TIA Proteins Are Necessary but Not Sufficient for the Tissue-specific Splicing of the Myosin Phosphatase Targeting Subunit 1*

Supriya Shukla‡, Wessel P. Dirkse†, Katherine M. Joyce‡, Caroline Le Guiner-Blanvillain§, Richard Breathnach§, and Steven A. Fisher‡¶

From the Departments Of ‡Medicine (Cardiology) and ¶Physiology and Biophysics, Case Western Reserve University school of Medicine, Cleveland, Ohio 44106-4958 and §§INSERM U463, Institute de Biologie-CHR, 9 Quai Moncousou, 44093 Nantes Cedex 1, France

We are using the tissue-specific splicing of myosin phosphatase targeting subunit (MYPT1) as a model to investigate smooth muscle phenotypic diversity. We previously identified a U-rich intronic enhancer flanking the 5’ splice site (IE1), and a bipartite exonence/exon suppressor, that regulate splicing of the MYPT1 central alternative exon. Here we show that T-cell inhibitor of apoptosis (TIA-1) and T-cell inhibitor of apoptosis-related (TIAR) proteins bind to the IE1. Co-transfection of TIA expression vectors with a MYPT1 mini-gene construct increase splicing of the central alternative exon. TIA proteins do not enhance splicing when the palindromic exonic splicing enhancer (ESE) is mutated, indicating that TIAS are necessary but not sufficient for splicing. The ESE specifically binds SRp55 and SRp20 proteins, supporting a model in which both SR and TIA proteins binding to their cis-elements are required for the recruitment of the splicing complex to a weak 5’ splice site. Inactivation of TIA proteins in the DT40 cell line (TIA-1−/−/TIAR−/−) reduced the splicing of the central alternative exon of the endogenous MYPT1 as well as stably transfected MYPT1 minigene constructs. Splicing of the MYPT1 3’ alternative exon and the MLC17 alternative exon were unaffected, suggesting that TIA proteins regulate a subset of smooth muscle/nonmuscle alternative splicing reactions. Finally, reduced RNA binding and reduced expression of the TIA and SR proteins in phasic (gizzard) smooth muscle around hatch coincided with the switch from exon inclusion to exon skipping, suggesting that loss of TIA and SR enhancer activity may play a role in the developmental switch in MYPT1 splicing.

Smooth muscle (SM) tissues show considerable phenotypic and functional diversity. Much phenotypic diversity in SM tissues is generated by the alternative splicing of exons (1–4). Nearly 60% of vertebrate genes are estimated to undergo alternative splicing (5, 6). Whereas there are a few well characterized genes where alternative splicing is governed by the presence or absence of a single regulatory protein (6), in many genes regulated splicing of exons involves a combinatorial interplay of positive and negative cis-regulatory elements and trans-acting proteins (3, 7). These regulatory interactions affect the splice site selection by the splicing machinery and thus govern the inclusion or exclusion of alternative exons.

Our laboratory is using the MYPT1 gene (a subunit of smooth muscle myosin phosphatase) as a model gene to understand how SM phenotypic diversity is generated through alternative splicing of exons for several reasons. In birds and mammals, isoforms of MYPT1 are generated by cassette-type alternative splicing of exons in the central and 3′-end of the transcript. The splicing of the alternative exons is tissue-specific and developmentally regulated. Around the time of hatching (embryonic day 21 (ED21)), the MYPT1 isoform in the chicken gizzard (phasic fast contracting smooth muscle phenotype) completely switches from central exon-in and 3′-exon-out to central exon-out and 3′-exon-in. In contrast, the chicken slow tonic contracting aorta smooth muscle phenotype throughout development expresses the MYPT1 isoform in which the central alternative exon is included and the 3′ alternative exon is skipped (8, 9). The tissue-specific expression of the MYPT1 splice variants is thought to be an important determinant of smooth muscle functional diversity (9, 10).

We previously used mutation and deletion analysis of MYPT1 minigene constructs to identify cis-regulators of splicing of the chicken MYPT1 central alternative exon (from here on referred to as the MYPT1 alt exon). We identified several cis-enhancer elements (Fig. 1B) in the vicinity of the alternative exon 5′-splice site, including a proximal U-rich intronic sequence, a 67-nucleotide intronic enhancer, and an exonic splicing enhancer, that are necessary for the splicing of the MYPT1 alt exon (11). The proximal intronic splicing enhancer (IE1) is a 19-nucleotide U-rich sequence including two UCUU motifs immediately adjacent to the 5′ splice site of the alternative exon. This sequence is a putative binding site for TIA-1 and TIAR (12–14) and PTB (15) proteins, suggesting a possible role of these proteins in the regulation of MYPT1 alt exon splicing. TIA-1 and TIAR are RNA-binding proteins regulating a number of functions in both nucleus and cytoplasm (16, 17). They have been shown to be the activators of constitutive as well as alternative splicing (12, 14, 18–20). PTB, also known as heterogeneous nuclear ribonucleoprotein-I, is a well known suppressor of splicing (21). In the current study, we use gain and loss of function experiments as well as RNA-protein binding experiments to show that TIA-1 or TIAR functioning through the U-rich enhancer element is necessary but not sufficient for the splicing of the MYPT1 alt exon. We also find that expression of...
the TIA proteins is developmentally regulated, suggesting a model in which the silencing of TIA expression during the development of the phasic fast (gizzard) smooth muscle phenotype contributes to the loss of splicing of the MYPT1 alt exon.

