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A Conserved Signal-Responsive Sequence Mediates Activation-Induced Alternative Splicing of CD45

Short Article

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Summary

Alternative splicing of nascent transcripts is a widespread mechanism for altering protein expression in response to extracellular stimuli. However, little is known about the sequences that mediate signal-induced alternative splicing, complicating efforts to identify genes whose splicing may be regulated in response to a particular stimuli. Here we define a sequence element that is both the primary determinant of CD45 variable exon exclusion following T cell stimulation by PMA and is sufficient to confer activation-induced skipping of a heterologous exon. Additionally, we show that this regulatory element has homology to sequences in other signal-regulated genes, suggesting that the alternative splicing of large families of genes may be regulated by common signaling pathways.

Introduction

Recent analysis of the human genome has demonstrated that the majority of human genes encode multiple distinct protein isoforms as a result of alternative splicing (Black, 2003). Variant protein isoforms encoded from a single gene typically have distinct functions within a cell. Thus, regulation of isoform expression, via the regulation of alternative splicing, is likely to play a widespread and profound role in determining cellular function. Indeed, several human diseases have now been causally associated with aberrant alternative splicing (Caceres and Kornblihtt, 2002).

Despite the abundance and significance of alternative splicing, we are only beginning to understand the factors and mechanisms by which splicing is regulated. In particular, there is a significant need for a greater understanding of how signaling pathways alter isoform expression. A growing number of examples have emerged of genes that are alternatively spliced in response to extracellular stimuli, including changes in isoform expression of the insulin receptor and PKCβ in response to insulin treatment, changes in neuronal BK ion channel expression in response to stress hormones or depolarization, and changes in the expression of CD44 and CD45 T cell regulatory proteins in response to antigen stimulation of T cells (Stamm, 2002). The biological significance of signal-induced changes in isoform expression is underscored by the fact that abnormal isoform ratios of the CD44 and CD45 genes, among others, have been linked to susceptibility to cancer or autoimmune diseases, respectively (Herrlich et al., 1993; Jacobsen et al., 2000). However, few of the genes now known to undergo signal-induced alternative splicing have been studied in any detail. For instance, although it is now possible to predict the presence and activity of some regulatory sequence motifs that influence basal or tissue-specific splicing of a given gene (Cartegni et al., 2002; Fairbrother et al., 2002; Ladd and Cooper, 2002), there has been only very limited characterization of sequences that confer signal-responsive isoform changes. Thus, we are not yet able to identify consensus sequences among signal-responsive regulatory elements or to predict what additional genes may be susceptible to signal-responsive splicing changes. Moreover, it remains unclear whether families of genes may be coordinately regulated in response to a particular stimuli at the level of alternative splicing in a manner analogous to signal-induced synchronized changes in transcription (Smale and Fisher, 2002).

As a model for dissecting signal-regulated alternative splicing, we are studying the induced exclusion of CD45 variable exons that occurs in response to antigen-induced activation of T cells (Hermiston et al., 2002; see Figure 1A). Previously we have demonstrated that activation-induced skipping of CD45 exon 4 can be mimicked in minigenes expressed in the T cell-derived JSL1 cell line upon treatment with the phorbol ester PMA (Lynch and Weiss, 2000). The JSL1 cell line provides a useful system for analyzing signal-induced alternative splicing, since few other cell lines of any tissue type have been shown to support alternative splicing in response to extracellular stimuli. Using this JSL1 cell line, we have demonstrated that constitutive activation of the small GTPase Ras, a downstream component of the PMA-induced signaling pathway, is sufficient to induce alternative splicing of CD45 (Lynch and Weiss, 2000). Moreover, we have identified several sequence elements within CD45 exon 4 that are important for determining the level of exon 4 inclusion in resting cells (i.e., basal splicing) (Lynch and Weiss, 2001). However, the sequences responsible for mediating activation-induced exon repression remain unexplored. In this study we identify the sequence motif that is responsible for the activation-induced skipping of each of the three CD45 variable exons. Importantly, a 60 nt fragment of CD45 exon 4 that contains this sequence motif is sufficient to confer activation-induced repression upon a normally constitutive exon. Finally, we show that this sequence motif is present in variable exons of other genes and is a predictor of exons that may be alternatively spliced in response to signaling events in a variety of tissues.

