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

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Background: Excitable cells show activity-dependent alternative splicing of ion channels.
Results: CaMKIV phosphorylates hnRNP L at Ser-513, which is essential for depolarization-repression of a Slo1 potassium channel exon and splicing factor U2AF65.
Conclusion: Depolarization controls alternative splicing of Slo1 channels through Ser-513 phosphorylation and inhibition of U2AF65.
Significance: This provides the first direct link between depolarization/CaMKIV and the constitutive spliceosome.

Molecular mechanisms of gene regulation underlying the activity-dependent long term changes of cellular electrical properties, such as those during memory, are largely unknown. We have shown that alternative splicing can be dynamically regulated in response to membrane depolarization and Ca^{2+}/calmodulin-dependent protein kinase IV (CaMKIV) activation, through special CaM kinase responsive RNA elements. However, proteins that mediate this regulation and how they are affected by CaMKIV are not known. Here we show that the regulation of the stress axis-regulated exon of the Slo1 potassium channel transcripts by membrane depolarization requires a highly conserved CaMKIV target serine (Ser-513) of the heterogeneous ribonucleoprotein L. Ser-513 phosphorylation within the RNA recognition motif 4 enhanced heterogeneous ribonucleoprotein L interaction with the CaMKIV-responsive RNA element 1 of stress axis-regulated exon and inhibited binding of the large subunit of the U2 auxiliary factor U2AF65. Both of these activities were abolished by a S513A mutation. Thus, through Ser-513, membrane depolarization/calcium signaling controls a critical spliceosomal assembly step to regulate the variant subunit composition of potassium channels.

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 (1–3). However, many molecular mecha-

nisms for the regulation of ion channel expression and function in this process remain unknown. Alternative pre-mRNA splicing of ion channels contributes greatly to their functional diversity (4, 5). 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 (4).

Transcripts from the *Slo1* gene (*Kcnma1*) undergoes extensive alternative splicing capable of producing hundreds of variant subunits and billions of BK channel tetramers (4, 6, 7). These channels, sensitive to both voltage and calcium (8), 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 (6, 9).

One of the most intensively studied Slo1 exons is the stress-axis regulated exon (STREX)[3] (10). It encodes a 58-amino acid peptide that confers many unique properties to the channel including higher sensitivity to Ca^{2+} and voltage (4, 10–12). Its regulation by membrane depolarization is thus expected to control the channel properties and the firing of following waves of action potentials (13), likely contributing to the fine-tuning of the long-term electrophysiological changes, such as memory in neurons (4, 14).

Inclusion of the STREX exon is repressed by depolarization in GH_3 pituitary cells and cerebellar neurons through L-type calcium channels and the Ca^{2+}/calmodulin-dependent protein kinase IV (CaMKIV)-responsive RNA element 1 (CaRRE1) (13, 15, 16). A CaRRE1-binding repressor has been identified as the heterogeneous ribonucleoprotein hnRNP L that interacts with

[3] The abbreviations used are: STREX, stress-axis regulated exon; BK channel, big potassium channel; CaMKIV, Ca^{2+}/calmodulin-dependent protein kinase IV; CaRRE, Ca^{2+}/calmodulin-dependent protein kinase IV-responsive RNA element; hnRNP L, heterogeneous ribonucleoprotein L; hnRNP LL, hnRNP L-like; RRM, RNA recognition motif.

CaRRE1 in a phosphorylation-dependent way (17). However, the proteins mediating the depolarization repression of STREX and their regulation by CaMKIV remain unknown.

In this report, we identified a conserved serine of hnRNP L as a critical CaMKIV target and determined that it has an essential role in the control of STREX inclusion by membrane depolarization.

