of selected exons upon knockdown of the splicing factors shown in A. Error bars represent standard deviations, and sample numbers are indicated under each pair of columns. ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$; two-tailed Student's t test.

Similarly as seen for hnRNP LL, knockdown of hnRNP K led to reduction of the constitutive level of STREX (Fig. 16B), suggesting they are activators or weaker repressors of STREX. Apparently, overexpression of hnRNP LL in HEK293T cells induced exon repression in the reporter minigene pDUP175ST containing the upstream 3' splice site of STREX (Fig. 17), suggesting it is a repressor. Further experiments are needed to confirm the role of hnRNP K.

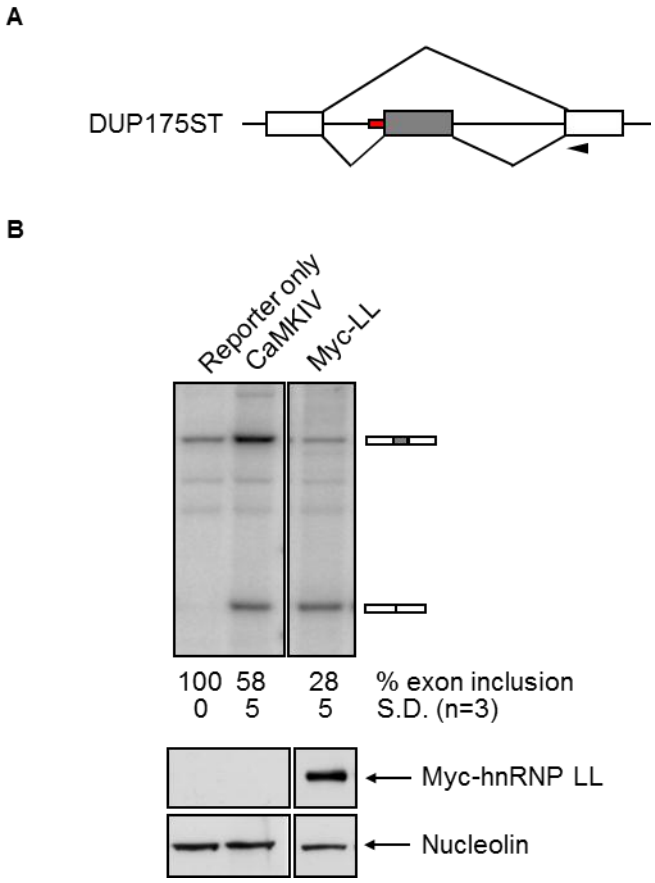

Figure 17. Overexpressed LL inhibited exon-inclusion in splicing reporter minigenes. A, a diagram of the splicing reporter minigene containing the 3' splice site of STREX upstream of the middle exon. The CaRRE1 is indicated by a red bar, the primer by a black arrowhead. B, a representative PAGE gel of [^{32}P]-labeled primer extension is shown at the top, with percentage of STREX inclusion included under each lane. Expression of Myc-hnRNP LL was examined by Western blots shown at the bottom. Nucleolin is a loading control. (Collaborated with Vincent G. Lobo)

The Nrg1 E18 was increased in cells knocked down of hnRNP L or LL, suggesting that both L and LL are repressors of E18 (Fig 16B). Similar to STREX, repression of the Nrg1 E18 by depolarization was nearly abolished upon knockdown of both hnRNP L and LL (n = 3 pairs, p = 0.13), and of both L and PTB to a lesser extent (n = 3 pairs, p = 0.09). Unexpectedly, reduction of PTB almost abolished E18 repression by depolarization (n = 3 pairs, p = 0.16) as well, possibly through the Py tract near the 3' end

of the intron (Fig. 15A). A similar effect was observed in cells depleted of hnRNP K, as well. Therefore, the Nrg1 E18 is also cooperatively controlled by the splicing factors. However, unlike STREX, a full repression of E18 by depolarization can be regulated by a single factor or two cooperative factors.

For the other two exons, the Pitpnb E11 and the D4Ertd22 E2, neither single knockdown of individual factors nor double knockdown abolished depolarization-induced exon repression, though depletion of hnRNP L (for both exons) or LL (the Pitpnb E11) led to less repression upon KCl-stimuli (Fig. 16B). Unexpectedly, the data for the Spna2 E36 was not convincing based on the current analysis; therefore, it was not presented.

Therefore, STREX and the other depolarization-responsive exons are differentially regulated by a group of hnRNPs. However, a full response to depolarization conferred by both hnRNP L and LL or PTB is specific to STREX and the Nrg1 E18. Because no protein level changes by depolarization were observed for the 4 hnRNPs (Fig. 16A), it is likely other mechanisms such as posttranslational modifications (e.g. hnRNP L Ser513) and/or protein relocalization are involved.

