Fas-activated Serine/Threonine Kinase (FAST K) Synergizes with TIA-1/TIAR Proteins to Regulate Fas Alternative Splicing*

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The factors and mechanisms that mediate the effects of intracellular signaling cascades on alternative pre-mRNA splicing are poorly understood. TIA-1 (T-cell intracellular antigen 1) and TIAR (TIA-1-related) proteins regulate alternative pre-mRNA splicing by promoting the use of suboptimal 5′ splice sites followed by uridine-rich intronic enhancer sequences. These proteins promote, for example, inclusion of Fas receptor exon 6, which leads to an mRNA encoding a pro-apoptotic form of the receptor at the expense of the form that skips exon 6, which encodes an anti-apoptotic form. Fas-activated serine/threonine kinase (FAST K) is known to interact with and phosphorylate TIA-1. Here we have tested the possibility that FAST K influences alternative pre-mRNA splicing by affecting the activity of TIA-1/TIAR. Depletion of FAST K form Jurkat cells leads to skipping of exon 6 from endogenous Fas transcripts. Conversely, FAST K overexpression enhances exon 6 inclusion of Fas reporters transfected in HeLa cells. Consistent with the possibility that the effects of FAST K are mediated by changes in the function of TIA-1/TIAR, the effects of FAST K overexpression (i) are largely suppressed by depletion of TIA-1 and TIAR and (ii) are significantly compromised by mutation of a TIA-1/TIAR-responsive enhancer present downstream of exon 6 5′ splice site. Furthermore, in vitro phosphorylation of TIA-1 by FAST K results in enhanced U1 snRNP recruitment. Interestingly, this enhancement is not due to increased binding of TIA-1 to the pre-mRNA. Taken together, the results connect Fas signaling with the activity of splicing factors that modulate Fas alternative splicing, suggesting the existence of an autoregulatory loop that could serve to amplify Fas responses.

Pre-mRNA splicing is a major control mechanism for modulating gene expression. Alternative splicing is a prevalent, versatile, and highly regulated process, by which a single mRNA precursor (pre-mRNA) can produce multiple different mRNAs and proteins. It is known that at least 74% of human multiexon genes encode alternatively spliced mRNAs and 80% of this alternative splicing events promote changes in the coded protein (for review, see Refs. 1 and 2). This phenomenon is a broad source of transcriptome and proteome diversity and is relevant to regulate critical cellular events, such as immune diversity, nervous system plasticity, cancer, or apoptosis (for review, see Refs. 3 and 4).

The molecular basis of alternative splicing control is linked to the process of splice site selection. Splice site selection depends on the balance between positive and negative sequence elements and on changes in the activity and/or amount of cellular splicing factors under physiological or pathological conditions (3, 4). The RNA-binding proteins TIA-1 (T-cell intracellular antigen 1) and TIAR (TIA-1 related protein) have been identified as splicing regulators relevant for a variety of alternative splicing decisions in mammals (5–11). These proteins contain three RNA recognition motifs and a glutamine-rich COOH-terminal domain (12). TIA-1/TIAR regulates the splicing of certain human and Drosophila pre-mRNAs (e.g. FGFR-2, msl-2, TIAR, CFTR (cystic fibrosis transmembrane conductance regulator), and Fas pre-mRNAs (5–11)) through their binding to uridine-rich stretches located immediately downstream of weak 5′ splice sites and facilitating 5′ splice site recognition by U1 snRNP through protein-protein interactions with the U1 snRNP-associated protein U1 C (5–10). TIAR can also enhance U6 snRNP assembly on a pseudo-5′ splice site followed by uridines, playing a role in alternative splicing of the calcitonin/CGRP gene (11).

Fas-activated serine/threonine kinase (FAST K) was identified as a TIA-interacting protein in a yeast two-hybrid screening (13). This kinase is constitutively phosphorylated on serine and threonine residues in Jurkat cells. In response to Fas ligation, FAST K is rapidly dephosphorylated and TIA-1 is concomitantly phosphorylated in serine residues (13). These results suggested that FAST K and TIA-1 may play a role in signaling Fas-induced apoptosis. The goal of this work was to determine whether FAST K can influence the activity of TIA-1/TIAR as splicing regulators. The results indicate that FAST K modulates the inclusion of Fas exon 6 through synergistic effects with TIA-1/TIAR as well as additional splicing factors, thus linking a signaling pathway with the activity of splicing factors and specific events in post-transcriptional gene regulation.

