Two Isoforms of the T-cell Intracellular Antigen 1 (TIA-1) Splicing Factor Display Distinct Splicing Regulation Activities

CONTROL OF TIA-1 ISOFORM RATIO BY TIA-1-RELATED PROTEIN

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TIA-1 (T-cell Intracellular Antigen 1) and TIAR (TIA-1-related protein) are RNA-binding proteins involved in the regulation of alternative pre-mRNA splicing and other aspects of RNA metabolism. Various isoforms of these proteins exist in mammals. For example, TIA-1 presents two major isoforms (TIA-1a and TIA-1b) generated by alternative splicing of exon 5 that differ by eleven amino acids exclusive of the TIA-1a isoform. Here we show that the relative expression of TIA-1 and TIAR isoforms varies in different human tissues and cell lines, suggesting distinct functional properties and regulated isoform expression. We report that whereas TIA-1 isoforms show similar subcellular distribution and RNA binding, TIA-1b displays enhanced splicing stimulatory activity compared with TIA-1a, both in vitro and in vivo. Interestingly, TIAR depletion from HeLa and mouse embryonic fibroblasts results in an increased ratio of TIA-1b/a expression, suggesting that TIAR regulates the relative expression of TIA-1 isoforms. Taken together, the results reveal distinct functional properties of TIA-1 isoforms and the existence of a regulatory network that controls isoform expression.

Gene expression regulation at the post-transcriptional level is a widespread feature in the human genome. Alternative splicing is a major control mechanism by which a single mRNA precursor (pre-mRNA) can produce different mRNAs in a combinatorial, but not stochastic, manner, contributing significantly in the generation of protein isoform diversity (~80% of alternative splicing events promote changes in the coded protein) (1–3). This process can change gene function in different tissues and developmental stages by producing multiple functional isoforms from a single gene (4, 5). It is known that at least 74% of human multiexon genes encode alternatively spliced mRNAs (6), suggesting that this phenomenon represents an efficient expansion of the vocabulary of the genome (7, 8). A major goal of the postgenomic era is to identify physiologically relevant alternative splicing events and determine where and when these occur, what the specific roles of individual isoforms are, and how they are regulated (9). Alternative splicing is also relevant to disease and therapy (2, 3, 10). It has been implicated in many processes, including regulation of immune system diversity (11) and nervous system plasticity (12), generation of aberrant splice variants in cancer (13), and apoptosis (14).

The vast majority of human introns present weak splicing signals with nonconsensus features at the splice sites, branch point, and/or polypyrimidine tract (5–7). Thus, often the authentic splicing motifs are indistinguishable from cryptic sequences hidden in human pre-mRNAs, raising the question of how the spliceosomal machinery is able to define an authentic splicing signal. Additional sequence elements known as “enhancers” and “silencers” are often needed to achieve the correct splicing of exons. These regulatory signals, which are located either in exons or introns, can be near (10–100 nucleotides) or very far away (>1000 nucleotides) from the splice sites (15). The balance between positive and negative regulation of splice site selection likely depends on DNA sequences and changes in the levels of cellular splicing factors under physiological or pathological conditions (2, 4, 15–19). These elements may represent the molecular basis to selectively and specifically regulate alternative splicing of a particular pre-mRNA in different cell types, at different stages of development, or at different stages of cell differentiation and/or proliferation in either normal or pathological conditions (3, 4, 10, 16).

The RNA-binding proteins T-cell intracellular antigen 1 (TIA-1)2 and TIA-1-related protein (TIAR) (20) contain three RNA recognition motifs (RRMs) and a glutamine-rich carboxy-terminal domain (21). Both proteins have been involved in nuclear and cytoplasmic RNA metabolism, i.e. pre-mRNA splicing and mRNA translation. In particular, TIA-1 has been identified as an important splicing regulator in mammals (22–27). This protein is closely related to Nam8p, a yeast protein involved in activation of weak 5′-splice sites followed by downstream U-rich sequences (28). TIA-1 regulates the alternative

Footnotes:

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2 The abbreviations used are: TIA-1, T-cell intracellular antigen 1; TIAR, TIA-1-related protein; NRK, normal rat kidney cell; FGF-2, fibroblast growth factor receptor 2; HMMP13, human metalloproteinases-13; snRNP, small nuclear ribonucleoprotein; RT-PCR, reverse transcriptase-PCR; GFP, green fluorescent protein; GST, glutathione S-transferase; RRM, RNA recognition motif; siRNA, small interference RNA.
pre-mRNA splicing of various human and *Drosophila* genes (FGFR-2, msl-2, TIA1, cystic fibrosis transmembrane conductance regulator, and Fas) (22–27) through binding to U-rich stretches, facilitating atypical 5’-splice site recognition by U1 small nuclear ribonucleoprotein (snRNP). TIA1 can also enhance U6 snRNP assembly on a composite regulatory element consisting of a pseudo-5’-splice site followed by uridines, playing a role in alternative splicing of the calcitonin/CGRP gene (29).