EXPERIMENTAL PROCEDURES

Plasmid Construction—Plasmids for CaMKIV-dCT (IV) and CaMKIV-dCTK75E (IVm) produce constitutively active and inactive CaMKIV enzymes, respectively, as described (13). The hnRNP L-FLAG expression plasmid was kindly provided by the laboratory of Dr. Stefan Stamm as in our previous study (17). hnRNP L and LL mutants were made by PCR using Pfu DNA polymerase, cloned into respective vectors, 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 (18). The lentiviral plasmid pFG12-shL (shL), as reported (17), targets the 3' untranslated region of hnRNP L. The pLKO.1-shLL against hnRNP LL (shLL, clone ID TRCN0000075101, sequence ccggCGACAGG-CTCTAGTGGAATTTctcgagAAATTCCACTAGAGCCTG-TCGtttttg, with the nucleotides targeting LL in uppercase) (19) was purchased from the OPEN Biosystem human shRNA library in the Manitoba Proteomic Centre. The 175ST-1 plasmid was made by cloning a PCR fragment from DUP175ST (13) between the AatII and BglII sites of a pGEM-T-based vector. The fragment starts from upstream primer DUP9a (5'-GTTT-AGTGAACCGTCAGATC-3') with an AatII site added to the 5' end to the BglII site in the second intron. For 175ST-1m, the CaRRE1 sequence was mutated to the corresponding fugu fish sequence Fugu_a (Fig. 1*C*) as reported (17). The plasmids were linearized with BglII before *in vitro* transcription using T7 RNA polymerase. The 175ST-1S and 175ST-1Sm templates were PCR-amplified from the plasmids using primers T7DUP6 (5'-TAATACCGACTCACTATAGGGAAGACTCTTGGGTTT-CTG-3') and 175exon86R (5'-CATGGTGTCTGTTTGAGG-TTG-3') inside the first and second exons, respectively. pET28a-hnRNP L was recloned from the hnRNP L-FLAG into pET28a by insertion of its open reading frame fragment at the EcoRI site.

Cell Culture, Western Blot Analyses, and RT-PCR—Rat GH_3 pituitary cells were maintained in F10 media plus 10% horse serum, 2.5% FBS and 1% penicillin/streptomycin/glutamine solution (Invitrogen). HEK293T cells are cultured in Iscove's modified Dulbecco's medium containing 10% FBS and 1% penicillin/streptomycin/glutamine solution for virus preparation. Western blot analyses were on the basis of the procedure as described (20). To detect the phospho-Ser-513 of hnRNP L, 1 mM Na_3VO_4 was preadded to the dry milk suspension to block protein phosphatases. Anti-hnRNP L (4D11), anti-hnRNP K (3C2), and anti-hnRNP F/H (1G11) were purchased from Santa Cruz Biotechnology, Inc. Anti-hnRNP LL (catalog no. 4783) was purchased from Cell Signaling Technology, Inc., and anti-FLAG (M2, F1804) was purchased from Sigma-Aldrich.

Semi-quantitative RT-PCR of endogenous STREX was performed on the basis of a previous procedure (13), except

an upstream rSlo1 (5'-GCCTGTCATGATGACGTCACA-GATC-3') and a ^{32}P-labeled downstream rSlo2 (5'-CCTCAT-GCCCCCATTACGTTGTT-3') primers binding to exons 18 and 19 of Slo1, as shown in Fig. 1*B*, were used for shorter PCR products. Briefly, 25–150 ng of cytoplasmic RNA was reverse-transcribed in a 10-μl reaction, of which 0.5 or 1 μl was amplified in a 12.5-μl reaction for 25 cycles. The products were resolved in 6% denaturing polyacrylamide gels, dried, and exposed to PhosphorImager plates. Band intensities were quantified using ImageJ (National Institutes of Health).

Lentiviral Vector-mediated RNA Interference/Complementation Assay—This was according to our published procedure (17) 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, and cppt2E-Myc-hnRNP LL and its S498A mutant) using PolyJet™ DNA *in vitro* transfection reagent (Signage®) 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 (17) 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 °C.

For transduction, rat GH_3 pituitary cells at a density of ~2 × 10^5 cells/well in a 24-well plate (Falcon) were transduced using shL- or shLL-carrying viruses for 3 h and 24 h later using both shRNA and protein-expressing ones and then transferred to a 12-well plate. On day 6, they 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 (21, 22) 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 × 10 cm cellulose TLC plates (EMD Chemicals, Inc.).