EXPERIMENTAL PROCEDURES

Reagents

Fas Constructs—PCR-based mutagenesis of the expression vector containing human Fas genomic sequences was carried

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‡ The abbreviations used are: TIA-1, T-cell intracellular antigen 1; TIAR, TIA-1-related protein; FAST K, Fas-activated serine/threonine kinase; U1R6, U-rich sequence located close to the Fas intron 6 5′ splice site; FGFR-2, fibroblast growth factor receptor 2; snRNP, small nuclear ribonucleoprotein; nt, nucleotide(s); HA, hemagglutinin; GST, glutathione S-transferase; siRNA, small interfering RNA; RT, reverse transcriptase; GFP, green fluorescent protein.
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out as described (8, 9). TIA-1b/TIARb cDNAs were fused in frame to the carboxyl-terminal part of GFP by using vector pE GFP-C1 (Clontech) (9). HA-FAST K expression plasmid was kindly provided by P. Anderson.

**Antibodies**—anti-U2AF65 (clone MC3), anti-TIA-1 (C-20, Santa Cruz Biotechnology), anti-TIAR (C-18, Santa Cruz Biotechnology), anti-FAST K (C-20, Santa Cruz Biotechnology), anti-GFP (JL-8, BD Biosciences), and anti-HA epitope (16B12, Covance).

**Cell Cultures and Transfections**

Jurkat and HeLa S2 cells were cultured in RPMI and Dulbecco’s modified Eagle’s medium, respectively, supplemented with 10% fetal calf serum. The 21-nt siRNA oligonucleotides used for targeting FAST K, TIA-1, and TIAR were synthesized and annealed as described previously (9, 14, 15). Jurkat and HeLa cells were transfected as described (9, 15). For double transfections, siRNAs were transfected first, followed 72 h later by transfection of plasmid DNAs.

**Protein and RNA Analysis**

Whole-cell Jurkat and HeLa extracts were prepared by resuspending the cells in lysis buffer and analyzed by Western blot (9). Cytoplasmic RNAs were prepared using the RNeasy kit (Qiagen) Cytoplasmic RNA was quantified by optical density at 260 nm and treated with RNase-free DNase (Promega). RNA (1.5 μg) was reverse-transcribed using specific primers and avian myeloblastosis virus reverse transcriptase (Promega) for 1 h at 42 °C. An aliquot (1/10) of the cDNAs was amplified by PCR using PT1 and PT2 oligonucleotide pairs. PT1 and PT2 oligonucleotides to analyze alternatively spliced products from human Fas minigene were described previously (6, 9). 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.

**Kinase Assays, Psoralen-mediated Cross-linking, and Electrophoretic Mobility Shift Assays**

For *in vitro* kinase assays, anti-HA-FAST K immunoprecipitates from HeLa cells transfected with HA epitope-tagged FAST K were incubated with purified GST or GST-TIA-1 as described previously (13). Psoralen-mediated cross-linking and mobility shift assays were performed as reported previously (6, 8, 9).

**RESULTS AND DISCUSSION**

To study the function of FAST K in the regulation of Fas alternative splicing, the expression levels of this protein, as well as of TIA-1 and TIAR, in Jurkat cells were specifically reduced by electroporation of 21-nt specific siRNAs against FAST K or TIA-1 and TIAR mRNAs. Samples from siRNAs-treated Jurkat cells were analyzed after 72 h by Western blot using antibodies against FAST K, TIA-1, TIAR, or U2AF65 (used as a loading control). 80–90% depletion (quantification by densitometer) of FAST K, TIA-1, and TIAR proteins was reproducibly achieved under these conditions (Fig. 1, A–C). From the same samples, cytoplasmic RNA was isolated and used to analyze the splicing patterns of the endogenous Fas gene by semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR). Consistent with previous results, TIA-1 and TIAR depletion promoted partial exon 6 skipping in Jurkat cells (Fig. 1D, lane 3). These results support a role for FAST K in promoting the synthesis of the mRNA encoding the Fas/CD95 membrane receptor isoform in Jurkat cells, with similar effects to overexpression of TIA-1/TIAR.

To gain an additional evidence for the role of FAST K as Fas splicing activator, HeLa cells were cotransfected with recombinant FAST K, TIA-1, or TIAR and a mutated human Fas minigene (U-20C), which contains a uridine to cytidine substitution 20 nucleotides upstream from the 3’ splice of intron 5, which results in decreased levels of exon 6 inclusion (Fig. 2A, lane 1), as reported previously (9). The ectopic expression of recombinant proteins as well as endogenous levels of TIA-1 and TIAR in