The Ser513 of hnRNP L is also essential for depolarization-induced repression of the Nrg1 E18

Studies from the last chapter demonstrated that phosphorylation of hnRNP L at Ser513 is critical for repression of the STREX by depolarization through the CA-repeat containing CaRRE1 element (311). Because hnRNP L is also an essential factor for the Nrg1 E18 repression and able to induce full response to depolarization along with LL, reminiscent of STREX (Fig. 9E), the Ser513 of L may have similar effect on the control

of E18. Therefore, splicing patterns of the Nrg1 E18 were examined by hot RT-PCR using the same samples as those for STREX (Fig. 11C). As shown in Fig. 18, complementation of either wild-type hnRNP L or its S513A mutant in GH_3 cells knocked down of endogenous L proteins showed no difference in inclusion level of E18 without depolarization. Upon depolarization, the wild-type hnRNP L induced significant reduction of E18 (n = 7 pairs, $p < 0.001$). By contrast, the S513A resulted in insignificant response to depolarization (n = 3 pairs, $p = 0.25$). Therefore, the hnRNP L Ser513 is also critical for inhibition of the Nrg1 E18 by depolarization.

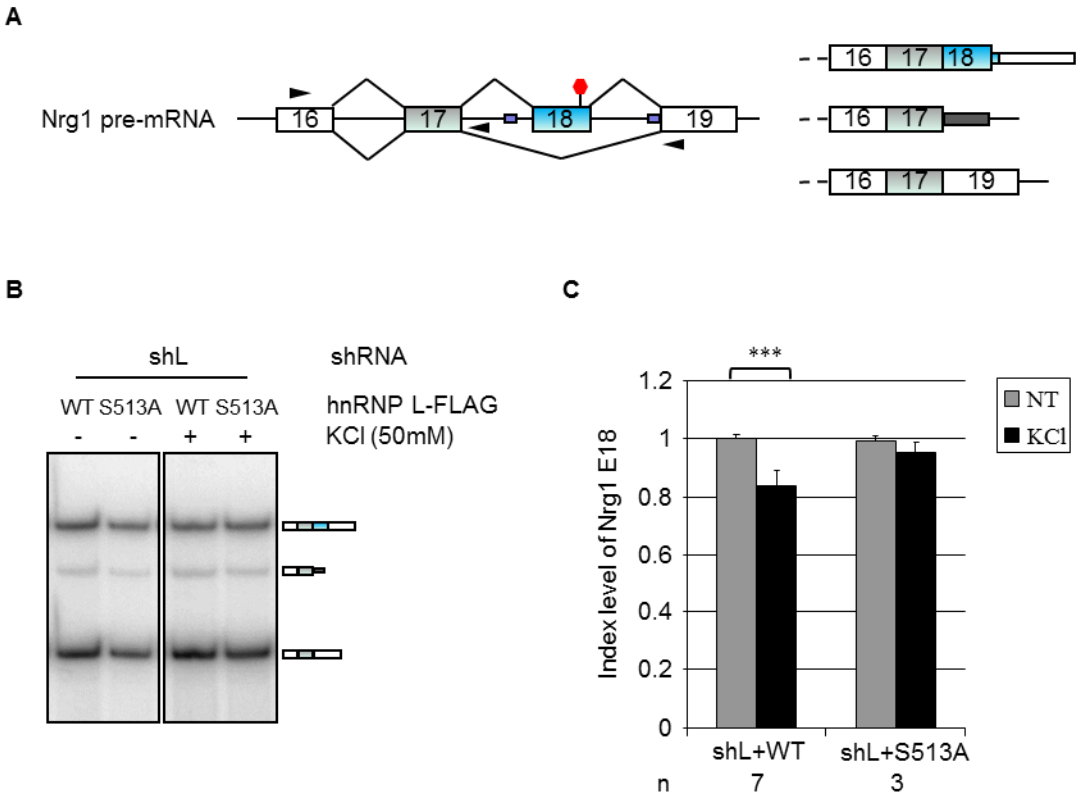

Figure 18. hnRNP L Ser513 is essential for the repression of the Nrg1 E18 by depolarization. **A**, a diagram of the splicing patterns of the Nrg1 around its 3' end (not to scale). Arrow heads: location of PCR primers; the 3 primers were used in a same PCR reaction. Purple bars: cacaca. Red hexagon: stop codon in E18. **B-C**, representative PAGE gels of [γ-^{32}P]ATP-labeled RT-PCR products of the endogenous Nrg1 transcripts of GH_3 cells expressing shRNA against hnRNP L (shL) and complemented with lentivirus-mediated expression of either hnRNP L-FLAG WT or the S513A mutant.