TIA1 has also been well characterized as a translational regulator. This protein has been implicated in stress-induced translational arrest, colocalizing after stress with poly(A)+ RNA in the cytoplasmic foci known as stress granules (30–33). TIA1 and TIAR are both able to bind to the 3’-untranslated regions of the translational regulatory AU-rich elements of tumor necrosis factor α (34, 35), human matrix metalloproteinases-13 (36), cyclooxygenase-2 (37), β-2-adrenergic receptor (38), mitochondrial cytochrome c (39), GADD45α (40), and β-F1-ATPase (41) mRNAs. This aspect of TIA1/TIAR function has recently been the focus of a broad and systematic analysis to identify mRNAs associated with both RNA-binding proteins (42, 43).

TIA1 and TIAR are also known to promote cellular (44–46) and virus-induced (47) apoptosis, to be implicated in viral replication (48), and to be required for DT40 cell viability (49). In addition, mice that lack TIA1 (or TIAR) show high rates of embryonic lethality, indicating an important role of these proteins during embryonic development (35, 50). TIA1 plays also important functions in apoptotic cell death and in adapting the cellular response to metabolic stress and inflammation (40, 42, 44, 45, 51).

In view of the biological relevance of these proteins we considered that it would be of interest to study the expression and functional properties of TIA1 and TIAR isoforms. We specifically addressed the contribution of TIA1a and TIA1b isoforms in the regulation of alternative splicing. The results show that TIA1b displays enhanced splicing regulatory activity compared with TIA1a. Furthermore, the expression of TIA1 and TIAR isoforms is often restricted to certain human tissues and cell lines, suggesting that expression of these proteins and their isoforms is tightly regulated to provide specific functions in splicing regulation. Consistent with this idea, we observe that TIAR influences the relative levels between TIA1 isoforms.

**EXPERIMENTAL PROCEDURES**

**Cell Cultures**—HeLa, normal rat kidney (NRK), and embryonic kidney 293 cells as well as mouse embryonic fibroblast wild-type knock-out for either TIA1 or TIAR (35) were grown in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with penicillin-streptomycin and 10% heat-inactivated fetal calf serum (Invitrogen).

**Constructs**—Plasmids containing human TIA1a cDNA were generated by PCR using *Pfu* Turbo DNA polymerase (Stratagene) (23). The WT and mutant U20C Fas minigenes were generated as described previously (23, 26). The FGFR-2 minigene RK97 was kindly provided by Dr. R. Breathnach (22). The constructs used to overexpress TIA1a and TIA1b cDNAs were fused in-frame to the carboxyl-terminal part of GFP using vector pEGFP-C1 (Clontech). The sequence of all constructs was verified by automated DNA sequencing.

**Recombinant Proteins**—Recombinant TIA1a and TIA1b proteins were expressed in *Escherichia coli* and purified from bacterial lysates, as glutathione S-transferase fusions (GST), using glutathione-Sepharose (Sigma) as described (23). The recombinant proteins were stored in buffer D (20 mM Heps, pH 7.6, 20% glycerol, 0.2 mM EDTA, 1 mM dithiothreitol, and 0.01% NP-40) plus 0.1 mM KCl at −70 °C until used. The purified fusion proteins were analyzed on a 10% SDS-polyacrylamide gel by staining with Cooomassie Blue and Western blot.

