Regulation of alternative pre-mRNA splicing by the ERK MAP-kinase pathway

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Differential gene expression through alternative pre-mRNA splicing is crucial to various physiological and pathological conditions. Upon activation of B and T lymphocytes during an immune response, variant isoforms of the cell surface molecule CD44 are generated by alternative pre-mRNA splicing. We show here that in primary mouse T cells as well as in the murine LB-17 T-cell line upregulation of variant CD44 mRNA species upon T-cell activation requires activation of the MEK–ERK pathway. By employing mutant signaling molecules and a novel luciferase-based splice reporter system we demonstrate that the Ras–Raf–MEK–ERK signaling cascade, but not the p38 MAP-kinase pathway, activates a mechanism that retains variant CD44 exon v5 sequence in mature mRNA. The findings demonstrate that a highly conserved pleiotropic signaling pathway links extracellular cues to splice regulation, providing an avenue for tissue-specific, developmental or pathology-associated splicing decisions.

Keywords: alternative splicing/CD44/MAP kinase/signal transduction/T cells

Introduction

By alternative pre-mRNA splicing, functionally different proteins can be generated from one and the same gene. Alternative splice sites in pre-mRNA are utilized under the influence of developmental stage-, sex- or tissue-specific differentiation (Chabot, 1996; Lopez, 1998; Smith and Valcarcel, 2000). Alternative splicing is thus an important mechanism of differential gene expression. Inappropriate splicing is involved in the development of various diseases, a fact documented by an increasing number of examples (Lopez, 1998; Philips and Cooper, 2000).

Establishment of stage- or tissue-specific splice patterns requires instruction of cells by extracellular cues, i.e. by soluble or cell-associated factors. Changes in alternative splicing of several target pre-mRNA transcripts upon stimulation by growth factors have been reported (Shifrin and Neel, 1993; Fichter *et al.*, 1997; Smith *et al.*, 1997; Liu and Kaczmarek, 1998). Also cytokines (Mackay *et al.*, 1994), hormones (Chalfant *et al.*, 1995; Xie and McCobb, 1998), drugs (Yao *et al.*, 1996), membrane depolarization

in neurons (Zacharias and Strehler, 1996) or antigenic stimulation of the T-cell receptor (TCR) (Screaton *et al.*, 1997; König *et al.*, 1998; Lynch and Weiss, 2000) causes splice changes. These extracellular cues are thought to act through the activation of signaling cascades that pass the information to the nucleus. As a result the relative abundance and/or activation state of splice factors (e.g. SR-proteins or hnRNPs) may be changed, which influences spliceosome assembly at alternative splice sites through interaction of the splice factors with *cis*-active regulatory elements on the pre-mRNA (Smith and Valcarcel, 2000). Even though evidence exists that protein kinase C (PKC) and Ras may regulate alternative splicing (Fichter *et al.*, 1997; Smith *et al.*, 1997; König *et al.*, 1998; Lynch and Weiss, 2000), signaling components between the cell surface and the nuclear splicing machinery have not yet been identified.

The CD44 family of type I transmembrane proteins provides a very interesting model to study the regulation of alternative splicing, since CD44 gene expression is extensively regulated by this mechanism and since the resulting CD44 isoforms are known to be involved in tumor progression, immune responses and embryonal development (Günthert *et al.*, 1991; Arch *et al.*, 1992; Sherman *et al.*, 1998). CD44 is encoded by one single gene that spans ~50 kb. The smallest isoform, CD44 standard (CD44s), is composed of a constant extracellular domain, encoded by exons 1–5 and constant transmembrane and intracellular domains, encoded by exons 15–19 in man and exons 16–20 in mice, respectively (Screaton *et al.*, 1992; Tölg *et al.*, 1993). Variant CD44 isoforms contain additional extracellular domains introduced by alternative splicing of exon 6 (v1–2) to exon 14 (v10) in man, and exon 6 (v1) to exon 15 (v10) in mice, respectively. Inclusion of variant domains influences binding of the CD44 molecule to ligands like hyaluronate (Stamenkovic *et al.*, 1991; Bennett *et al.*, 1995a; van der Voort *et al.*, 1995; Sleeman *et al.*, 1997) and growth factors (Bennett *et al.*, 1995b; Sherman *et al.*, 1998; van der Voort *et al.*, 1999; Herrlich *et al.*, 2000). Therefore, expression of different variant CD44 isoforms changes the functional properties of cells. CD44s is widely expressed in most tissues, whereas expression of variant CD44 isoforms is restricted to certain normal cell types, particularly to proliferating epithelia, and to many malignant tumors (Naor *et al.*, 1997). Moreover, activation of T lymphocytes by injection of allogeneic lymphocytes into adult rats (Arch *et al.*, 1992) or by TCR stimulation by an anti-CD3 antibody or by phorbol-ester treatment (Koopman *et al.*, 1993) results in the generation of alternatively spliced variant CD44 isoforms. As the activating extracellular stimuli for T cells are known, T cells provide a suitable model to investigate signal-regulated alternative splicing of CD44.