Shown in C is a bar graph of the normalized E18 inclusion levels (mean ± S.D., n = 7 or 3 for each pair of samples) in WT or S513A -complemented GH_3 cells knocked down of endogenous hnRNP L as in Fig. 11

Discussion

Depolarization-induced splicing is differentially regulated by multiple hnRNPs in a gene-specific manner

So far, only a limited number of regulatory splicing factors are known to mediat chronic depolarization-induced splicing, including Fox-1/2, hnRNP A1 and Sam68 (6,8,9). The current study demonstrated that hnRNP L, LL, PTB and likely hnRNP K are such mediators as well. Several depolarization-responsive alternative exons including the STREX and the Nrg1 E18 are differentially regulated by these factors.

At least two layers of differential regulation of alternative splicing are implicated here: first, splicing factors exert different effects on a same target exon; second, effect of one splicing factor is gene/exon-dependent. Both layers may reflect available regulatory elements surrounding a specific exon and likely their locations (Figs. 9C and 15A), which may lead to differential recruitments and organizations of specific sets of regulators and thus produce different effects (Fig. 16B). Together with the effect of hnRNP L Ser513 phosphorylation (Figs. 10-12), it is likely that the STREX and the Nrg1 E18 are controlled by, at least, two mechanisms: the combined effect from multiple regulatory factors and functional modulation of individual factors by depolarization. These regulations together provide, at least partly, the molecular basis for the fine-tuning of potassium channel activity or signal-transducing functions.

Although all the factors can control the splicing of the STREX and the Nrg1 E18, the possibility of involvement of additional factors cannot be ruled out. Examination of

the two exons as well as their flanking introns found one UGCAUG motif in STREX, one in the Nrg1 E18 and another in its downstream intron. It is known that Fox proteins regulate splicing through the UGCAUG element (84,223). Whether Fox proteins regulate the two exons through their UGCAUG motifs remains to be identified.

Cooperative repression of depolarization-regulated exons by two hnRNPs

An interesting observation from these studies is that hnRNP L likely confers a full response of the exons to depolarization in the presence of another cofactor, LL or PTB (Figs. 9E and 16B). The underlying mechanisms are currently unknown. Coordinated control of splicing by both hnRNP L and LL has been reported in studies of CD45 (135,159,253,254,339). Although both factors are repressors of the CD45 E4, they appear differentially required depending on cell status, with L being more important in naive T cells (253,340) and upregulated LL functioning dominantly upon activation (135,254). A recent study demonstrated in human B cells that the master regulator hnRNP LL and the coregulator L bind the CD45 exons 6 and 4 respectively and mediate simultaneous skipping of the exon 4-5-6 unit likely by a looping-out mechanism (159). In the current study, hnRNP L and LL repress STREX and the Nrg1 E18 with differential strengths under both resting and stimulated conditions. It remains to be investigated whether hnRNP L and LL repress the two exons through similar mechanisms (For example, they bind the two $(CA)_3$ motifs upstream of the Nrg1 E18 and E19 (Fig. 15A) respectively). Since no obvious changes in protein levels of hnRNP L and LL were observed upon depolarization (Fig. 16A), posttranslational protein modifications may be important for modulations of their interactions upon stimulation.

Implications of regulated splicing of the Nrg1 E18

The alternative 3' ends in upstream and downstream exons produce NRG1 proteins with cytoplasmic tails of variable lengths ((341) and Fig.14). The cytosolic domain of NRG1 has important functions in neuronal survival upon depolarization through proteolytic release and relocalization of this domain to the nucleus, which represses expression of several apoptosis-related proteins (342). Alternative usage of the 3' ends re-organizes the cytoplasmic tail of NRG1, which may affect its back-signaling to the nucleus. Thus, it is likely that, to fulfill the needs for NRG1 isoforms with a longer cytoplasmic domain, hnRNP L and the other regulators facilitates the production of such transcripts by inhibiting usage of the upstream 3' ends (Figs. 15B and 18B). Inclusion of the Nrg1 E18 introduces an extra stop codon (Fig. 18A), which possibly inhibits the use of the longer E19. Repression of E18 by hnRNP L upon depolarization increases the use of E19 and so the production of the isoform with a longer cytoplasmic tail. We also observed repression of the Nrg1 variant ended at its E17 by depolarization and hnRNP L appears to be a mediator (Figs. 15B and 18). This may provide another mechanism to ensure the use of the longer E19. Considering the $(CA)_3$ motifs preceding E18 and E19, it would be interesting to examine whether and how hnRNP L and possibly LL as well could regulate the selective usage of the 3' ends through the CA repeats. Moreover, it may be important to investigate the functional impacts of the preferential use of E19 by depolarization in GH_3 cells.