Results

The ESS1 Silencer from Exon 4 Is Necessary and Sufficient for Activation-Induced Exon Skipping

To identify the sequences responsible for mediating activation-induced exon skipping of CD45 variable exons,

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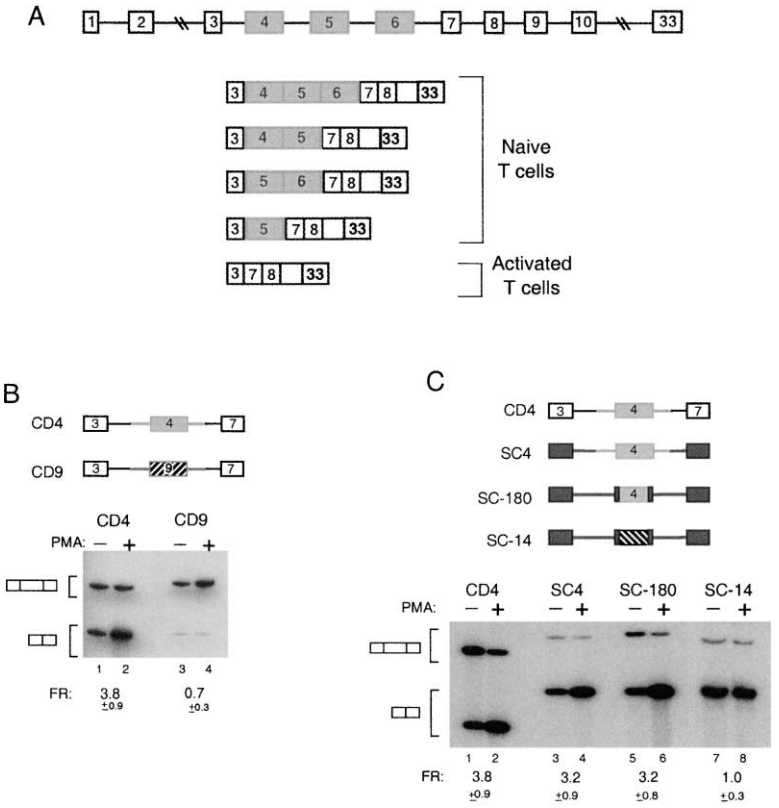

Figure 1. Sequences Internal to CD45 Exon 4 Are Necessary and Sufficient for PMA-Induced Exon Skipping

(A) A schematic of the CD45 gene and the five isoforms that are expressed in human T cells. Exons and introns are represented by open or shaded boxes or lines, respectively.
(B) RT-PCR analysis of RNA derived from minigenes in which CD45 regulated exon 4 or constitutive exon 9 are flanked by constitutive CD45 exons 3 and 7.
(C) RT-PCR analysis of RNA derived from minigene chimeras of CD45 and human β-globin. Sequences derived from β-globin are depicted as dark boxes and thick lines. For all experiments minigenes are stably expressed in JSL1 cells under resting (−PMA) or activated (+PMA) conditions, and analyzed as described in the Experimental Procedures.

we made a series of chimeric minigenes and examined the mRNA isoform expression derived from these minigenes stably expressed in the JSL1 cell line. All of the minigenes used in this study with the prefix CD have a test exon flanked by the CD45 constitutive exons 3 and 7 (which naturally flank variable exons 4, 5, and 6), whereas constructs with the prefix SC have the text exon flanked by heterologous exons derived from the human β-globin gene. Intron sequences are derived from the respective flanking exons as shown in the figures and described in the Experimental Procedures. Consistent with our previous studies (Lynch and Weiss, 2000), skipping of exon 4 in the context of the CD4 minigene is increased 3- to 4-fold upon treatment of JSL1 cells with PMA (Figure 1B, CD4). For all constructs in this study the fold repression values shown are the average of multiple stimulations of at least four to eight independent clones that stably express the given minigene. Previously we reported PMA-induced alternative splicing as the percentage of three-exon product detected (Lynch and Weiss, 2000). However, in this study we quantitate the PMA-induced alternative splicing as fold repression (FR), defined as the change in the ratio of three-exon to two-exon product between resting and activated cells. This method of quantitation is more reliable than previous methods because it standardizes constructs that have exceptionally high or low levels of basal three-exon product. Therefore, using fold repression values we can directly compare the signal responsiveness of minigene constructs that have differing basal levels of exon inclusion due to differences in splice site strength or the presence of previously described constitutive splicing regulatory elements (Lynch and Weiss, 2001). In addition, the function of CD45 is influenced by the ratio, and not the absolute amount, of the high and low molecular weight isoforms (see Discussion). Thus, measuring the difference in the ratio of the two isoforms is most consistent with the biological importance of CD45 alternative splicing.

Replacement of exon 4 with constitutive CD45 exon 9 abolished PMA-induced changes in splicing (Figure 1B, CD9), whereas replacement of the flanking exons 3 and 7 with constitutive exons from the human β-globin gene did not impede PMA-induced skipping of exon 4 (Figure 1C, SC4). The lack of PMA responsiveness of the CD9-derived RNA is not attributable to the overall efficiency of splicing, since we can detect clear activation-induced exon skipping in other RNAs with high basal inclusion (see Figure 3A, SC5). Thus, we conclude that constitutive CD45 exons 3 and 7 are neither necessary for the skipping of exon 4 nor sufficient to confer activation-dependent exclusion of an intervening exon. Instead, we find that sequences internal to exon 4 are sufficient to confer activation-induced skipping of a heterologous exon. Minigene SC-180 (Figure 1C) consists entirely of β-globin-derived sequences, with the exception of the internal 180 nucleotides (nt) of exon 4 that have been inserted between β-globin splice sites in the central exon. This chimeric central exon of SC-180 is highly responsive to PMA-induced repression, whereas an analogous exon derived from CD45 constitutive exon 14 is not (Figure 1C, SC-180 versus SC-14).