**Protein Analysis**—Whole-cell extracts were prepared by resuspending the cells in lysis buffer (50 mM Tris-HCl, pH 8.0, 140 mM NaCl, 1.5 mM MgCl₂, 0.5% Nonidet P-40 plus a mixture of protease inhibitors), freeze-thawing three times, and centrifugation at 10,000 rpm for 5 min in a microfuge at 4 °C. Resulting supernatants were recovered and stored at −70 °C. Protein concentration was determined with the Bradford reagent (Bio-Rad protein assay) using bovine serum albumin as standard. For Western blot analysis, equal amounts of protein from the supernatants were loaded on 10% SDS-PAGE gels and Western blots carried out using nylon membranes and the following antibodies: anti-TIA1 (C-20; Santa Cruz Biotechnology), anti-TIAR (C-18; Santa Cruz Biotechnology), anti-GFP (Clontech), anti-α-tubulin (Sigma), and anti-U2AF65 (MC3) (52). The blots were developed using ECL reagent (Amerham Biosciences) according to the manufacturer’s instructions. Immobilon INSTA-Blot membranes containing samples of several human tissues (brain, heart, small intestine, kidney, liver, lung, skeletal muscle, testes, spleen, pancreas, and ovary) and cell lines (HeLa, Jurkat, Daudi, 293, Rh 30, A375, T98G, HCT-116, and Hep-G2) were obtained from Calbiochem. Total RNAs from human tissues were obtained from Ambion and Clontech.

**Fluorescence Microscopy**—After transfection (24 h), the cells were analyzed by fluorescence microscopy using a confocal microscope (ZEISS) equipped with a GFP excitation filter. Digital image acquisition and processing were performed with a ZEISS camera and ZEISS software package, respectively.

**Preparation of HeLa Nuclear Extracts and Psoralen-mediated Cross-linking Assays**—HeLa nuclear extract was prepared as described previously by Dignam *et al.* (53). Psoralen-mediated cross-linking assays were carried out as reported previously (26).

**Band Shift Assays**—5’-32P-end-labeled synthetic msl-2 RNA oligo containing 10 nucleotides of exonic sequence and 36 nucleotides of intronic sequence or 32P-full-labeled Fas RNA probe containing 23 nucleotides of human Fas exon 6 and 35 nucleotides of human Fas intron 6 5’-splice site were incubated with different molar amounts of GST-TIA1a or GST-TIAR1b (from 10⁻⁸ to 10⁻⁶ M) in a total volume of 15 μl of buffer D with 0.1 mM KCl in the presence of tRNA (200 ng/μl) for 30 min on ice. After incubation, the samples were resolved by electrophoresis on non-denaturing 5% polyacrylamide gels, dried, and exposed to film (26).

**siRNA Duplex Preparation and Transfections of siRNAs**—The sequences of the 21-nt RNA oligonucleotides used for targeting TIA1 and TIAR were synthesized and annealed as described previously (26, 41). HeLa cells were transfected at 30% confluency.
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FIGURE 1. Alternative splicing of human TIA-1 exon 5 and predicted protein isoforms. A, alternative splicing of human TIA-1 exon 5. Exons are represented by boxes, introns by thick lines, and patterns of alternative splicing by thin lines. Alternative TIA-1 isoforms generated by inclusion or skipping of exon 5 are indicated. Numbers indicate the sizes (in nucleotides) of intronic and exonic sequences from human TIA-1 gene (54). B, domain structure of human TIA-1 isoforms. The eleven-amino acid peptide encoded by exon 5 is shown in bold. TIA-1 proteins include three RNA Recognition Motifs (RRM domains) and a carboxyl-terminal glutamine-rich domain. Numbers indicate amino acid residues corresponding to each RRM and carboxyl-terminal domain of the TIA-1b isoform.