For peptide mapping of *in vitro*-phosphorylated peptides by CaMKIV, 20 ng of GST-CaMKIV (BioMol) was incubated with each of the peptides ERSSSGLLEW (Ser-513) and ERSS(p-S)-GLLEW (p-S, phospho-Ser-513) plus purified Myc-CaMKK2 (~10 ng) (22) 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 °C for 30 min, mixed with SDS loading buffer, denatured at 95 °C 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 the synthetic phospho-Ser-513 peptide ERSS(p-S)-GLLEW (Fig. 2*B*), similarly as described previously (21), in the Alpha Diagnostics, Inc. The antiserum showed at least 4 times of immunoreactivity for the phospho-peptide over the non-phosphopeptide 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. 3*A*.

Immunodepletion, UV Cross-linking, and Immunoprecipitation—Immunodepletion was performed according to a published procedure in the presence of 0.5 M NaCl (23). The resulting nuclear extract was used directly for UV cross-linking at a final NaCl concentration of 90–180 mM.

UV cross-linking and immunoprecipitation were performed on the basis of our described procedure (17), with several modifications. For UV cross-linking, HeLa (~22 μg) nuclear extract was incubated with 7.5×10^5 cpm of $[\alpha\text{-}^{32}\text{P}]$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 of anti-U2AF65 (MC3, Sigma-Aldrich) antibodies bound to protein G-Sepharose beads (Pierce) were incubated with 2–4 volumes of UV cross-linking reactions with rotation at 4 °C overnight. The beads were then washed three times with 1 ml of radioimmune precipitation assay buffer (containing 150 mM NaCl). The resulting proteins were processed as described (17).

Expression and Purification of Recombinant His-hnRNP L—One liter of overnight culture of pET28a-hnRNP L plasmid-transformed *Escherichia coli* Rosetta-gami 2 (DE3) pLysS (Novagen) was induced with 0.3 mM isopropyl 1-thio-β-D-galactopyranoside for 3 h at 37 °C before harvest by centrifugation. The bacteria were then resuspended and sonicated in 4 ml of cold PBS containing 8 M urea and 20 mM 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 a sequential wash with decreasing concentrations of urea (6 M, 4 M, 2 M, 1 M, and 0 M) in cold PBS. His-hnRNP L was eluted with 1 ml of PBS buffer (containing 1 M imidazole, 1 M 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 cross-linking 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. 4E).

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 CaRRE1 to repress STREX splicing (Fig. 1) but that knockdown of hnRNP L alone did not abolish the depolarization effect (17), suggesting the involvement of other factors such as hnRNP L-like (*LL*) (19, 24). 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. 1D). 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. 1E). 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 either 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 (17), hereafter we will focus on hnRNP L as an example to delineate the pathway from CaMKIV to the spliceosome.

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 found previously that hnRNP L was phosphorylated when coexpressed with a constitutively active CaMKIV(17). The phosphorylation resulted in increased binding to CaRRE1 and is phosphatase-sensitive (17). We thus mutated hnRNP L at several conserved amino acid residues within CaMKIV consensus sequences (Arg-*X*-*X*-Ser/Thr) (25, 26), including Ser-326 and Ser-513 (Fig. 2, *A* and *B*). The effect of the mutations on phosphorylation by CaMKIV in HEK293T cells was analyzed by phosphopeptide mapping. Mutation of Ser-326 did not result in different patterns of phosphopeptides from that of the wild-type hnRNP L (Fig. 2C). However, mutation of Ser-513, whose consensus peptide is highly conserved among chordates and arthropods (Fig. 2B), completely abolished the major phosphopeptides (Fig. 2C). Therefore, Ser-513 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-Ser-513 (ERSS(p-S)GLLEW; p-S, phosphorylated Ser-513) and unphosphorylated Ser-513 (ERSSSGLLEW). The CaM kinases in the reaction were phosphorylated in the absence of peptide substrates as expected (Fig. 2D, *left panel*). Addition of the peptide P-Ser-513 as a substrate resulted in a similar pattern (Fig. 2D, *center panel*). In contrast, addition of the unphosphorylated Ser-513 peptide resulted in a strong ^{32}P-labeled spot not seen in the background (Fig. 2D, *right panel*, compared with the *left panel*). Therefore, Ser-513 is a direct target of CaMKIV.