MATERIALS AND METHODS

Tissue Culture and Transfection of Plasmid DNA—Smooth muscle cells (SMCs) were isolated from embryonic chicken gizzards and cultured in Dulbecco’s modified Eagle’s medium/F-12 with 10% heat-inactivated fetal bovine serum (HyClone Laboratories) as previously described (8, 11). 293 cells (Quantum Biotechnology) were grown in monolayer in Dulbecco’s modified Eagle’s medium supplemented with 10% serum, as recommended by the manufacturer. Cells were transfected with plasmid DNA using LipofectAMINE as a carrier as previously described. The cells were transfected in 24-well plates with 0.1 μg/well expression vector containing wild type or mutant MYPT1 minigene expression vectors under the control of the Rous sarcoma virus promoter (11) (Fig. 1B) and 0.01–0.1 μg of full-length TIA-1 or TIAR (16) or PTB (15) expression vectors. The pUC19 plasmid (0.0–0.09 μg) was included to maintain a constant amount of plasmid DNA used in each transfection. RNA was isolated 24–48 h after transfection using Promega SV RNA isolation kit, following the manufacturer’s protocol.

Cell Culture, Electroporation, and RNA Isolation from DT-40 Cells—DT-40 cells were cultured and electroporated as described by Le Guiner et al. (22). For electroporations, 30 μg of a minigene construct were mixed with 3 μg of a plasmid carrying a bacterial resistance marker. Cells were selected in medium containing 50 μg/ml blasticidin, and RNA was prepared and reverse-transcribed as described by Le Guiner et al. (20).

RT-PCR of RNA—RNA samples were analyzed by RT-PCR for the presence of exon-included and exon-skipped MYPT1 minigene transcripts as described previously (11). In these reactions, a 3′ primer is used that specifically anneals with the bovine growth hormone polyadenylation sequence present only in the exogenous MYPT1 minigene transcript. The RT-PCR products were separated on a 6% native polyacrylamide gel, and fluorescent signals of the exon-included and exon-excluded products were quantified with a Storm 860 Imager (Amersham Biosciences) and Amersham Biosciences ImageQuant software. Splice variants of MLC1, MYPT1 central, and MYPT1 C-terminal were examined in DT40 cells and DT40 TIA−/−; TIAR−/− (referred as TIAmut cells hereon) (20) using primers as previously described (11). For RT-PCR, 23–27 (for transfected gizzard cells), 30–35 (for endogenous genes from DT40 cells), and 60–65 (for DT40 stably transfected cells) cycles of amplification were used. RT reactions were done with 0.5–1.5 μg of total RNA, and one-twentieth to one-one hundredth of the RT reaction used in the PCR. For every sample, negative control reactions were performed in which the avian myeloblastosis virus RT enzyme was omitted from the RT reaction. To demonstrate the linearity of the occurrence of exon inclusion, samples were performed using 0.5 and 1.5 μg of input mRNA from each RT reaction. As previously shown (11), this resulted in severalfold increase in the total signal without any significant change in the ratio of the exon-included to exon-skipped products (not shown).

Preparation of Nuclear and Whole Tissue Extracts—Nuclear and whole tissue extracts were prepared from embryonic and posthatched chick gizzard and aorta tissues using the Nuclear Extract Kit (Active Motif, Carlsbad, CA) as described by the manufacturer. Samples were assayed for protein concentrations using BCA protein assay kit (Pierce).

Synthesis of Labeled RNA Oligonucleotides—The 5′-end amine-modified RNA oligonucleotides containing a wild type or a mutant IE1 or an ESE sequence were purchased from Oligo Etc. (Wilsonville, OR). The oligonucleotides were biotin-labeled with a sulfo-succinimidyl-6-biotinamido-hexacontane by Pierce, following the procedure as described by the manufacturer. The labeled oligonucleotides were purified by ethanol precipitation followed by polyacrylamide gel purification on a 19% gel.