DNA Transfections, RNA Isolation, and RT-PCR Analysis—Exponentially grown HeLa and 293 cells were transfected at 50–70% confluence with 0.5 μg of WT/mutant (U-20C) Fas or FGFR-2 minigenes, respectively, and 1.5 μg of GFP-reporter plasmids using Lipofectin (Invitrogen) following the manufacturer’s instructions for adherent cell lines. Cells were then incubated 24–48 h before protein and RNA purifications. Cytoplasmic RNAs were prepared using the RNeasy kit (Qiagen), quantified by OD at 260 nm, and analyzed by RT-PCR. After 25 cycles, the products were analyzed on 2% agarose gels. Control experiments with different input amounts of RNA indicated that the amplification was quantitative under these conditions (23, 26). The oligonucleotide sequences used in RT-PCR analysis were: hTIA-1.sense (5′-GCCTAAGACTCTACGTCGGTAACC-3′) and hTIA-1.antisense (5′-GTGGCAAGAAGACGCCGGATTTATATCTTC-3′) for human TIA-1 gene; hTIAR.sense (5′-ATGGAAAGACGCGACGGCACCAGGCAGC-CCCCCGACTC-3′) and hTIAR.antisense (5′-TCTGGACTCAATCCCACAACACATGG-3′) for human TIAR gene; hβ-actin.sense (5′-AAAGACGTGACTCCCAACACACATGGG-3′) and hβ-actin.antisense (5′-GTCACTACTCCTGCTTGGCTGA-3′) for human β-actin gene; mTIA-1.sense (5′-GCCCAAGACTCTATAGTGCCCAAGACTCTATACGTCG-3′) and mTIA-1.antisense (5′-GGTGCAAAAGCAGC-CTGGACTCAAATCCCC-3′) for mouse TIA-1 gene; mTIAR.sense (5′-GCCCAAGACTCTATAGTGCCCAAGACTCTATACGTCG-3′) and mTIAR.antisense (5′-ATGGAAAGACGCGACGGCACCAGGCAGC-CCCCCGACTC-3′) for mouse TIAR gene.

RESULTS

Human TIA-1 isoforms are generated from a unique gene by exon 5 alternative splicing (Fig. 1A). Exon 5 inclusion generates TIA-1 isoform a, whereas exon skipping produces the shorter b isoform (Fig. 1A). The eleven-
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Differential expression of TIA-1 and TIAR isoforms in HeLa and NRK cells. A and B, TIA-1 and TIAR isofrom distribution in HeLa and NRK cells. Total protein extracts (25 and 50 μg) were fractionated on 10% SDS-polyacrylamide gels and analyzed by Western blot using antibodies specific for TIA-1 or TIAR proteins were used to evaluate their relative levels of expression in human tissues and cell lines. Tissue-specific differences in expression were observed for both proteins as well as differences between their respective patterns of expression (Fig. 2); when the resolution of the blot was sufficient to distinguish different isoforms as species of different mobility, their relative expression also varied. For example, whereas the levels of TIA-1 expression in brain tissue were low compared with kidney, testes, or ovaries, the tissue-specific differences in isoform expression (Fig. 2) were also observed for TIAR, TIAR and the other relatives of TIA-1, TIAR isoforms, and U2AF65 are indicated on the right. C, RT-PCR analysis of cytoplasmic RNA isolated from HeLa and NRK cells. Molecular mass markers in base pairs are shown on the left. Relative positions of the two TIA-1 and TIAR isoforms are also indicated. D, splicing patterns of WT and mutant (U-20C) Fas minigenes in HeLa and NRK cells. Cells were transfected with a minigene containing Fas genomic sequence between exons 5 and 7 (WT) or with the same minigene containing a uridine to cytosine at position −20 on intron 5 (U-20C) that results in higher levels of exon skipping. Accumulation of the alternatively spliced transcripts was determined by quantitative RT-PCR using primers corresponding to vector sequences. Size markers in base pairs are shown on the left. The sizes expected for the alternatively spliced transcripts are schematically indicated on the right.

Antibodies specific for TIA-1 or TIAR proteins were used to evaluate their relative levels of expression in human tissues and cell lines. Tissue-specific differences in expression were observed for both proteins as well as differences between their respective patterns of expression (Fig. 2); when the resolution of the blot was sufficient to distinguish different isoforms as species of different mobility, their relative expression also varied. For example, whereas the levels of TIA-1 expression in brain tissue were low compared with kidney, testes, or ovaries, the opposite trend is observed for TIAR (Fig. 2, A and B). To more clearly document changes in isoform expression, reverse transcriptase and polymerase chain reaction (RT-PCR) analyses were performed. Differences in TIA-1a versus b ratios were observed (compare for example kidney versus testis, lanes 5 and 9 of the upper panel in Fig. 2C). Although changes in mRNA abundance generally correlated with protein levels for TIA-1 (compare Fig. 2A and the upper panel of 2C), this was not always the case for TIAR, suggesting the operation of additional mechanisms of translational or protein stability control. The analysis of cell lines provided additional evidence for differences in isoform expression (Fig. 2, D and E). For example, isoform a was predominant in most cell lines expressing TIA-1, whereas isoform b was more abundant in the T cell Jurkat cell line (Fig. 2D). TIAR isoform a was predominant in Hep-G2 cells and isoform b in HCT-116, whereas the other cell lines tested expressed the two isoforms in roughly a 1:1 ratio. Changes in isoform expression ratio are also detectable in tissue samples (Fig. 2, A–C). Taken together, these observations suggest that TIA-1 and TIAR proteins and their isoforms are differentially expressed in different cell types.