Ser-513 Is Essential for Depolarization-regulated Splicing of Endogenous STREX in GH$_3$ Cells—To verify the phosphorylation of endogenous Ser-513 and its regulation in cells, we made phospho-Ser-513-specific antibody and validated its specificity by Western blot analyses (Fig. 3A). 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-Ser-513 epitope.
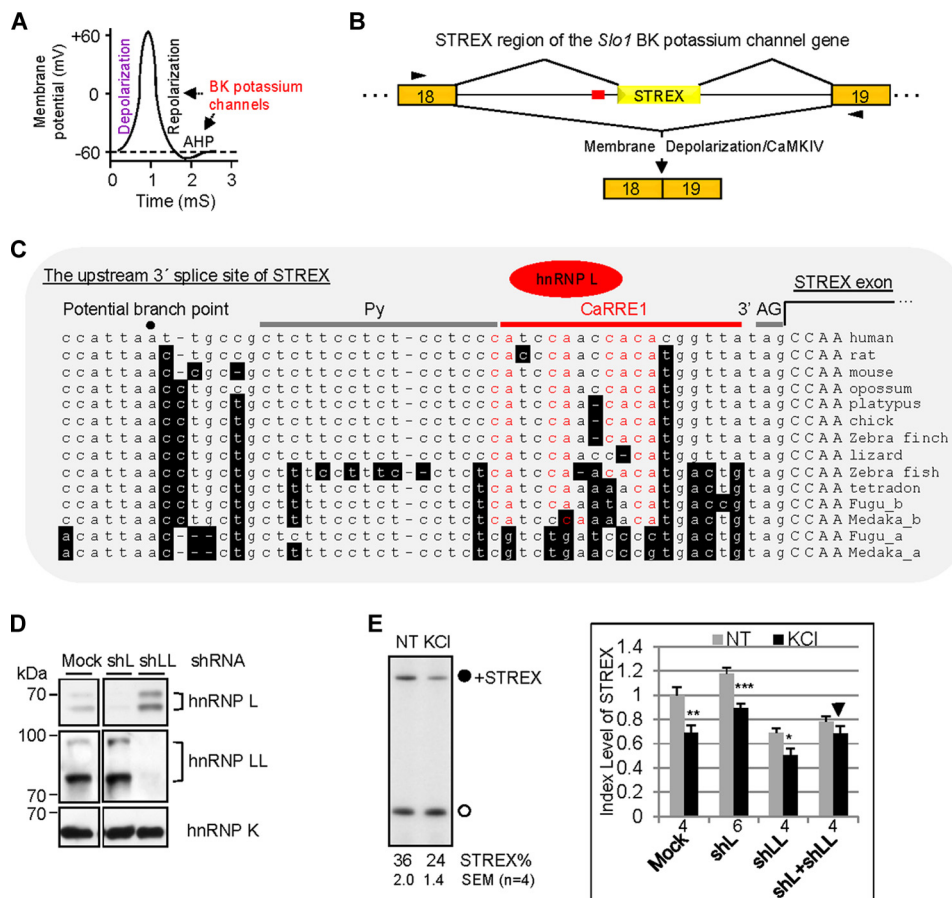
FIGURE 1. **Essential role of hnRNP L and L-like proteins in depolarization-induced repression of the STREX exon of Slo1 BK potassium channel transcripts.** *A*, diagram of the physiological context of the splicing regulation in neurons or endocrine cells, including GH$_3$ cells. Shown is the relationship 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 STREX-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 GH3 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 unusual "insertion" of the element 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 shown in *red*. Nucleotides different from the human sequence are *shaded*. Fugu and medaka fish each have two paralogous Slo1/STREX (Rohmann'09), one of which (*Fugu_a*) does not contain the CaRRE1 element (15). *D*, efficiency and specificity of the lentivirus-mediated RNA interference against hnRNP L (*shL*) or LL (*shLL*). Shown are Western blot analyses 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 detected specifically by anti-hnRNP L and anti-LL antibodies and knocked down by the specific shRNA, respectively. The sizes of the hnRNP LL bands, bigger than the ~60 kDa human hnRNP LL in the datasheet of the supplier are likely from alternative splicing in the rat GH$_3$ cells. A 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, 50 mM) GH3 cells. On the right is a *bar graph* (mean ± S.E., *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. The *black arrowhead* points to the loss of significant changes in the KCl-treated samples compared with the paired NT samples. ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$; two-tailed Student's *t* test.