The internal 180 nucleotides of CD45 exon 4 contain two major elements that determine the basal level of

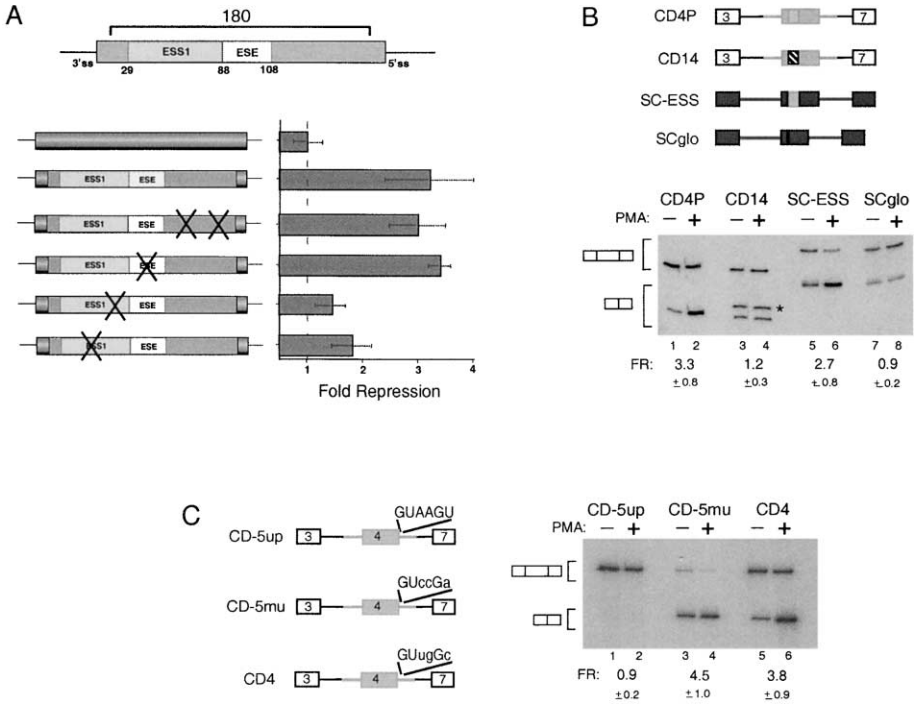

Figure 2. The 60 nt ESS1 Element Is Necessary and Sufficient for PMA-Induced Exon Skipping

(A) Fold repression values from RT-PCR analysis of SC-180 (Figure 1B) constructs that contain mutations of 20 nt regions of exon 4 sequences as described (Lynch and Weiss, 2001).

(B) RT-PCR analysis of minigene constructs is shown. CD4P is identical to CD4 (Figure 1) except that it contains the restriction enzyme sites used to make CD14. The band indicated by an asterisk is a cryptic splice product. SC-ESS and SCglo differ only in the respective presence or absence of the ESS1 sequence.

(C) RT-PCR analysis of minigene constructs differing at the 5′ss. Lowercase letters correspond to deviation from the optimal 5′ss consensus sequence.

exon inclusion in resting cells, namely, a purine-rich exonic splicing enhancer (ESE) and an exonic splicing silencer (ESS1) (Lynch and Weiss, 2001). As shown in Figure 2A, mutation of the ESE had no deleterious effect on the fold switch of the SC-180 construct, nor did mutations further downstream of this element. In contrast, mutations that partially delete the exonic splicing silencer (ESS1) not only increase basal inclusion of the resulting exon (Lynch and Weiss, 2001; see Figure 3C) but also result in a reduction in the activation-induced skipping of this exon (Figures 2A and 3C). Furthermore, substitution of the full 60 nt ESS1 element within the CD4 minigene, with sequences from a constitutive CD45 exon, completely eliminate any effect of PMA activation on exon inclusion (Figure 2B, CD14). Strikingly, we find that the 60 nt ESS1 sequence is alone sufficient to confer PMA-induced exon repression within a heterologous exon, as shown by minigene SC-ESS (Figure 2B). Together, these data indicate that the ESS1 sequence is the primary activation-responsive sequence (ARS) responsible for the activation-dependent repression of CD45 exon 4. Sequences involved in mediating signal-responsive splicing have been identified in only a very few other systems, and none of these previously identified sequences share any significant homology with the CD45 ESS1. Thus, we propose that the CD45 ESS1 splicing regulatory element represents a new class of signal-responsive splicing regulatory sequences.