To further document these observations and their potential functional implications, expression of these splice regulators was analyzed in HeLa and NRK cells in parallel with the patterns of alternative splicing of Fas minigenes. Fas pre-mRNA splicing is known to be affected by variations in the activity of TIA-1/TIAR (23, 26). The overall levels of TIA-1, at both mRNA and protein levels, were higher in HeLa than NRK cells, with isoform b being predominant in HeLa and isoform b being the only detectable species in NRK (Fig. 3, A and C). TIAR was expressed at roughly the same levels in the two cell lines, but once again, differences in isoform expression were clearly detectable: whereas HeLa cells expressed both TIARa and b in equal levels, TIARb was the predominant isoform detected in NRK cells (Fig. 3, A and C). To assess whether the differential expression of TIA-1/TIAR and their isoforms results in different levels of TIA-1/TIAR activity in splicing regulation, a minigene containing Fas genomic sequences between exons 5 and 7 was transfected in HeLa and NRK cells and the pattern of exon 6 inclusion/skipping was analyzed by semiquantitative RT-PCR. Previous work (23, 26) showed that both TIA-1 and TIAR promote exon 6 inclusion by binding to uridine-rich sequences and facilitating U1 snRNP binding to regulated 5′-splice sites in this transcript. A mutant minigene containing a mutation in the intron 5 3′-splice site region that induces substantial levels of exon 6 skipping (U-20C) was also used. No differences in the patterns of alternative splicing of either wild type or U-20C Fas minigenes were detected between HeLa and NRK cells (Fig. 3D), indicating that differences in expression between TIA-1 and TIAR isoforms do not result in changes in alternative splicing in one of the known targets of these splicing regulators. Given previous observations that changes in the levels of TIA-1/TIAR proteins, either by depletion or overexpression, affect Fas exon 6 inclusion (23, 26), the similar patterns of Fas alternative splicing in HeLa and NRK cells suggest the possibility that the different isoforms display different activity as splicing regulators.

To test this hypothesis, the subcellular localization, RNA binding, and splicing regulatory activity of the a and b isoforms of TIA-1 were compared. The subcellular distribution of TIA-1a and TIA-1b isoforms was studied by the pattern of fluorescence of GFP fusions of these factors in HeLa cells. GFP-TIA-1a and GFP-TIA-1b fusions were predominantly detected in the nucleus with nucleolar exclusion and diffuse fluorescence in the cytoplasm. No differences in localization between TIA-1a and TIA-1b were evident in these experiments (data not shown). The same conclusion was obtained using standard subcellular fractionation procedures to separate nuclear and cytoplasmic compartments using detergents and differential centrifugation for either endogenous or ectopically expressed GFP-tagged proteins (data not shown). We conclude that TIA-1a and TIA-1b isoforms are localized in the nucleus and in the cytosol, consistent with the known shuttling of TIA-1 and TIAR.
between these compartments (43–45, 54, 55), without significant differences between them.

Next, the activity of TIA-1 isoforms in regulating Fas alternative splicing was tested by co-expression of each isoform (as a GFP fusion) with the U-20C Fas minigene reporter. The a and b isoforms were expressed at similar levels (Fig. 4A). As previously reported (26), overexpression of TIA-1 caused a significant increase of exon 6 inclusion. Interestingly, the apparent specific activity of TIA-1b was significantly and reproducibly higher than that of TIA-1a (compare lanes 2 and 3 in Fig. 4B). Similar results were obtained for another TIA-1-dependent alternative splicing event, inclusion of K-SAM exon of the FGFR-2 gene (22), in another cell line (293 cells). Once again, equivalent levels of TIA-1a and b expression resulted in greater levels of K-SAM exon inclusion for TIA-1b than for TIA-1a (Fig. 4, C and D).

Taken together, the results using both Fas and FGFR-2 minigenes suggest that TIA-1b is more active than TIA-1a as splicing regulator.