We next examined whether the phospho-Ser-513 was induced by depolarization with the phospho-Ser-513-specific antibody. The phospho signal was strongly detected at 1 and 3 h after depolarization by KCl treatment of GH$_3$ cells (Fig. 3*B*) in comparison to the loaded hnRNP L protein level in the same blots. Therefore, increased Ser-513 phosphorylation can be induced by membrane depolarization.

To determine whether increases in Ser-513 phosphorylation play a role in splicing, we used lentiviral systems to express either hnRNP L-FLAG or its S513A mutant together with shL (to deplete endogenous L proteins) in GH$_3$ cells (Fig. 3*C, left panel*). Without treatment (*NT*), the STREX levels in cells expressing hnRNP L-FLAG or its mutant are similar (Fig. 3*C, right panel*). Upon depolarization by KCl, the STREX level in

cells expressing the wild-type hnRNP L-FLAG was reduced significantly ($n = 7$ pairs, $p < 0.001$). However, this reduction was abolished in cells expressing the S513A mutant ($n = 6$ pairs, $p = 0.98$). Therefore, Ser-513 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 Ser-513 phosphorylation in splicing repression, we examined the effect of phosphorylation on the interaction of hnRNP L with the CaRRE1 RNA on the basis of the location of Ser-513 within the RNA recognition motif 4 (RRM4, Fig. 2*A*). For this experiment, we purified unphosphorylated, phosphorylated, or S513A mutant hnRNP L-FLAG from HEK293T cells by immunoprecipita-
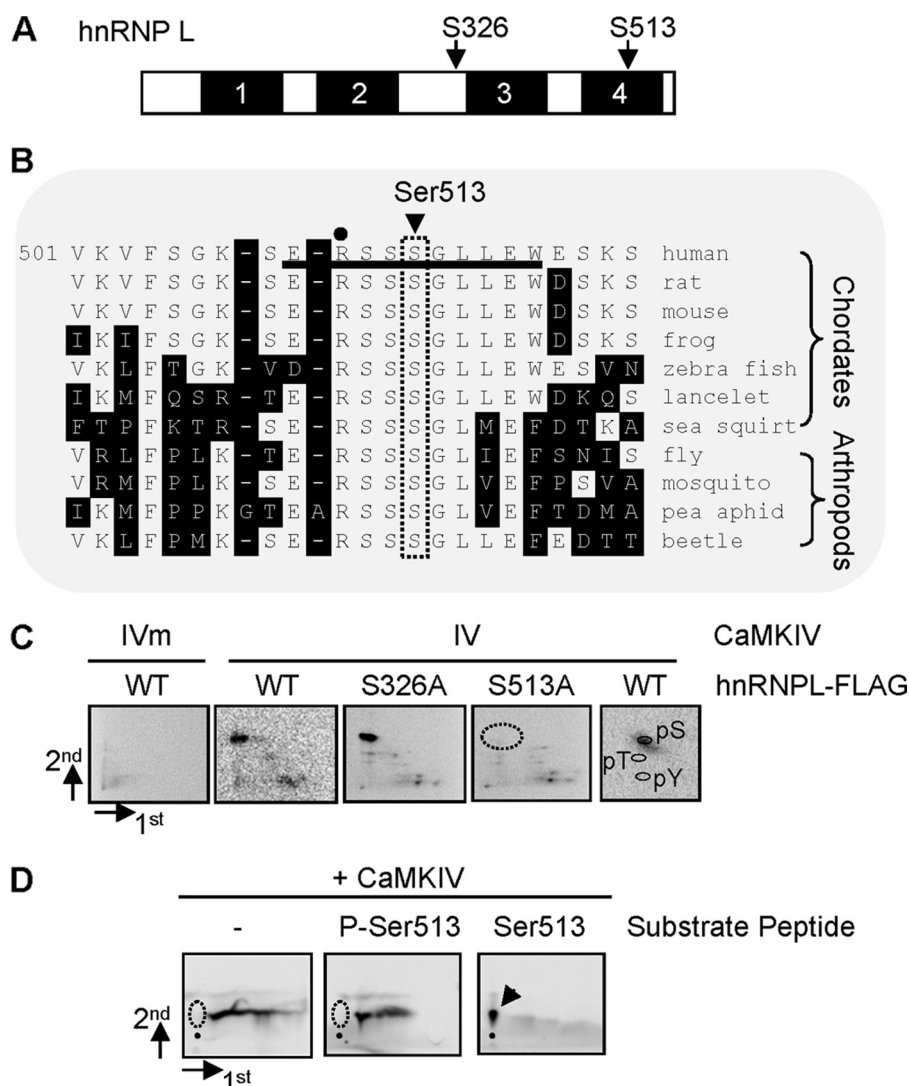
FIGURE 2. **The highly conserved serine 513 of hnRNP L is a major phosphorylation target of CaMKIV.** *A*, diagram of the RRM domains (*dark*) of hnRNP L protein and locations of two potential CaMKIV target serines (not to scale). *B*, serine 513 (*S513* or *Ser-513*) is highly conserved between phyla chordata and arthropoda. Shown are amino acid sequences around Ser-513 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-pSer-513 antibody. The common names of the species are indicated to the right. Their binomial nomenclatures are as follows: 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*; and beetle, *Tribolium castaneum*. *C*, two-dimensional phosphopeptide mapping of [^{32}P]orthophosphate-labeled 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 with a *dotted line*. The positions of standard phosphoserine (*pS*), phospho-threonine (*pT*), and phospho-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 "Experimental Procedures" for peptide sequences. The background signals with substrate peptides are apparently from the phosphorylation/autophosphorylation of CaM kinases used. The *dotted circles* in the *two left panels* indicate the absence of the ^{32}P-labeled-phospho-Ser-513 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-Ser-513 peptide are indicated with *black dots*.