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**FIGURE 1.** siRNA-mediated knockdown of FAST K expression promotes exon 6 skipping of endogenous Fas gene. Protein extracts (20 μg, A–C) and cytoplasmic RNA (1.5 μg, D) from untransfected (A–D, lanes 1) or TIA-1– or FAST K siRNA-treated Jurkat cells (A–D, lanes 2 and 3) were purified and analyzed by Western blot (A–C, with anti-Fast K, anti-TIA-1, or anti-TIAR antibodies, respectively) or by RT-PCR (D) as described. Alternative splicing products from human Fas minigene-specific primer pairs corresponding to exons 5 and 7 (6). Molecular mass markers for protein (kDa in A–C) and DNA (bp in D) are indicated on the right by arrowheads. PCR amplification products (D) corresponding to the Fas alternatively spliced products are indicated at the right by boxes.
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FIGURE 2. Overexpression of recombinant FAST K activates exon 6 inclusion from a mutant Fas minigene. A, analysis of alternatively spliced products derived from an U-20C Fas minigene (9) covering the genomic region between exon 5 and exon 7 co-transfected with GFP (lane 1), GFP-TIA-1 (lane 2), GFP-TIAR (lane 3), or HA-FAST K (lane 4) expression plasmids into HeLa cells. RNA isolated from post-transfected HeLa cells (24 h) was analyzed by semi-quantitative RT-PCR and the products of amplification fractionated on 2%-agarose gel electrophoresis. Positions of predicted alternatively spliced products are indicated at the right by boxes. B, representative Western blots are shown to illustrate the ectopic expression levels of GFP, GFP-TIA-1, GFP-TIAR, and endogenous TIA-1 (upper panel, lanes 1–4, respectively) as well as recombinant HA-FAST K and endogenous TIAR (lower panel, lanes 1–4). Molecular weight markers are indicated on the left. The positions of the expressed recombinant proteins are indicated on the right by arrowhead.

transfected cells was assessed by Western blot analysis (Fig. 2B). As expected, overexpression of TIA-1 or TIAR resulted in increased exon 6 inclusion (Fig. 2A, lanes 2 and 3), consistent with the notion that both proteins are Fas splicing activators that promote exon 6 inclusion (9). Interestingly, overexpression of FAST K also resulted in increased exon 6 inclusion (Fig. 2A, lane 4) consistent with the role of this kinase as an activator of exon 6 inclusion. Taken together, the siRNA-mediated knockdown of FAST K in Jurkat cells (Fig. 1) and its overexpression in HeLa cells (Fig. 2) argue for a function of FAST K as a regulator of the alternative splicing event that promotes Fas exon 6 inclusion.

To investigate whether FAST K-dependent splicing activity is mediated by the activities of the splicing regulators TIA-1 and/or TIAR, a combination of overexpression of FAST K and depletion of TIA-1, TIAR, or TIA-1 and TIAR experiments were performed (Fig. 3). The results indicated that siRNA-mediated knockdown of TIA-1, TIAR, and TIA-1 plus TIAR resulted in gradual skipping of exon 6 from the U-20C Fas minigene (Fig. 3A, lanes 2–4). Overexpression of FAST K in cells depleted of any one of these proteins resulted in a significant increase of exon 6 inclusion (Fig. 3A, lanes 5–7). However, the depletion of both TIA-1 and TIAR significantly compromised the exon inclusion-promoting effects of FAST overexpression (Fig. 3A, lane 8). These results show that FAST K effects on Fas splicing regulation are mediated, at least in part, through the function of splicing factors TIA-1 and TIAR. The observation that some increase in exon 6 inclusion was observed under conditions of TIA-1 and TIAR depletion could be explained either by incomplete siRNA-mediated depletion of these factors or by effects of FAST K on other splicing factors that regulate this splicing event.

To further document these conclusions, the complementary approach of testing the effect of FAST K on a Fas minigene containing mutations at an intronic enhancer sequence known to mediate the stimulatory effects of TIA-1/TIAR (9). This minigene (mURI6) contains mutations in the conserved uridine-rich sequence located downstream of the 5′ splice site of intron 6 (URI6) resulting in a prevalent exon 6 skipping (Fig. 3A, lanes 9–12). Whereas overexpression of TIA-1, TIAR, or FAST promoted inclusion of exon 6 using the U-20C Fas minigene (Fig. 3B, lanes 3–5), overexpression of TIA-1 or TIAR did not result, as expected, in higher levels of exon inclusion in the mURI6 Fas minigene (Fig. 3B, compare lanes 3 and 4 and 7 and 8, respectively). Overexpression of FAST K resulted in levels of exon inclusion around 30–40% (Fig. 3B, compare
lanes 5 and 9). We conclude that mutation of the sequences that mediate the effects of TIA-1/TIAR on exon inclusion decreases the effects of FAST K overexpression (compare lanes 5 and 9 in Fig. 3B), consistent with the effects of reducing the levels of these factors (Fig. 3A). The non-insignificant levels of exon 6 inclusion in the mURI6 upon FAST K overexpression could be due to either mediated by TIA-1/TIAR acting through other sequences (6) or to effects of FAST K on other splicing factors and associated splicing regulatory motifs.