RNA Affinity Assay—RNA affinity assay was carried out following the methods described by Blynn et al. (22). Briefly, binding reactions were performed in the 250 μl of binding buffer consisting of 100 mM HEPES, 10 mM MgCl2, 500 mM KCl, 50% glycerol, 10 mM dithiothreitol, 20 mM creatine phosphate, 2 mM ATP, and 2.0 mM bovine serum albumin (10 mg/ml), 10 μg t-RNA, 200 pmol of biotin-labeled RNA, and 250 or 400 μg of NE. After a 10-min incubation at 30 °C, the reaction mixture was added to 10–100 μl of streptavidin Magnesium particles (Promega, Madison, WI), prewashed five times with the binding buffer without t-RNA. The reaction mixture was incubated with prewashed beads for 15 min at 30 °C to allow binding of the protein-RNA complex to the beads. The beads-RNA-protein complex was then washed five times with binding buffer. After final wash, 30 μl of 2× protein sample buffer was added to the complex and incubated for 10 min at 30 °C to allow dissociation of the protein from the complex. 10 μl of each protein sample (one-third of the total sample) was analyzed by SDS-PAGE and Western blotting.

Protein Analysis—Proteins were separated on 4–12% gradient NuPage Novex bis-tris gels (Invitrogen) and either visualized by silver staining or electrophroblotted onto a polyvinylidene difluoride membrane (Amersham Biosciences). Membranes were blocked in 5% nonfat dry milk (w/v) in wash buffer for 1 h and probed with antibodies M1, 29 against TIA-1, 6E3 against TIAR (23), and mAb 104 against SR proteins (24) and SF2-specific antibody (25). The secondary antibody was a goat anti-mouse IgG conjugated with horseradish peroxidase (1:1800; Bio-Rad). Signals were detected using the ECL chemiluminescence kit (Amersham Biosciences).

RESULTS

TIA-1 and TIAR trans-Activate MYPT1 Central Exon Splicing—Transfection of the MYPT1 wild type minigene expression plasmid (Fig. 1A) into cultured gizzard SMCs resulted in 89 ± 2% exon-included transcripts as we previously reported (11) (Fig. 2A). Co-transfection of a TIA-1 expression plasmid increased the level of exon-included product from 89 ± 2% to 98 ± 2% (Fig. 2A, lanes 1 and 2). Co-transfection of a TIAR expression plasmid had a similar but slightly less potent effect (95 ± 3% in wild type Fig. 2A, lane 3). In contrast, co-transfection with a PTB expression plasmid resulted in a small shift toward the exon-inclusion in the cultured SMCs (85 ± 4%, Fig. 2A, lane 4).

In order to determine whether the ability of TIA-1 to enhance splicing of the MYPT1 alt exon was cell type-specific, transfections were repeated in the mammalian nonmuscle 293 cell line. In these cells, transfection of the MYPT1 minigene expression plasmid resulted in ~50% exon-included transcripts (Fig. 2B, lane 1), as previously reported. Co-transfection of TIA-1 expression plasmids increased exon inclusion to 85 ± 4% of transcripts (Fig. 2B, lane 2). Thus, TIA-1 or TIAR, but not PTB, can enhance splicing of the MYPT1 central alt exon in multiple cell types.

Trans-Activation Effect of TIA Is U-rich IE1-dependent—To test whether the observed enhancer effect of TIA on splicing is functioning through the U-rich IE1, TIA-1 or TIAR expression plasmids were co-transfected with MYPT1 minigene constructs where the IE1 is deleted (ΔUCU1U1 + 2) or mutated (Cmut2 and Cmut1 + 2) (Fig. 1B). Deletion of the 19-nt U-rich sequence results in a significant loss of exon splicing in cultured SMCs (19 ± 4% exon inclusion) (see Fig. 3A, lane 3). Co-transfection with the TIA-1 expression plasmid had no effect on exon splicing (15 ± 2% exon-in) (Fig. 3A, lanes 3 and 4). Similarly, mutation of the U-rich sequence to C-rich (Cmut1 + 2) also resulted in exon skipping and failure of TIA-1 co-transfection to enhance splicing (15 ± 2% versus 18 ± 4%) (Fig. 3A, lanes 5 and 6). When the U-rich sequence was partially mutated (Cmut2), a smaller shift toward exon exclusion occurred, and this could be entirely rescued by co-expression of TIA-1 (60 ± 4 versus 92 ± 2% exon-included) (Fig. 3A, compare lanes 7 and 8). Nearly identical results were obtained with co-transfection of a TIAR expression plasmid and the MYPT1 mini-gene constructs (data not shown). Thus, TIA-1 and TIAR trans-activation of MYPT1 alt exon splicing is IE1-dependent.