In a previous study we showed that the 5′ splice site (ss) of CD45 exon 4 is inherently weak and is required for basal silencing by the ESS1 (Lynch and Weiss, 2001). To determine whether the strength of the 5′ss is also important for the ARS function of the ESS1, we tested the role of the 5′ss in the PMA responsiveness of CD4. Strengthening of the 5′ss abolishes PMA-induced exon skipping, whereas changing the sequence of the 5′ss from one weak sequence to another has no effect (Figure 2C, CD-5up and CD-5mu). Therefore, although there is no requirement for a particular sequence at the 5′ss, a weak splice site is required for PMA-induced regulation, suggesting that the ESS functions to repress splicing upon PMA treatment by inhibiting the recognition of a weak splice site by the splicing machinery. It is worth noting that the 5′ss within the SC-ESS construct and flanking CD45 exons 5 and 6 (see Figure 3) also deviates significantly from the optimal consensus sequence.

A Conserved Sequence Mediates Activation-Induced Skipping of All Three CD45 Variable Exons

We next wanted to more precisely define the sequences within the ESS1 element that determined the ARS function. Previously we have shown that a single base change within ESS1 (C77G) dramatically reduces the basal splicing silencing activity of ESS1, resulting in an increased inclusion of exon 4 (Lynch and Weiss, 2001). However, this mutation does not result in a significant

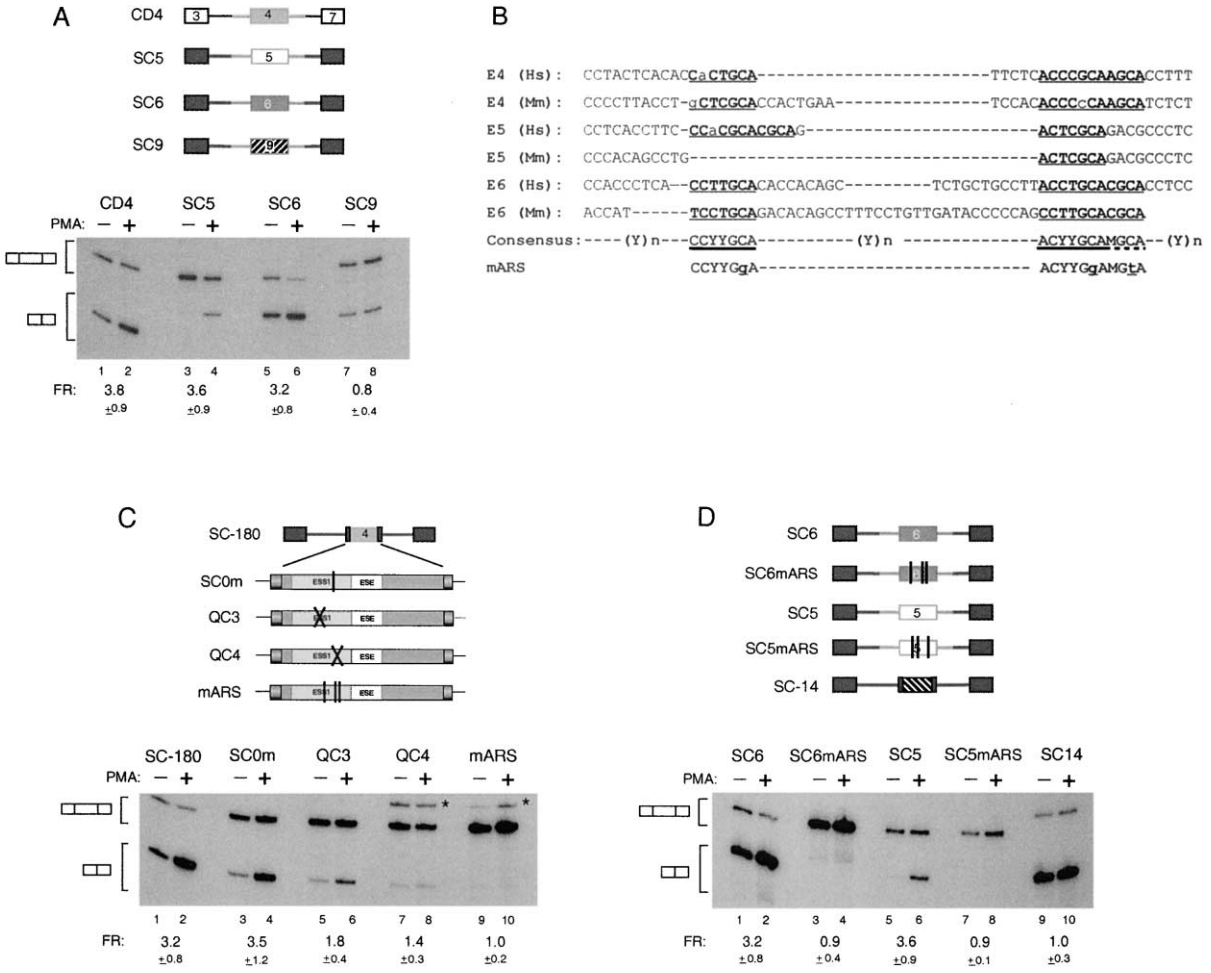

Figure 3. Identification of the ARS Consensus Sequence in CD45 Variable Exons

(A) RT-PCR analysis of splicing of CD45 variable exons 5 and 6 flanked by β-globin exons.