tion, verified them by Western blot analysis (Fig. 3*A*), and examined their cross-linking efficiency with the CaRRE1 RNA probe in solution (Fig. 4, *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.

Because the CaRRE1 element is "inserted" between the polypyrimidine tract and 3′ AG (Fig. 1*C*) (15,17), binding sites for the constitutive heterodimeric splicing factors U2AF65 and U2AF35 (27), respectively, it is reasonable to speculate that regulated hnRNP L binding to CaRRE1 inhibits interaction of U2AF65 with the upstream polypyrimidine 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. 1*C*) and examined U2AF65 interaction with the longer CaRRE1-containing RNA probe 175ST-1S and its mutant 175ST-1Sm (Fig. 4*C*). Compared with the wild type, the mutant
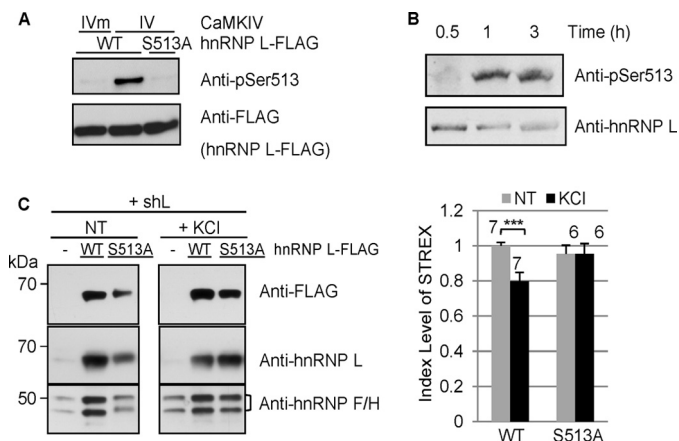
FIGURE 3. **Essential role of Ser-513 in depolarization-induced repression of the STREX exon in GH3 cells.** *A*, specificity of the anti-phospho-Ser-513 antibody. Shown is a Western blot analysis of protein samples of HEK293T cells coexpressing hnRNP L-FLAG (WT) or its S513A mutant (S513A) with CaMKIVm or CaMKIV using antibodies against phospho-Ser-513 or FLAG. *B*, effect of depolarization on phospho-Ser-513 in GH3 cells. Shown are Western blot analyses of depolarized (KCl, 50 mM) GH3 samples at different time points after KCl addition using antibodies against phospho-Ser-513 (*anti-pSer513*) or hnRNP L. *C*, effect of S513A mutation on depolarization-induced repression of STREX exon in GH3 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*, *bar graph* of the normalized STREX inclusion levels (mean ± S.E., n = 7 or 6 for each pair of samples as indicated) in the hnRNP L-FLAG WT or S513A mutant-complemented GH3 cells knocked down of hnRNP L. For RT-PCR, primers as in Fig. 1*E* were used.