TIA Enhancer Activity Is ESE-dependent but Independent of the Downstream IE2—We next tested whether the trans-activating ability of TIA-1, functioning through the IE1, is dependent on the exonic and downstream intronic cis-enhancers (ESE and IE2), since mutation of these cis-elements had significant effects on splicing independent of the IE1. Replacement of the chicken MYPT1 alt exon sequence with the corresponding rat MYPT1 exon sequence results in the deletion of 18 nt (including an 11-nt palindromic ESE) and substitution mutations of

13669

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36 nt (8). It should be noted that this exon in the rat (and other mammals) is constitutively spliced and thus may lack regulatory elements (8). The transcripts processed from this minigene construct have minimal exon inclusion, and there is no effect of co-transfection of TIA expression plasmids (Fig. 3B, lanes 1 and 2). This indicates that the cis-enhancer elements within the exon are required as co-activators for the TIA-1-dependent IE1.

To more specifically define the co-activator, we tested the effects of mutation of two previously identified cis-regulators of splicing of the alternative exon. WT sequences are shown on the first line with regulatory sequences in boldface type. Deletions and mutations of the IE1 (U-rich sequence) and ESE are shown below the WT sequence. The ESE contains an 11-nt perfect palindromic sequence (boldface type), which is mutated to destroy the putative SR protein-binding site (Mut pal1) or to destroy both half-sites of the palindromic sequence (Mut pal1 + 2). Mut A alters the sequence immediately upstream of the palindrome. These mutation and deletion constructs were previously created and characterized (11).

**Fig. 1. Diagram of MYPT1 minigene expression construct and mutations and deletions.** A, the MYPT1 minigene is shown. Solid box, alternative exon; open boxes, flanking exons. The horizontal lines connecting these boxes represent introns. Sizes of exons and introns are not drawn to scale. Upstream is the Rous sarcoma virus promoter, and downstream is the bovine growth hormone polyadenylation signal (gray box) in the pRcRSV plasmid. The arrows indicate the approximate locations of the oligonucleotides that were used in all of the RT-PCRs in this study. B, diagram of previously identified cis-regulators of splicing of the alternative exon. WT sequences are shown on the first line with regulatory sequences in boldface type. Deletions and mutations of the IE1 (U-rich sequence) and ESE are shown below the WT sequence. The ESE contains an 11-nt perfect palindromic sequence (boldface type), which is mutated to destroy the putative SR protein-binding site (Mut pal1) or to destroy both half-sites of the palindromic sequence (Mut pal1 + 2). Mut A alters the sequence immediately upstream of the palindrome. These mutation and deletion constructs were previously created and characterized (11).
TIA Regulation of MYPT1 Splicing

This effect is similar to the effect of partially disabling the TIA cis-element Cmut2 (see Fig. 3A, lane 7; 60 ± 4% exon inclusion) and suggests that the remaining TIAR allele can partially support MYPT1 alt exon splicing, consistent with the co-transfection assays. Stable transfection of DT40 WT with the Cmut1 + 2 MYPT1 minigene plasmid resulted in a large shift toward exon skipping with only 15 ± 3% exon-included transcripts (Fig. 4, lane 3), similar to that observed in the transient transfection of smooth and nonmuscle cells. This same construct in the TIAmut DT40 cells generated 5 ± 4% exon-included transcripts (Fig. 4, lane 4), suggesting that the U-rich flanking sequence remaining in this construct has residual TIA-dependent enhancer activity (consistent with TIA protein-binding studies; see below).

TIA Proteins Bind to the U-rich IE1 and SR Proteins Bind to the ESE—The results of co-transfection experiments suggested that TIA activation of splicing of MYPT1 alt exon required both the IE1 and the ESE. We next carried out RNA-protein binding assays to determine what was binding to these elements and whether the co-dependence resulted from a requirement for TIA enhancer function is not due to binding of the ESE by TIA. The assay provides direct evidence that ESE is not required for TIA binding to IE1 and that the requirement of an intact ESE for TIA enhancer function is not due to binding of the ESE by TIA. We next identified factors that bind to the ESE. Systematic evolution of ligands by exponential enrichment (26, 27) analysis of ESE defines it having an optimal binding site for SRp40 and SRp20 (not shown). We carried out RNA affinity binding assays with nuclear extract from ED15 gizzard and probed for SR proteins by Western blot using the mAb 104 antibody that recognizes the RS domain of SR proteins (24). SR proteins of 55 and 20 kDa bound to ESE sequence (Fig. 5B, lanes 2 and 3, respectively). Immunostaining confirmed the identity of this band as TIA-1 (Fig. 5B, lane 1) and TIAR (not shown). On Western blot TIA-1 appeared as a single band at 42 kDa binding to the IE1. Immunostaining also revealed weak binding of TIA to mutIE1 with ~15% as much binding as the to wild type IE1 sequence (Fig. 5B, lane 6). The assay provides direct evidence that ESE is not required for TIA binding to IE1 and that the requirement of an intact ESE for TIA enhancer function is not due to binding of the ESE by TIA.