Summary

These studies identified a group of hnRNPs that were unknown in depolarization-regulated alternative splicing previously. These hnRNPs, particularly hnRNP L, LL and PTB, differentially regulate alternative splicing of a group of exons. Among them, hnRNP L is a critical repressor for the STREX and the Nrg1 E18 likely through coordinate control with hnRNP LL and/or PTB.

CHAPTER VII

Conclusion

1. CaMKIV/PKA-responsive RNA elements controlling alternative splicing were isolated by using in vivo selection, and consensus motifs that are similar to the previously characterized CaRRE1 and CA repeats, as well as novel A-rich elements, were identified.

   The CaRRE1-like elements provide a basis for further refinement of CaRREs. Further characterization of the elements may help define more accurately coregulated genes and thus functional clusters of genes upon depolarization. This selection also provides an approach that could be applied to other signal pathways.

2. Ser513 of hnRNP L was identified as a critical site for L-mediated exon repression by depolarization. Depolarization/CaMKIV-induced phosphorylation of the Ser513 increases hnRNP L interaction with CaRRE1 and reduces U2AF65 binding to the 3' splice site, which likely causes exon skipping.

   This provides a direct functional link between Ca^{2+} signaling and a specific phospho-serine of a splicing regulator. Moreover, the regulation from membrane depolarization to the splicing machinery presents a molecular mechanism underlying cellular activity-triggered modulation of compositions and functions of ion channels and signal-transducing molecules.

3. Together with hnRNP L, a group of hnRNPs were identified as regulators of depolarization-induced splicing, with differential effects on a group of alternative exons.

Regulation of STREX and the additional exons by these hnRNPs (including their posttranslational modifications, such as phosphorylation of the hnRNP L Ser513) provides a layer of fine-tuning of activities and functions of the regulated proteins. This modulation of protein functions are likely important for electrophysiological properties of excitable cells (the STREX) and cell signaling (the Nrg1 E18), which may subsequently impact on physiological functions such as memory formation and hormone secretions.

In summary, these studies contribute to further understanding of molecular mechanisms underlying cell signaling-controlled gene expression and provide a basis for further studies of the role of alternative splicing in cell physiology.

CHAPTER VIII

Future Directions

Identification of Ser513 of hnRNP L as a critical site in depolarization-induced splicing raises the question of how phosphorylation within an RNA recognition motif enhances RNA binding. Based on the observation that RRMs of hnRNP L interact with each other (331), it is possible that phosphorylation of the Ser513 in the RRM4 promotes RNA binding of other RMMs of L. Therefore, a major direction could be investigations of the effects of Ser513 phosphorylation on interactions among RRMs of hnRNP L and their impacts on RNA binding.

This study also identified additional hnRNPs including hnRNP LL and PTB as essential regulators of depolarization-induced splicing. Identification of their binding sites on the target pre-mRNAs, such as the *Slo1* and the Nrg1, could be a future direction for further elucidation of the splicing control by the hnRNPs, though it is also possible that they may function through protein-protein interactions. It has recently been reported that the co-repression of the CD45 exon 4-5-6 cassette is repressed through interactions between hnRNP L and LL binding exons 4 and 6 respectively (159). It is also been observed that hnRNP L and PTB cooperates to mediate active translation of the Cat-1 arginine/lysine transporter mRNA in response to amino acid starvation (124). Thus, it would be interesting to investigate whether they form complex and how they coordinate with each other to regulate the depolarization-target exons such as the STREX and the Nrg1 E18.

The BK potassium channels are important for electrical properties of excitable cells (263,264). The inclusion of the STREX exon increases the channel sensitivity to the Ca^{2+}/voltage and other stimuli as well as inhibition by PKA (56). The shifts in channel isoforms is then expected to control the channel properties and the firing of following waves of action potentials (56). Since we have shown that the STREX is regulated by a specific set of splicing factors upon depolarization, it would be interesting to investigate in future if the depolarization-regulated channel activity could be modulated by the splicing regulators. Moreover, since the BK channel and the Nrg1 have been implicated in secretion of growth hormone and prolactin, respectively (13,278,338), it could be very interesting to examine if their regulated activities take part in the control of the secretion of these hormones.

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