(B) Sequence comparison of human and mouse CD45 exons 4, 5, and 6 highlighting conservation of imperfect repeat. M is defined as a C or A. Mutated bases for the mARS construct are also shown.

(C) RT-PCR analysis of SC-180 derivative minigenes. Asterisks indicate bands derived from minigene DNA. Mutations, represented by bars or X's, are as described in the Experimental Procedures or shown in (B) (mARS).

(D) RT-PCR analysis of variable exons 5 and 6 with or without the mARS mutation shown in (B).

decrease in the PMA-dependent changes in splicing (i.e., ARS function) since we find that exon skipping of a CD4-derived minigene containing the C77G mutation is increased from 3% to 10% upon treatment with PMA (Lynch and Weiss, 2000). Similarly, in this study we show that the C77G mutation in the context of the SC-180 minigene increases basal expression of the three-exon product from 20% to 65% but has no effect on PMA-induced repression (Figure 3C, SC-180 versus SC0m). Therefore, the sequence at nucleotide 77 does not appear to be a critical determinant of the ARS function of the ESS1 element.

Similar to exon 4, the inclusion of the two other CD45 variable exons, exons 5 and 6, is also repressed upon T cell activation or PMA stimulation of JSL1 cells (Figure 1A). Insertion of these exons into the β-globin-derived minigene demonstrates that sequences within exons 5 and 6 themselves are sufficient to mediate their own activation-induced repression (Figure 3A, SC5 and SC6). Since the induced skipping of exons 4, 5, and 6 appears to be mechanistically similar, we looked for regions of sequence homology between the ESS1 of exon 4 and sequences within exons 5 and 6. As shown in Figure 3B, we find an imperfect repeat that is conserved between the ESS1, exon 5, and exon 6, which we have termed the ARS consensus sequence. The ARS consensus sequence is additionally conserved in the alternatively spliced murine exons 4, 5, and 6 (Trowbridge and Thomas, 1994). We find no close match to this imperfect repeat in any of the constitutive exons within CD45, although there is a single repeat of this sequence in murine exon 7 which has been shown in mice to be variably excluded together with exons 4, 5, and 6 upon stimulation (Chang et al., 1991). Remarkably, mutation of three of the most conserved bases of the ARS consensus is sufficient to completely abolish the PMA responsiveness of the SC-180 minigene (Figures 3B and 3C, mARS). In contrast, as described above, the C77G mutation in exon 4 that changes a less conserved nucleotide of the consensus has no effect (Figure 3C, SC0m), while

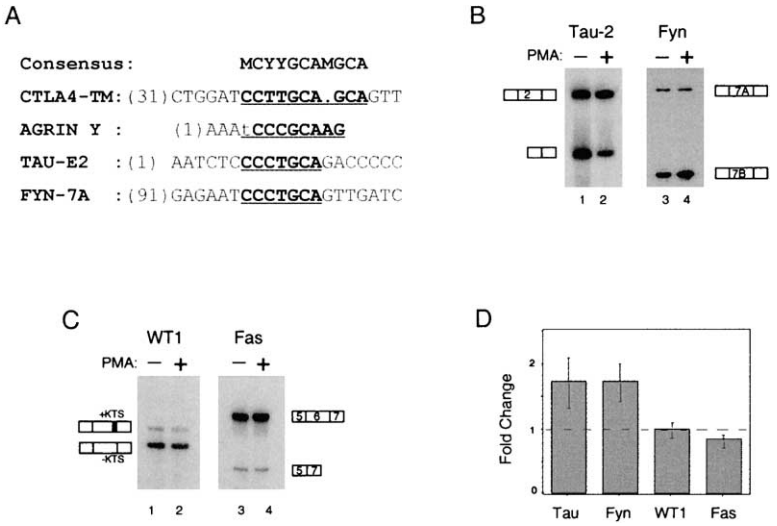

Figure 4. Other Alternatively Spliced Genes Contain Homology to the ARS Consensus Element within Their Variable Exons

(A) Alignment of ARS consensus sequence with sequences from alternative TM exon in CTLA-4, Y exon in Agrin, exon 2 from Tau, and exon 7A in Fyn. The position within exon is indicated by a bracketed number. In the case of Agrin the full exon is shown; for others a 20 nt region is shown. Bold and underlining indicate ARS consensus; lowercase corresponds to deviation from ARS.

(B) RT-PCR analysis of variable exon inclusion in the endogenous Tau and Fyn genes expressed in JSL1 cells ± PMA stimulation.

(C) RT-PCR analysis of variable exon inclusion in the endogenous WT1 and Fas genes expressed in JSL1 cells ± PMA stimulation.

(D) Fold change in isoform ratio upon PMA stimulation for genes shown in (B) and (C). Values for fold change are derived from stimulations of at least 20 independent cell clones.

substitution of 20 nucleotides containing either half of the imperfect repeat partially, but not completely, reduces PMA-induced exon skipping (Figure 3C, QC3 and QC4). Notably, substitution of the longer copy of the consensus repeat (QC4) is more deleterious to the PMA-induced exon skipping than is mutation of the shorter version (QC3).