We next identified factors that bind to the ESE. Systematic evolution of ligands by exponential enrichment (26, 27) analysis of ESE defines it having an optimal binding site for SRp55 with less of a match for SRp40 and SRp20 (not shown). We carried out RNA affinity binding assays with nuclear extract from ED15 gizzard and probed for SR proteins by Western blot using the mAb 104 antibody that recognizes the RS domain of SR proteins (24). SR proteins of 55 and 20 kDa bound to ESE sequence and not to the U-rich IE1 sequence (control) nor to beads only (Fig. 5D, lanes 1 and 2, respectively). All of the major SR proteins are detected in ED15 gizzard nuclear extracts (Fig. 5D, lane 9) or ED15 gizzard purified SR preparations (data not shown), indicating that SRp55 and SRp20 are specifically binding to the ESE.
TIA Binding to IE1 and SR Binding to ESE Follows the Developmentally Regulated Pattern of MYPT1 Alt Exon Splicing—We next sought to determine whether there was a relationship between binding of the TIA and SR proteins to the enhancer sequences and the developmental switch from MYPT1 alt exon splicing to exon exclusion in the gizzard smooth muscle. Affinity binding assays were carried out using the NEs collected from four different periods of development with the following splicing pattern of MYPT1 alt exon: 1) ED15 – 100% exon-included; 2) ED19 – 78% exon included; 3) D1 – 40% exon-included; 4) D6 – 10% exon-included. No significant difference was observed in the binding of TIA to the IE1 between the NE from ED15 and ED19 (Fig. 5, B (lane 5) and C (lane 1)). However, there was a dramatic decrease in TIA-IE1 binding in NE from D1 (Fig. 5C, lane 3). No binding of TIA proteins was detected with the NE from D6 tissue (Fig. 5C, lane 7), even when 4-fold increased amounts of nuclear proteins were used in the binding reaction. A silver stain of the NEs used in these experiments (Fig. 5C, lanes 7–9) shows approximately equal amounts of intact protein in each sample. A similar pattern was observed for SRp55 and SRp20 binding to the ESE (Fig. 5D, compare lanes 1, 3, 5, and 7).

Down-regulated Expression of TIA and SR Proteins in Developing Chick Gizzard Correlates with Developmental Changes in MYPT1 Central Exon Splicing—The stage-dependent binding of TIA and SR proteins to the IE1 and ESE, respectively, prompted us to examine the expression of the TIA and SR proteins during smooth muscle phenotypic specification. We determined the abundance of these proteins in whole tissue extracts (WTEs) and nuclear fractions from ED15, ED19, D1, and D6 chicken gizzard. In WTEs from ED15 and ED19, TIA-1 was detected as a singlet band and TIAR as a doublet at 42 kDa (Fig. 6A, lanes 1 and 2), as previously reported in avian species (20). Additionally, in ED19, D1, and D6 WTEs, a band migrating at ~55 kDa was also observed. This band of unknown identity has been previously reported (28, 29). There was a significant decline in the levels of expression of TIA1 and...
TIAR proteins in the gizzard from days 1–6 after hatching, when examined in WTEs or nuclear subfraction protein extracts. Silver staining of protein extract showed similar amounts of intact protein in each sample (Fig. 5C). When 4-fold more of the D6 nuclear extract protein (40 μg) was loaded onto a gel, weak signals for TIA1 and TIAR were observed (not shown). We also examined the expression of SR proteins in whole tissue extracts, nuclear extracts, and purified SR preparations (24) from gizzard tissues spanning the same developmental period. Western blot analysis of WTEs using the antibody mAb 104 for SR proteins revealed abundance of SRp55, SRp40, and SRp30 in the samples. SRp30 proteins appeared as a doublet. The slower migrating band (SRp30A) was identified as SF2 with a specific monoclonal antibody (25) (not shown). SRp20 and SRp75 were much less abundant but also were present at all stages of development. Similar patterns were observed in nuclear extracts and purified SR preparations probed with either the mAb 104 or SF2-specific antibodies (not shown). In either the whole tissue or subfraction extracts, there was a 2–4-fold decrease in the abundance of the SR proteins from ED19 to the adult gizzard. There were no significant