To further investigate the relative activities of TIA-1 isoforms, in vitro assays were carried out using recombinant purified GST fusions of these proteins. RNA binding was tested using electrophoretic mobility shift assays (Fig. 5). Two RNAs corresponding to the 5′-splice site regions of msl-2 exon 1 and Fas exon 6, known to respond to TIA-1, were used (Fig. 5A). Binding of TIA-1a and TIA-1b to these RNAs was essentially identical (Fig. 5, B and C). We conclude that the RNA binding properties of TIA-1a and b do not differ significantly.

Next, the splicing-stimulatory activity of TIA-1 isoforms was tested using in vitro psoralen-mediated cross-linking assays. TIA-1/TIAR modulates the splicing of a number of vertebrate and Drosophila primary transcripts, such as FGFR-2, msl-2, TIAR, cystic fibrosis transmembrane conductance regulator, and Fas pre-mRNAs (22–29).

FIGURE 4. TIA-1 isoforms enhance Fas pre-mRNA splicing in vivo. A and C, Western blot analyses of the expression levels of GFP (lane 1), GFP-TIA-1a (lane 2), and GFP-TIA-1b (lane 3) proteins as well as endogenous TIA-1 (lanes 1–3) in co-transfected HeLa (A) and 293 (C) cells. Positions of ectopically expressed GFP and TIA-1 isoforms and endogenous TIA-1 protein are indicated by arrowheads on the right. B and D, effect of TIA-1a and TIA-1b overexpression on alternative splicing of mutant U-20C Fas (B) and FGFR-2 (D) reporter minigenes. Analysis of alternatively spliced products derived from a mutant U-20C Fas (26) and RK97 FGFR-2 (22) minigenes cotransfected with pEGFP-C1 (lane 1), pEGFP-C1-TIA-1a (lane 2), or pEGFP-C1-TIA-1b (lane 3) plasmids into HeLa (B) and 293 (D) cells. RNAs isolated from post-transfected (24 h) HeLa and 293 cells were analyzed by semiquantitative RT-PCR and the products of amplification separated by 2% agarose gel electrophoresis. Positions of predicted alternatively spliced products are indicated at the right side by boxes. The results are means ± S.E. for two to three independent experiments.

FIGURE 5. RNA binding affinity of TIA-1a and TIA-1b binding to msl-2 and Fas 5′-splice site regions. A, RNA sequences of msl-2 and Fas 5′-splice sites used in electrophoretic mobility shift assays (EMSA) are shown in detail. B, EMSA using various concentrations of purified recombinant GST-TIA-1a and GST-TIA-1b proteins (10⁻⁹, 10⁻⁸, 10⁻⁷, and 10⁻⁶ M) and 5′-³²P-labeled msl-2 ribooligo is shown. C, EMSA using GST-TIA-1a and GST-TIA-1b proteins (10⁻⁹, 10⁻⁸, 10⁻⁷, 3 10⁻⁷, 6 10⁻⁷, and 10⁻⁶ M) and radioactively labeled Fas 5′-splice site region RNA is shown. In panels B and C, RNA-protein complexes were analyzed on native 6% polyacrylamide gel, dried, and exposed to film. The positions of the free RNAs and RNA-protein complexes are indicated on the right.
downstream of weak 5′-splice sites and facilitate 5′-splice site recognition by U1 snRNP through a protein-protein interaction involving the U1 snRNP-associated protein U1C (22–28). Consistent with the higher apparent specific activity of TIA-1b in transient transfection assays (Fig. 4), TIA-1b was reproducibly more efficient than TIA-1a in promoting U1 snRNP recruitment to Fas intron 6 5′-splice site (Fig. 6, compare lanes 3 and 4 with 5 and 6). Taken together, the results of Figs. 4 and 6 argue that TIA-1 isoforms have distinct activities as splicing regulators.

Previous results have shown that TIA-1 can regulate the levels of mRNA isoforms of TIAR to induce expression of mRNAs containing premature termination codons, thus suggesting a mechanism to balance the relative activity of the two families of factors. The presence of a conserved uridine-rich motif adjacent to the TIA-1 exon 5 5′-splice site (Fig. 7A) suggested the possibility that TIA-1/TIAR regulate the relative ratio between TIA-1 isoforms. To test this hypothesis, the relative expression of TIA-1a and b was analyzed in cells where expression of TIA/TIA-1 proteins was reduced using siRNAs (HeLa cells) or by double knock out of the genes (MEFs) as described (26). Both reduction in the levels of TIA and knock out of the gene resulted in increased TIA-1b/TIA-1a ratio (upper panels in Fig. 7B, lane 2 versus 4 and Fig. 8C, lane 1 versus 3). Clear changes in the b/a isoform ratio were also obvious when RNA levels were analyzed (Fig. 7, D and E, lanes 1 versus 3), strongly arguing that TIA promotes TIA-1 exon 5 inclusion and therefore accumulation of TIA-1a mRNA. Depletion of TIA-1, in contrast, did not significantly affect the ratio between TIAR isoforms (Fig. 7, B–E).