Given that the cellular activity of splicing factors can be regulated by its abundance, cellular localization, the availability of specific regulatory molecules, and/or post-translational modifications (2–4), the effects of FAST K on Fas alternative splicing could be potentially explained by any of the mechanisms mentioned above. One possible scenario, based on the fact that FAST K and TIA-1 interact with each other and TIA-1 is phosphorylated by this kinase, is that TIA-1/TIAR phosphorylation results in increased levels of these factors in the nucleus, TIA proteins are known to shuttle between nucleus and the cytoplasm (16), or a nuclear compartment engaging in functional interactions with splicing factors and/or nascent transcripts. A second, not mutually exclusive, possibility is that phosphorylation increases the biochemical activity of these factors in splicing regulation. To test this possibility, recombinant purified GST-TIA-1 was phosphorylated in vitro by incubation with immunoprecipitates of HA-epitope tagged FAST K expressed in HeLa cells, as described previously (13) (data not shown). Incubation under the same conditions in the absence of ATP was carried out as a control. Next, the activity of the phosphorylated and mock-phosphorylated GST-TIA-1 in promoting U1 snRNP binding was analyzed. Two weak 5’ splice sites followed by uridine-rich sequences were used, Fas exon 6 5’ splice site region and a 5’ splice site region from Drosophila gene male specific lethal-2, which were both previously characterized as a TIA-1-dependent 5’ splice site regions (6). Base pairing between U1 snRNA and the 5’ splice site was detected using psoralen-mediated UV cross-linking, as reported previously (6, 9). Phosphorylated TIA-1 protein induced increased cross-linking of U1 snRNA with the 5’ splice site.
site, both in nuclear extracts and using partially purified U1 snRNP (Fig. 4A–C). Significantly, the activity of the phosphorylated protein was found to be reproducibly higher than that of its mock-phosphorylated counterpart (Fig. 4, A–C, compare lane 3 with lane 4). Taken together, these results indicate that TIA-1 phosphorylation by FAST K results in higher U1 snRNP recruitment activity. Interestingly, this enhancement is not due to increased binding of the phosphorylated protein to its cognate sequences (Fig. 4D), suggesting that the effect of TIA-1 phosphorylation is to stimulate U1 snRNP binding through improved interaction with U1 C (6, 8) or some other recruiting mechanism.

Other splicing factors that can be phosphorylated and have capacity to recruit U1 snRNP, such as SR proteins, SR-protein like factors, or even hnRNPs, may be specific substrates of FAST K, possibly explaining the potentially TIA-independent splicing regulatory effects of this kinase.

Inclusion of Fas exon 6 results in the synthesis of the mRNA encoding the proapoptotic form of the Fas receptor at the expense of the soluble form that inhibits programmed cell death (17, 18). Human Fas (Apo-1/CD95) mRNA encodes a type 1 transmembrane protein that mediates apoptosis upon ligation of the Fas ligand (FasL) (17). It seems plausible that a signaling pathway implicating FAST K, TIA-1, and/or TIAR, and probably additional splicing factors, may be operating as part of a positive autoregulatory loop whereby initial triggering of the Fas signaling pathway can lead to the accumulation of higher levels of membrane-bound receptor, which, in turn, can lead to higher levels of Fas-mediated activation. Such a scenario can operate, for example, during the activation of T cells, a process that requires an increase in exon 6 inclusion (17, 19). Indeed, defects in exon 6 inclusion lead to lymphocyte hyperproliferation and autoimmune lymphoproliferative syndrome (20, 21).

Although signaling cascades are known to affect alternative splicing decisions (22), in almost no case has it been possible to connect specific molecules in the signaling route with particular splicing factors being affected and having predictable effects on alternative splicing events of physiological significance. The balance between Fas 6 exon inclusion and skipping is tightly regulated in a number of other physiological and pathological situations. For example, increased levels of soluble Fas are found in the serum of patients with autoimmune diseases such as systemic lupus erythematosus, adult T lymphoma, lymphocytic leukemia, as well as in other diseases such as cirrhosis, hepatoma, hepatic C infections, and multiple sclerosis (18, 20, 21). An important goal for future studies will be to test whether some of these pathological conditions arise from deficient regulation of exon 6 inclusion caused by the unbalanced activities of FAST K/TIA-1/TIAR signaling pathway. Additional studies will also be required to assess the effects of FAST K on other cellular pre-mRNAs (e.g. CTFR exon 9, FGFR-2 exon Iib, . . . ) whose alternative splicing events are known to be regulated by TIA-1 and/or TIAR in either normal or pathological conditions (5, 10).

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