To further confirm the general role of the ARS consensus element we also made mutations in exons 5 and 6 corresponding to those of exon 4 mARS (Figure 3B). As shown in Figure 3D, mutation of three nucleotides of the ARS consensus in either exon 5 or exon 6 completely eliminates PMA-induced exon skipping. The effect of the mARS mutation in exon 5 is particularly striking, since in this context the mutations have no effect on the already high level of basal splicing (Figure 3D, lanes 5 versus 7), yet fully abolish activation-induced exon skipping. Therefore, the ARS function of the consensus sequence does not strictly depend on the presence of basal silencing activity.

The Presence of the ARS Consensus Sequence Is Predictive of Exons Whose Splicing Is Altered by Stimulation

Splicing regulatory sequences are typically highly degenerate, and their function is usually highly influenced by context (Black, 2003), which complicates attempts to identify regulatory elements by virtue of primary sequence homology alone. However, the abundance and significance of signal-induced splicing suggest that commonalities may be present. Given the context dependence and typical combinatorial nature of splicing regulatory sequences (Black, 2003; Cartegni et al., 2002), we predict that it is unlikely that the ARS consensus sequence alone is sufficient to confer signal-induced exon skipping in all contexts. However, the above results do indicate that the ARS consensus sequence is necessary for the PMA responsiveness of the CD45 variable exons. We therefore looked for homology to the CD45 ARS consensus sequence in exons known to be alternatively spliced in response to PMA or in response to Ras activation, which we have previously implicated as a component of the CD45 signal-induced regulation pathway (Lynch and Weiss, 2000). Surprisingly we find a close match to the ARS consensus in the transmembrane (TM) exon of the T cell regulatory protein CTLA-4 (Figure 4A), which has been well-documented to be inducibly included in CTLA-4 transcripts in response to PMA stimulation of T cells (Magistrelli et al., 1999; Oaks et al., 2000). In addition, we find a match in the Y exon of the matrix protein Agrin, known to be included in a Ras-dependent manner in neuronal cells (Smith et al., 1997). Many splicing regulatory sequences have previously been shown to function as either enhancers or silencers depending on their position and broader sequence context (Black, 2003). For example, the splicing regulator Tra-2 functions as a splicing activator when bound to sequences downstream of an intron in the *Drosophila* doublesex (*dsx*) gene but functions as a repressor of splicing when bound to a similar sequence located upstream of an intron within its own pre-mRNA (Chandler et al., 2003; Lynch and Maniatis, 1996). This difference in activity has been attributed to Tra-2 recruiting other activating proteins to *dsx* but inhibiting the function of a neighboring enhancer in the *tra-2* pre-mRNA. Therefore, it is not necessarily surprising that the ARS sequence might enhance inclusion of the CTLA-4 and Agrin exons in response to PMA/Ras signaling yet repress inclusion of the CD45 exons. Although neither Agrin nor CTLA-4 is expressed in our cell line, we were encouraged by the finding of the ARS consensus in several known signal-responsive exons. We therefore screened an alternative splicing database (http://cgsigma.cshl.org/new_alt_exon_db2/) for the presence of the ARS consensus to determine whether other exons that contained a match to the ARS sequence might also be regulated in response to PMA. The microtubule-associated protein Tau and the Src-kinase family member Fyn both contain alternative exons with homology to a single copy of the ARS consensus, and both are expressed endogenously in our JSL1 cells line. Remarkably, analysis of the splicing of the endogenous Tau and Fyn genes reveals a modest but detectable

change in the inclusion of the ARS-containing exons between resting and PMA-activated cells (Figures 4B and 4D). Tau exon 2 is included almost 2-fold more efficiently upon activation, whereas Fyn 7A is repressed in favor of exon 7B following T cell activation by approximately 2-fold. To date, we have not analyzed any ARS consensus-containing alternative exons that do not reproducibly show a notable change in splicing upon PMA activation. In contrast, analysis of two non-ARS containing alternative exons in the WT1 and Fas genes reveals no detectable change in splicing of these exons upon PMA stimulation (Figures 4C and 4D). Thus, we conclude that the presence of even a single copy of the ARS consensus within a known alternative exon is, with reasonable frequency, predictive of activation-induced changes in alternative isoform expression.

Discussion

The ESS1 Is an Activation-Responsive Splicing Regulator

In summary, we find that the ESS1 element of CD45 exon 4 functions as a discrete activation-responsive splicing regulatory element. To date, the only other sequence that has been conclusively shown to confer signal-responsive regulation of splicing within a fully heterologous context is the CaMK responsive RNA element (CaRRE) from the STREX exon of the slo gene (Xie and Black, 2001). Although the CaRRE and ESS1 elements both influence splicing in a signal-responsive manner, they are clearly functionally distinct. The CaRRE responds to Ca^{2+}/calmodulin-dependent protein kinase (CaMK IV) (Xie and Black, 2001), whereas the ESS1 responds to PMA/Ras (Lynch and Weiss, 2000). Furthermore, there is no sequence similarity between the CaRRE and ESS1 elements, and the CaRRE is localized within the 3′ splice site upstream of the regulated exon, whereas the ESS1 functions within the exon. Importantly, although ESS1 differs from the CaRRE, the ESS1 does have homology to sequences in other signal-responsive exons. We show here that activation-regulated exons in the Agrin, Tau, Fyn, and CTLA-4 genes have homology to the critical ARS consensus sequences of ESS1. Thus, the ESS1 sequence could be the first in a family of related activation-responsive splicing regulatory elements that control the signal-induced expression of a variety of genes.