probe cross-linked more strongly to U2AF65 in HeLa nuclear extracts. This difference suggests that the presence of CaRRE1, and likely hnRNP L as well, is inhibitory to U2AF65 binding to the upstream polypyrimidine tract (Py).

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. 4*D*). Upon UV cross-linking of these extracts with 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. 4*E*, *left panel*). In contrast, U2AF65 binding was enhanced upon depletion of endogenous hnRNP L (Fig. 4*E*, *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 Ser-513 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 with the unphosphorylated one (Fig. 4*F*). More importantly, the S513A mutation abolished this effect. Therefore, Ser-513 phosphorylation of hnRNP L enhances its ability to inhibit U2AF65 binding to the 3′ splice site.

Taken together, the UV cross-linking experiments (Fig. 4) indicate that Ser-513 phosphorylation enhances hnRNP L binding to the CaRRE1 element and interferes with U2AF65 binding to the upstream polypyrimidine tract.
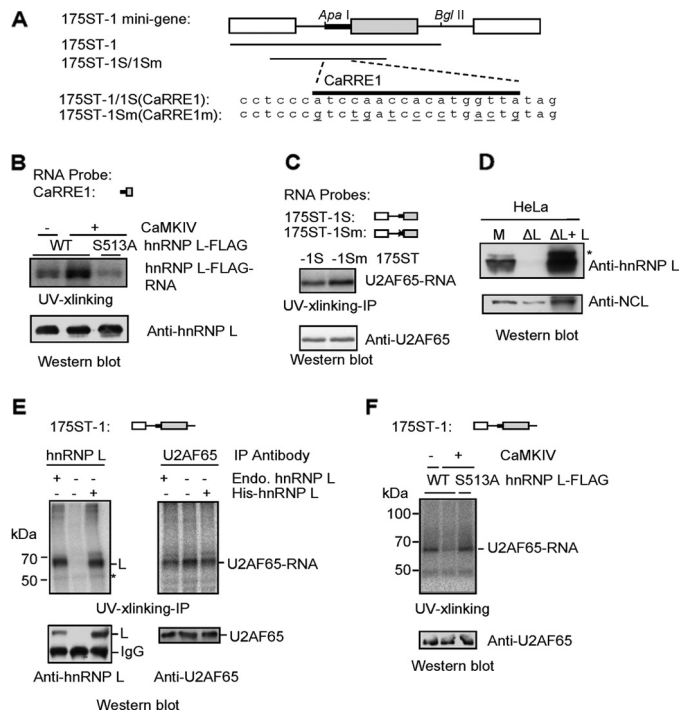
FIGURE 4. **Effect of Ser-513 phosphorylation on hnRNP L interaction with CaRRE1 and U2AF65 binding to the upstream 3′ splice site of STREX.** *A*, diagram of the 175ST-1 plasmid. *Thin lines* represent introns, and the *boxes* represent exons. The *thick line* corresponds to the 3′ splice site of STREX 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 "Experimental Procedures" for details). In 175ST-1Sm, the CaRRE1 element was changed to the corresponding Fugu_a sequence. The mutated nucleotides are *underlined*. *Heavy bar*, CaRRE1 element as in Fig. 1*C*. *B*, Ser-513 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-labeled 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. 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 Ser-513 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. The 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 are representative of at least three experiments.
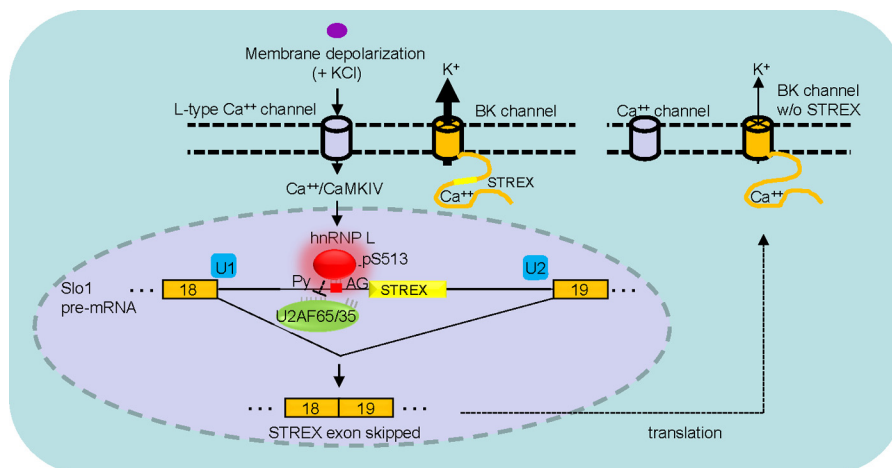
FIGURE 5. **Summary for the role of Ser-513 phosphorylation of hnRNP L in mediating depolarization-regulated splicing of the Slo1 BK channels.** Membrane depolarization of GH3 or neuronal cells by high concentrations of KCl (25–50 mm) activates the L-type calcium channels (15) and the Ca^{2+}/CaMKIV pathway (13), which leads to Ser-513 phosphorylation (Fig. 2). This modification enhances hnRNP L binding to the CaRRE1 element between the polypyrimidine (*Py*) 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 protein kinase A and, thus, different electrical properties that are expected to change the cellular excitability upon following waves of stimulation.