**DISCUSSION**

Alternative splicing is a relevant mechanism to establish the architecture of biological complexity in higher eukaryotes determining development-, cell- and tissue-specific expression of various transcript forms from a single gene. It is well established that protein isoforms generated by alternative splicing can have distinct, sometimes even antagonistic, functions because alternatively spliced regions affect diverse functional features of the protein, including, for example, binding properties to DNA, RNA, or other proteins, intracellular localization, enzymatic activity, regulation, and stability (56). An outstanding question, however, is the extent to which alternative splicing causes functional differences in the majority of the vast range of transcripts generated by this mechanism (57), particularly when the differences between isoforms are limited to small peptides. Splicing factors and regulators are a family of proteins where contributions of alternative splicing to protein diversity are particularly remarkable. Important examples include the Wilms’ tumor suppressor WT1, which plays relevant functions in development (58, 59); alternative splicing generates two main isoforms that differ by the presence or absence of a three-amino acid (KTS) insertion within the zinc finger domain and that have distinct functions in vivo (58). Whereas the KTS isoform acts as a transcriptional regulator, the +KTS isoform is a poor transcription regulator but interacts and co-localizes with splicing factors, suggesting a role for this isoform in RNA processing (58, 60, 61). Other examples include members of the heterogeneous nuclear ribonucleoprotein (hnRNP) family of proteins. hnRNP D0 contains two RRM domains, and an alternatively spliced peptide of nineteen amino acids is known to affect RNA sequence binding specificity (62). hnRNP C1 is thirteen amino acids shorter than the C2 isoform, and both proteins differ considerably in their RNA binding affinities in vitro (63) and in vivo (64).

To contribute new information about functional differences between alternatively spliced splicing factor isoforms, we decided to evaluate the functional impact of alternative splicing on isoforms of the splicing factor TIA-1. Our results suggest redundant roles for TIA-1 and TIA proteins and at the same time reveal differences in splicing activity between TIA-1 isoforms. Most importantly, these data indicate that cross-regulatory interactions between TIA-1/TIAR and their isoforms can act as a mechanism to buffer changes in expression and provide uniform levels of regulatory activity.

Variations in the concentration of general splicing factors and regulators, including members of the SR protein and heterogeneous nuclear ribonucleoprotein protein families, have been proposed as mechanisms to achieve cell type- or developmental stage-specific splicing (5). For example, SR protein ASF/SF2 is crucial for postnatal heart remodeling in the mouse, regulating a select set of tissue-specific alternative splicing events in neonatal cardiomyocytes (65). In this study we report that the relative abundance of TIA-1, TIAR, and their isoforms differs in human tissues and cell lines, with TIAR showing a somewhat more restricted pattern of expression. The final splicing outcome will of course depend also on additional factors that synergize or antagonize TIA-1/TIAR activities. For example,
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A

Sequences of TIA-1 exons 5:
Human: 5'-GTAGTACGGTGTGACACCGAGGTTCAAG-3'
Mouse: 5'-GTAGTACGGTGTGACACCGAGGTTCAAG-3'

Sequences of TIA-1 intron 6 5' splice sites:
Human: 5'-GTAATTGTATCTTAAACATCAA-3'
Mouse: 5'-GTAATTGTATCTTAAACATCAA-3'