Despite a lack of sequence similarity between ESS1 and the CaRRE, both elements play a role in controlling basal and signal-induced levels of splicing (Xie and Black, 2001; this study). Such dual-role activity is likely to be a common theme among splicing regulatory elements since mammalian alternative splicing changes are rarely all-or-none events, but rather are changes in the ratios of competing isoforms (Black, 2003). Thus, it is reasonable to expect that splicing regulatory systems are designed to exert a minor influence in the basal state that sensitizes the splicing to subsequent and more robust effects upon stimulation. Indeed, in the case of the endogenous CD45 gene, there is a low level of expression of the smallest isoform lacking all variable exons in resting T cells, which is significantly increased upon stimulation (Trowbridge and Thomas, 1994; Lynch and Weiss 2000). Such an expression profile is consistent with the action of a weak basal splicing silencer that converts to a stronger silencer in response to activation. Within the ESS1, the basal and signal-induced silencing activities appear to be partially overlapping since several of the mutations we show (i.e., QC3, QC4, mARS in exons 4 and 6) disrupt both the basal and activation-induced silencing of exons. However, the C77G mutation within the ESS influences the basal inclusion but not the signal-induced repression of exon 4, and the mARS mutations within exon 5 abolish signal-induced activity but have no apparent effect on basal splicing. These data suggest that the basal and signal-dependent activities of ESS1 are not completely redundant and may involve distinct proteins with separate binding specificities. Future studies as to the specific proteins required for each activity will be required to fully understand the interplay between the activities of the ESS1 in resting and activated cells.

A Suggestive Role for ARS-Mediated Splicing Regulation in T Cell Homeostasis

The discovery of the ARS consensus sequence in both the CTLA-4 and CD45 regulated exons suggests that these genes may be regulated by a common pathway downstream of T cell activation. Such commonalities in the regulation of CTLA-4 and CD45 are particularly intriguing given the overlap in the functional significance of the alternative splicing of these two genes. CD45 phosphatase activity is required to maintain T cell stimulation (Hermiston et al., 2002). The longer isoforms of CD45, i.e., those encoded by mRNAs containing exons 4, 5, and 6, exist as a monomer on the cell surface. When in this monomeric form, the intracellular phosphatase domain of CD45 is fully active. However, skipping of exons 4, 5, and 6 results in a smaller isoform of CD45 that homodimerizes on the cell surface, resulting in steric inhibition of its phosphatase domain (Xu and Weiss, 2002). Therefore, the stimulation-induced switch from the larger to smaller CD45 isoform is believed to help prevent continuous activation of a T cell following an initial stimuli (Hermiston et al., 2002). Since the smaller isoforms are predicted to dimerize with the larger isoforms, the overall amount of active CD45 phosphatase is likely determined primarily by the ratio of the small to large isoform on the cell surface rather than the absolute level of the larger isoform (Hermiston et al., 2002). A second mechanism for preventing T cell hyperactivity following an initial antigen challenge is by increasing the cell surface expression of CTLA-4. CTLA-4 shuts off T cell activation by inhibiting a required costimulatory signal from the molecules CD80/86 (Magistrelli et al., 1999). Thus, the stimulation-induced inclusion of the CTLA-4 TM exon helps to shut off further T cell signaling by increasing the expression of the plasma membrane-associated form of CTLA-4. In summary, the regulated inclusion of the CTLA-4 TM exon and the regulated exclusion of the CD45 variable exons both play a role in maintaining T cell homeostasis following an initial antigen challenge. Strikingly, disruption of the normal isoform expression pattern of both CD45 and CTLA-4 has been associated with susceptibility to autoimmune diseases in humans (Jacobsen et al., 2000; Ueda and al.,

2003). These data suggest the possibility that T cells use a program of coordinated signal-induced alternative splicing events to regulate their function in a manner analogous to the coordinated transcriptional regulation of cytokine genes. It should be noted that there are likely to be at least two pathways leading from T cell signaling to regulated changes in splicing, in that we find no homology between ESS1 and sequences within the previously described regulated CD44 exon 5, and the time course following T cell stimulation upon which splicing changes in CD44 are first detected (7 hr) is different from that of CD45 and CTLA-4 (24–48 hr) (Konig et al., 1998; Lynch and Weiss, 2000; Oaks et al., 2000).