DISCUSSION

In summary, these data demonstrate that a highly conserved CaM kinase target Ser-513 of hnRNP L is essential for membrane depolarization to regulate the alternative splicing of BK potassium channels. The Ser-513 phosphorylation controls the interaction of a critical component at the early steps of spliceosome assembly with the pre-mRNA (Fig. 5). This not only provides a direct functional link between a calcium 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, 13, 15, 17). This regulation forms a unique molecular basis for how depolarization could modulate the splice variant compositions and functions of potassium channels.

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. 5). As proposed previously (4, 13), this regulation likely contributes to the fine-tuning of the long term changes such as electrophysiological memory in neurons (14).

With this critical site identified, how the Ser-513 phosphorylation within an RNA recognition motif enhances RNA binding by hnRNP L would be an interesting question for future investigations. Interestingly, Skrisovska *et al.* (28) 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 Ser-513 phosphorylation promotes the binding of the RRMs to their cognate sequence elements.

The highly conserved nature of the target Ser-513 site (Fig. 2*B*), the extensive alternative splicing of BK and other ion channels (4), and the many other roles of hnRNP L in RNA metabolism (29–37) together make it worthwhile to explore a wider role of phospho-Ser-513 in excitable cells.

HnRNP L, as a global alternative splicing regulator (35), 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 (35) 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 (36). For the exon 20 of the *TJP1* transcripts, hnRNP L acts through a CA-rich element within the polypyrimidine tract of the 3′ splice site to compete with U2AF65 (37). For STREX, hnRNP L inhibits the binding of U2AF65 (Fig. 4*E*). However, because the CaRRE1 element bound by hnRNP L is mainly downstream of the polypyrimidine tract (Fig. 1*C*), the mechanism of this inhibition remains to be investigated.

Besides hnRNP L, its paralogue hnRNP L-like (*LL*) is also required for the regulation of STREX splicing by depolarization (Fig. 1*E*). Moreover, PTB binds to the upstream 3′ splice site of STREX to repress its inclusion[4] (17). 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 other factors and their modifications in the regulation is necessary to uncover the underlying molecular mechanisms.

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