Fig. 5. TIA proteins bind to IE1, and SRp55 and SRp20 bind to the ESE. An RNA affinity binding assay was carried out with biotinylated RNA oligonucleotides containing a 19-nt wild-type or mutant IE1 sequence, the ESE sequence, or beads only (A). NEs for binding reactions were collected from four different stages of chicken gizzard development (ED15, ED19, D1, and D6). Biotinylated RNA was incubated with the NE under binding conditions (see “Materials and Methods”). Nuclear proteins bound to the RNA were mixed with streptavidin columns, and bound proteins were eluted and analyzed by SDS-PAGE and Western blotting as described under “Materials and Methods.” The oligonucleotide containing the IE1 served as a control for the ESE binding reaction (C) and vice versa. Nuclear extracts were also mixed with SA beads (beads alone) as a control for nonspecific binding. B, a silver stain and Western blot analysis of proteins binding to the IE1 sequence using ED15 NE. Western blot analysis was carried out using anti-TIA-1 antibody ML29 and anti-TIAR 6E3 antibody (not shown). Western blot analysis of TIA-IE1 binding using NEs from different developmental stages, as described above. A silver stain of total NEs used in this study is also shown. D, a Western blot analysis of purified proteins binding to the ESE, using protein-specific mAb 104. Nuclear extracts were used from different developmental stages (described above).

Fig. 6. Down-regulated expression of TIA and SR proteins during chicken gizzard development. Western blot analysis of TIA-1, TIAR, and SR proteins in WTEs (20 μg/sample) and NEs (10 μg/sample) from ED11–ED15 to adult gizzard. A, Western blot analysis of WTEs isolated from ED15, ED19, D1, and D6 gizzard tissues using anti-TIA-1 ML29 (top), anti-TIAR 6E3 (middle), and anti-GAPDH (bottom; as an internal control) antibodies. B, Western blot analysis of gizzard tissue NEs prepared from the stages described above, using anti-TIA ML29 (top) or anti-TIAR 6E3 (bottom). Silver-stained gel of these protein samples is shown in Fig. 5. C, Western blot analysis of WTEs of gizzard isolated from ED11, ED15, ED21, and adult chicken using SR protein-specific mAb 104 antibody (top) and β-actin antibody (bottom; as an internal control). Similar results were obtained with nuclear extracts or purified SR protein preparations from these tissues (not shown).
changes in the relative abundance of each SR protein within a sample.

Regulation of Splicing by TIA Proteins Is Specific to the MYPT1 Central Alternative Exon—As many as 60% of gene transcripts undergo alternative splicing of exons. It was therefore of interest to determine whether the TIA proteins regulate splicing of additional alternative exons. Because many of the gene transcripts that are expressed in smooth muscle cells are also expressed in nonmuscle cells, we could examine this question in the DT40 cell lines in which TIA proteins were either intact or inactivated (TIA-1<sup>−/−</sup> TIA<sup>−/−</sup>). Splicing of the endogenous MYPT1 central alt exon exhibited a significant difference between the two cell lines. In WT DT40 cells, a single RT-PCR product was obtained at 450 bp, representing the central exon-included isoform (8). In DT40 TIAmut cells, the RT-PCR yielded two products at 450 and 325 bp corresponding to the exon-included and the exon-skipped products, respectively, in a ratio of 65:35 (Fig. 7A, lanes 1 and 2). The ratio of the two products was constant over a range of input RNA (data not shown). In contrast, splicing of the MYPT1 3′ alternate exon, which is regulated in the opposite manner (i.e. included when the central alt exon is excluded, and vice versa), was unaffected by TIA disruption (Fig. 7B, lanes 1 and 2). This is somewhat expected, since there is no U-rich sequence in the vicinity of this exon. We also examined the splicing of an additional transcript, the MLC<sub>17</sub> alternative exon. The tissue-specific pattern of splicing of this alternative exon is similar to that of the MYPT1 central alt exon, but the developmental switch in MLC<sub>17</sub> splicing in the gizzard occurs from ED10 to ED16, whereas for MYPT1 it occurs around hatching (ED21) (11, 30). The splicing of this alt exon was also unaffected by TIA disruption (Fig. 7C, lanes 1 and 2). These results establish the MYPT1 central alt exon as a <em>bona fide</em> and specific target of the splicing regulatory activity of TIA proteins.

**DISCUSSION**

We are studying the regulation of alternative splicing of MYPT1 alternative exons as a window into the generation of smooth muscle phenotypic diversity. We previously demonstrated that splicing of MYPT1 alt exon is highly regulated (8) and that exon inclusion is dependent on a cis-enhancer complex consisting of a U-rich IE1, a 67-nucleotide MSE3-like IE2, and a ESE juxtaposed to a exonic splicing-silencer (11). In the present study, we demonstrate that TIA-1 and TIAR bind to the 19-nucleotide U-rich IE1 and enhance splicing of the central MYPT1 alt exon. However, TIA is not sufficient for splicing of the alternative exon, since it fails to enhance splicing when a nearby SR protein binding ESE is mutated. Both TIA and SR proteins can bind to the respective cis-sequences in the absence of the other sequence. This suggests that these cis-enhancers are cooperative in the recruitment of the splicing proteins to the alternative exon 5′ splice site. Finally, expression and binding of TIA-1, TIAR, and SR proteins was down-regulated in developing chick gizzard smooth muscle concomitant with the switch from splicing to skipping of the MYPT1 alt exon, suggesting that these proteins may regulate the tissue-specific splicing of alternative exons in smooth muscle.