B

FIGURE 7. Alternative TIA-1 splicing is regulated by TIAR. A, the sequences corresponding to the exon 5 and 5’-splice site (5'ss) of intron 6 (at least until 25 nt downstream to the 5'ss) from mouse and human TIA-1 genes are indicated. The sequences were obtained from ENSMUST0000005753 and ENST00000282574 Ensembl for mouse and human TIA-1 genes, respectively. Also indicated are the RNA sequences of intron 6 5’-splice sites, including the underlined uridine-rich intronic sequences on intron 6 from human and mouse TIA-1 pre-mRNAs. B, siRNA-mediated reduction of TIA-1/TIAR levels in HeLa cells. Total protein (5 μg lane 1) or 25 μg (lanes 2–4) from untransfected or siRNA-transfected HeLa cells was analyzed by Western blot using antibodies against TIA-1 (upper panel), TIAR (middle panel), or U2AF65 (lower panel). ~90% depletion of TIA-1 or TIAR protein levels was achieved under these conditions. The positions of molecular mass markers and human TIA-1 and TIAR isoforms are indicated. C, analysis of TIA-1 and TIAR isoform patterns in wild type mouse embryonic fibroblasts (MEFs) (WT, +/+), knocked out for TIA-1 (TIA-1, −/−) or for TIAR (TIAR, −/−). Total protein (20 μg) from each cell type was analyzed by Western blot using antibodies against TIA-1 and U2AF65 (loading control) (upper panel) or TIAR and α-tubulin (loading control) (lower panel) as indicated. D, analysis of endogenous TIA-1 and TIAR alternatively spliced transcripts in HeLa cells upon TIA-1/TIAR silencing. RNA isolated from cells transfected with the indicated siRNAs was analyzed by RT-PCR and the products of amplification fractionated by electrophoresis in agarose gels. Positions of size markers and the predicted alternatively spliced RNA products are indicated. E, analysis of endogenous TIA-1 and TIAR alternative splicing in mouse embryo fibroblasts. RNA isolated from cells as in panel C was analyzed by RT-PCR and the products of amplification separated as in panel D. The positions of size markers and mouse TIA-1 and TIAR RNAs are indicated.

Hu proteins have been recently reported to antagonize TIA-1/TIAR to achieve neuron-specific alternative splicing (66). In addition, post-translational modifications can modulate the activity of regulatory factors. TIA-1 phosphorylation by the Fas-activated FAST kinase, for instance, increases TIA-1 activity and enhances inclusion of Fas exon 6, thus creating a positive feedback loop by which Fas signaling induces higher levels of the proapoptotic form of the receptor (67).

The domain structure of TIA-1 and TIAR comprises three RRM domains and a carboxyl-terminal glutamine-rich domain. Binding of TIA-1 to uridine-rich sequences requires RRM2 and 3 (24). The eleven amino acids that distinguish TIA-1a from b are located in the boundary between RRM1 and RRM2. It was therefore conceivable that the presence or absence of these residues distinguishes the RNA binding properties of the two isoforms. Although we cannot rule out that this is the case for specific RNA substrates, the results of Fig. 6 argue against this possibility. Binding of TIA-1 downstream of weak 5’-splice site helps to recruit U1 snRNP through protein-protein interactions involving the glutamine-rich domain of TIA-1 and the U1-specific protein U1 C (22–26). Deletion analyses revealed an additional contribution of the non-consensus RRM1 motif through an unknown mechanism (24). It is therefore possible that the residues that distinguish the two isoforms of TIA-1 modulate the U1 snRNP-recruiting activity displayed by RRM1. TIAR differs from TIARb by an additional seventeen-amino acid peptide insertion inside RRM1 that has been suggested to play a role in RNA binding specificity (20) or, by analogy with TIA-1, may alter the splicing activation function of the TIAR.

Autoregulatory loops are common to set up gene networks involved in, for example, transcriptional control (68). It has been reported that splicing factor SC35 regulates splicing of its own pre-mRNA to generate alternative transcripts with different rates of decay (69). We report here that TIAR depletion in human and mouse cells results in partial skipping of TIA-1 exon 5 and therefore in an increase in TIA-1-b/a isoform ratio, which should result, according to the results of Figs. 5 and 7, in increased TIA-1 levels of activity. We therefore propose that this mechanism can serve as a regulatory feedback circuit between the two genes to compensate a reduction in the levels of TIAR. Interestingly, TIA-1 does not seem to exert an equivalent activity to modulate the ratio between TIA isoforms. It is nevertheless important to point out that both TIA-1 and TIAR pre-mRNAs contain cryptic exons that, when included, interrupt the open reading frames encoding these proteins and therefore cause decreased levels of these factors. Inclusion of these exons is, in turn, regulated by TIA-1 and TIAR activities (49). Taken together with our observations, these results reveal intricate gene networks involved in regulating TIA-1/TIAR function, further arguing for the physiological importance of these factors.

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