Prediction of Activation-Induced Splicing Regulation

In addition to the putative role of ARS-dependent splicing in the regulation of T cell function, the finding of ARS-like sequences within regulated exons from the primarily neuronal genes Agrin and Tau suggests that there are common splicing regulatory pathways in the neuronal and immune systems. Clearly, the ability to predict genes that undergo alternative splicing in response to specific cell stimuli would dramatically enhance our understanding of the broader relevance of signal-regulated splicing in influencing cellular function. In this study we demonstrate that the presence of the ARS consensus sequence within a variable exon is at least partially predictive of those exons whose inclusion is altered by cellular signaling pathways. Consequently, these studies are likely to provide the basis for the identification of many more genes that undergo signal-induced regulated alternative splicing in a variety of tissues and thus expand our appreciation of the prevalence and importance of signal-induced alternative splicing.

Experimental Procedures

Minigenes

The CD4 minigene was described previously as MG4 (Lynch and Weiss, 2000), in which exon 4 is flanked by its nearest constitutive neighbors, namely exons 3 and 7. For CD9, CD45 exon 9 and 150 nt of flanking intron were isolated by PCR and inserted into BglII sites that were engineered into the middle of each intron within the CD4 construct. Minigenes with the prefix SC are derived from the Dup175 construct (Xie and Black, 2001) in which we have engineered BglII sites into the middle of each intron, as well as a PstI site in the central duplicated exon, to yield the construct Glo1. SC4 was constructed by substituting the BglII fragment containing CD45 exon 4 and flanking intron from construct CD4 for the central BglII-flanked exon in Glo1. SC-180 and SC-14 were constructed by inserting PCR-derived 180 nt fragments of CD45 exon 4 or exon 14, respectively, into the PstI site of Glo1. SC-ESS was constructed by inserting the 60 nt ESS1 sequence, plus additional β-globin-derived filler sequence into the PstI site of Glo1. The mutations in SC-180 shown in Figure 2A are identical to the QC3-7 mutations described previously (Lynch and Weiss, 2001). CD4P differs from CD4 in that we have engineered PstI sites at either end of the ESS1 sequence of exon 4. For construct CD14 the ESS1 sequence has been removed from CD4P and replaced with a 60 nt fragment of CD45 exon 14. Constructs SC5, SC6, and SC9 were generated as described above for SC4, with inserts containing regions inclusive of CD45 exons 5, 6, and 9, respectively. Mutants mARS, SC0m, CD-5up, and CD-5mu were made by PCR-based mutagenesis of SC-180 or CD4, respectively.

Cell Culture

JSL1 cells, described previously (Lynch and Weiss, 2000), were cultured in RPMI+ 5% fetal calf serum at 37°C in 5% CO2. Stable cell lines expressing the minigenes were created by transfecting 10–20 million cells with 10 μg minigene plasmid by electroporation. Cells were allowed to recover for 48–72 hr in high serum media (10% fetal calf serum) and then plated at varying cell density in high serum media containing the selection drug Zeocin (Invitrogen). After 2 weeks of growth under drug selection, individual clones were isolated, expanded, and screened by RT-PCR for expression of the desired minigene. For stimulations we treated three to four independent clones of each minigene with or without PMA (20 ng/ml) for 60 hr at a starting cell density of 0.3 million cells/ml. Cells were then harvested, washed in PBS, and total RNA was extracted from each cell population using RNABee (Tel-Test).

RT-PCR

For all the RT-PCR assays we used a low-cycle PCR protocol described in detail previously (Lynch and Weiss, 2000, 2001) such that the signal we detect is linear with respect to input RNA. Quantitation, done by densitometry using a Typhoon Phosphorimager (Amersham Biosciences), is the average of at least eight independent assays. For all constructs, the identity of the RT-PCR products was confirmed by cloning and sequencing. In addition, representative clones were checked for alternative splicing of the endogenous CD45 gene to confirm that cells were properly stimulated. All minigenes were analyzed using the vector-specific primers ACT (5′-GGTTCGGCT TCTGGCGTGTGACCG-3′) and GE3R (5′-GCGAGCTTAGTGATACTT GTGGGCC-3′). Primers for the analysis of the endogenous Tau, Fyn, WT1, and Fas genes are as follows: TauF, 5′-GTCCTCGCCTCT GTCGACTATCAGG-3′; TauR, 5′-GCAGCTTCGTCTTCCAGGCTGG-3′; Fyn7A, 5′-GTCTCTGCTGCCGCCTAGTAGTTCCC-3′; Fyn7B, 5′-GCT GGGTCAGGGGTGTTTCGCTG-3′; FynRT, 5′-CACCACTGCATAGAGC TGGA-CCAGC-3′; WT1+KTS, 5′-GAATTCAAGCTTCCAGGACTCAT ACAGGTAAAAC-AAGTG-3′; WT1-KTS, 5′-CCACACCAGGACTCAT ACAGGTG-3′; WT10R, 5′-GTGAGGAGGAGTGGAGAGACAGAC-3′; FasF, 5′-GTATGTGAACACTGTGA-CCCTTGCACC-3′; FasR, 5′-GTG GTGATATATTTACTCAAGTCAACATCAG-3′.

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