**TIA-1/TIAR Binds to U-rich IE1 and Activates MYPT1 Alt Exon Splicing in an ESE-dependent Manner**—We previously identified a 19-nt U-rich sequence just downstream of the alternative exon 5′ splice site that is necessary for splicing of the alternative exon. This sequence also contains two UCUU sequences that are consensus for PTB binding (15). In this study, we show that co-transfection of TIA-1 or TIAR expression vectors increases exon inclusion, whereas co-transfection of PTB has minimal effect, suggesting that TIA-1 and TIAR function in a redundant manner to enhance MYPT1 alt exon splicing. That TIA-1 and TIAR are redundant in this context is also suggested by the loss-of-function experiments. Co-transfection of a dominant negative TIA-1 plasmid construct with the WT MYPT1 construct into 293 cells had no effect on exon splicing despite nearly complete elimination of TIA-1 expression, presumably due to continued expression of TIAR. In DT40 cells, complete abrogation of TIA-1 expression and partial abrogation of TIAR resulted in a 35% reduction in splicing of the endogenous alt exon. Thus, splicing of the endogenous alt exon is TIA-dependent, and the presence of even one of four functional TIA alleles can support splicing of the alt exon. The effect of inactivation of all four TIA alleles could not be studied in this system, since it results in cell lethality and will thus require a conditional inactivation approach. Interestingly, a partial mutation of the U-rich cis-element sequence in the minigene construct produces the same approximate effect on alt exon splicing (35% exon out) as occurs with the inactivation of three of four TIA alleles (35% exon out), and there was generally a close relationship between the effects of mutations on TIA binding assays and exon splicing.

TIA-1 and TIAR genes have also been inactivated in mice. Mice with both TIA-1- and TIAR-disrupted genes die before embryonic day 7, whereas mice with disrupted TIAR show 90% embryonic lethality (17). TIA-1 and TIAR inactivation produces different developmental phenotypes in mice. This may be due to the unique patterns of expression in developing mouse tissues or alternatively to nonredundant functions that were not evident in our <em>in vitro</em> experiments. TIA proteins also are involved in translational control, stress responses, and regula-
tion of cell death, and it remains to be determined which of these functions are required for cell and embryo viability (17, 23, 31). Further, TIAR-deficient mice fail to develop spermatogonia, indicating that TIAR is important for primordial germ cell activity (17, 29).

In this and a prior study, we have shown that TIA is necessary but not sufficient for splicing of the MYPT1 central alternative exon. Mutation of an 11-nt perfect palindromic sequence just upstream of the 5′ splice site and/or additional 5′ sequence significantly reduces splicing of the alt exon in the presence of an intact U-rich IE1, a TIA-1 binding sequence. Co-transfection of TIA expression vectors did not restore splicing when the palindrome was mutated but did restore splicing when other sequences in the minigene construct were mutated, indicating a co-dependence of the IE1 and ESE for alt ex splicing. We did not experimentally define the molecular nature of this co-dependence, but several models can be considered. The model we favor is one in which both TIA binding to the IE1 and SRp55 and SRp20 binding to the ESE are required to recruit U1 snRNP complex to the 5′ splice site (with the caveat that we have yet to show that SRp55 and SRp20 transactivate splicing of this exon) (Fig. 8). This model is based on the observations in splicing of Fas (13), MSL-2 (32), FGFR-2 (14), and TIA itself (19) that show that TIA binds to the U-rich intronic sequence located immediately downstream from a weak 5′ splice site and promotes recruitment of U1 snRNP to the weak 5′ splice site (18). Why both the ESE and IE1 are required is suggested by modeling of the secondary structure of the MYPT1 pre-mRNA (Fig. 8). The modeling suggested that this region forms a stem-loop with the splice site base-paired with the ESE. Thus, it would be proposed that trans-acting factors binding to the ESE are required to unmask the splice site and that without this TIA-1 cannot recruit U1 snRNP to the splice site. This model will require experimental testing. Alternatively, it is possible that TIA and SR binding to their cis-elements are cooperative in the intact MYPT1 pre-mRNA and that this is not evident when short RNA oligonucleotides are used to assay for protein binding. Finally, the assembly of splicing complexes involves

Fig. 8. Proposed model for TIA and SR protein regulation of splicing of MYPT1 central alt exon. Exons are shown as boxes, and introns are shown as horizontal lines connecting exons. A, top left, TIA binds to the U-rich IE1, and SRp55 and SRp20 bind to the ESE in tonic phenotype SMCs. These proteins may relieve the secondary structure that masks the splice site sequence and recruit the U1 to the weak 5′ splice site, thereby promoting splicing. B, TIA and SR proteins fail to bind to the IE1 and ESE, respectively, possibly due to either low levels of expression of these proteins in phasic phenotype (posthatched gizzard) SMCs or expression of a tissue-specific suppressor protein binding to the ESE and/ or to the IE1. The alternative exon is skipped.
the assembly of many proteins into a splicing complex, and the
further identification of these proteins is required for a more
complete understanding of the regulated splicing of this exon.

**Developmentally Regulated Expression of TIA—**The specific
silencing of splicing of MYPT1 central alt exon around hatching
in the gizzard (phasic smooth muscle) provides an attractive
target for understanding how smooth muscle phenotypic diver-
sity is generated through the regulated splicing of exons. The
switch from exon inclusion to exon skipping could involve the
loss of the action of enhancer elements and/or the appearance
of the action of repressor elements. In our previous study, we
identified an exonic suppressor sequence juxtaposed between
the ESE and IE1 that when mutated results in complete loss of
exon skipping in posthatched gizzard smooth muscle cells. This
suggests that the specific silencing of MYPT1 alt ex splicing in
these cells may be due to a tissue-specific repressor element.
We also found that the enhancer function of the ESE and IE1
was much more susceptible to disruption in the posthatched
gizzard SMCs as compared with the cultured SMCs. Thus,
mutations of the IE1 and the ESE that only partially reduced
exon splicing in the cultured SMCs, where the endogenous exon
is included, nearly completely eliminated exon splicing in the
migene transcript in the posthatched gizzard smooth muscle,
where the endogenous exon is excluded (11). In the current
study, we show reduced binding of TIA to the IE1 and SRp55
and Srp20 to the ESE with a time course that parallels the
switch from exon inclusion to exon exclusion. Also concordant
was a reduction in the level of expression of TIA-1 and SR
proteins from this developmental period. One model for these
data is that reduced expression of these proteins results in
weakened enhancer activity rendering exon splicing suscepti-
ble to repression. An alternative explanation would be that
binding of repressor proteins interfere with the binding of the
transactivators to these sequences in the RNA-protein binding
assays. The IE1 contains two UCUU motifs, a consensus bind-
ing site for PTB, a known repressor of splicing. Thus, it is
possible that TIA-1 and PTB compete for binding to this site,
with the relative level of expression or affinity of these proteins
for this site determining whether it functions as an enhancer or
repressor of splicing. Another explanation would be that
antagonistic activities of PTB and ETR3 to regulate the splicing of
tRNAs (21). The ratio of nuclear ETR3 and PTB is proposed to be
determinative for tRNAs exon inclusion in embryonic muscle,
thereby increasing the amount of ETR3 reverses PTB repression.
In another study, antagonistic activities of CELF and PTB
were found to be responsible for the differential splicing of an
α-actinin alternative exon in smooth muscle *versus* nonmuscle
cells (33). With regard to the ESE, the binding of SR proteins
in the current study was tested with an oligonucleotide that con-
tained the overlapping exon splicing silencer. It is thus con-
ceivable that a protein that is specifically expressed in the
phasic gizzard smooth muscle interferes with SR protein bind-
ing to the ESE. Further testing of the role of TIA and SR
proteins in regulated splicing of exons and smooth muscle
phenotypic specification will require their targeted deletions in
these tissues.

In summary, we have proposed a model in which cooperative
action of TIA proteins and SRp55 and Srp20 are required to
transactivate splicing of MYPT1 alt exon in smooth muscle and
nonmuscle cells. Genetic depletion of TIA proteins causes sig-
ificant reductions in splicing of the MYPT1 central alt exon.
Splicing of the MYPT1 3′ alternative exon and another smooth
muscle/nonmuscle alternative exon is unaffected by TIA deple-
tion. These results demonstrate the role of TIA and SR proteins
in the regulated splicing of alternative exons and also highlight
the incredible regulatory complexity in the generation of
smooth muscle phenotypic diversity. Further research is re-
quired to identify how a multitude of positive and negative
regulators of splicing interact to produce smooth muscle phe-
notypic diversity.

**Acknowledgments—**We thank Dr. Nancy Kedersha for providing an-
tibodies for TIA proteins; Dr. Hua Lou for providing expression plas-
mids for TIA-1, TIAR, and PTB; and Dr. Claudia Villalba for analysis of
SR protein expression.

**REFERENCES**