The small GTPase Ras has been shown to be activated in response to stimulation of the antigen receptor in T cells (Downward *et al.*, 1990) and has been characterized as the central molecule in T cells coupling upstream signaling to a number of different signal transduction pathways including the three major mitogen-activated protein kinase (MAP kinase) cascades. The activity of these MAP kinases, the extracellular signal-related kinase (ERK), the Jun N-terminal kinase (JNK) and p38, is regulated through distinct, hierarchically organized modules of kinases consisting of a MAP kinase kinase (MAPKK, MKK or MEK) and a MAP kinase kinase kinase (MAPKKK). Effectors for Ras include the serine/threonine kinase Raf-1, which via MEK activates ERK, and the GTPase Rac, which via different MKKs activates JNK and p38 (Izquierdo *et al.*, 1993; Genot *et al.*, 1996, 1998; Zhang *et al.*, 1999; Dong *et al.*, 2000; Weiss *et al.*, 2000; reviewed by Genot and Cantrell, 2000; Chang and Karin, 2001).

We have shown previously that phorbol-ester stimulation or introduction of activated Ras into the mouse T-lymphoma cell line LB-17 can induce usage of CD44 exon v5 in RNA expressed from a minigene. The retention of exon v5 sequence depended on signal-responsive splice regulatory elements within the exon (König *et al.*, 1998). The goal of the present study is to understand how extracellular stimuli, e.g. activation of the TCR by antigen, are linked to the regulation of alternative splicing. Since the activation of MAP-kinase pathways is a necessary step downstream of Ras in T-cell activation (Ward *et al.*, 1997; DeSilva *et al.*, 1998), we investigated whether MAP-kinase activation is involved in the upregulation of CD44 variant expression, and we found this to be the case. In a model T-cell line into which a novel luciferase-based splice reporter system had been incorporated, the Ras–Raf–MEK–ERK signaling pathway was sufficient and necessary to establish the inclusion of the CD44 v5 exon sequence in mature mRNA.

The findings show that extracellular cues can be converted into nuclear changes in splice patterns through a highly conserved cellular protein kinase cascade. Furthermore, they implicate signaling-induced phosphorylation events as regulatory principle in alternative pre-mRNA splicing.

Results

Inhibition of MEK–ERK pathway activation interferes with upregulation of CD44 variant expression in primary T cells

Activation of T lymphocytes leads to changes in alternative splicing of different target genes including CD44 (Arch *et al.*, 1992; Sreaton *et al.*, 1997; Lynch and Weiss, 2000). To test which CD44 variants are induced after TCR stimulation we activated primary mouse T lymphocytes with anti-CD3 ϵ /anti-CD28 antibodies and measured CD44 variant expression by exon-specific RT–PCR 6 h after induction (Figure 1). We found increased expression of splice variants carrying single variant exons, namely CD44 v3, CD44 v4, CD44 v5, CD44 v6, CD44 v7 and CD44 v8 (Figure 1B and D, compare lanes 4–9), and the appearance of transcripts carrying two and three variant exon sequences, respectively, e.g. CD44 v4/5 and v5/6 (Figure 1B and D, compare lanes 5 and 6). These changes

in alternative splicing also occurred when protein synthesis was blocked by emetine (data not shown), suggesting regulation by preformed factors. A similar splice pattern mainly of isoforms carrying single variant exons was obtained by treatment of the cells with the phorbol-ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA) mimicking TCR stimulation (Figure 1C and D, compare lanes 4–9).

The change in alternative splicing upon TCR activation is obviously mediated by signal transduction processes. We therefore investigated the major signaling cascades that are likely to be activated after anti-CD3 ϵ /CD28 co-stimulation. Indeed, the co-stimulatory treatment led to phosphorylation of the ERK1/2 and p38 MAP kinases, as determined by western blot analysis with phosphospecific antibodies (Figure 1G, compare lanes 1 and 2). JNK phosphorylation could not be detected during the relevant time period after co-stimulation (Figure 1H, lanes 1–5). Phorbol-ester treatment, in contrast, resulted in JNK activation (Figure 1H, compare lanes 1 and 6). Our data on ERK and p38 MAP-kinase activation, which are consistent with previously published data (Zhang *et al.*, 1999; Weiss *et al.*, 2000), would be compatible with their involvement in the TCR-dependent upregulation of CD44 variant expression.

To investigate whether activation of the ERK or the p38 MAP-kinase pathway, or of both, accounts for TCR-dependent induction of CD44 variant expression, we blocked either the activation of MAP kinase ERK kinase (MEK1/2) with the specific inhibitor U0126 (DeSilva *et al.*, 1998; Favata *et al.*, 1998), or p38 kinase activity with the inhibitor SB203580 (Lee *et al.*, 1994; Ward *et al.*, 1997) (Figure 1G). Inhibition of the MEK pathway by U0126 led to a significant reduction of variant exon-specific RT–PCR products (compare Figure 1D and E, lanes 4–9). Inhibition of p38 signaling with SB203580, however, did not interfere with the generation of CD44 variants (Figure 1F).

Quantitative interpretation of the exon-specific RT–PCR is limited even though it was conducted under non-saturated conditions. Nevertheless, the data suggest that the MEK–ERK pathway is involved in the generation of variant CD44 mRNAs upon T-cell activation by TCR and CD28 stimulation.

A novel splice reporter system in a T-cell model to study regulated alternative splicing of CD44

To dissect the signaling pathways regulating alternative splicing, we made use of a cell line in which we can modulate signaling by transfection experiments. We chose the transfectable murine T-lymphoma cell line LB-17 2.3, for which we have already shown that CD44 splice regulation resembles that in primary T cells. LB-17 cells express CD3 only very weakly (Zahalka *et al.*, 1993 and our own observations) but expression of endogenous CD44 splice variants can be induced by phorbol-ester treatment (König *et al.*, 1998), as in primary T cells. In this cell line, we previously established a minigene splice reporter containing CD44 exon v5, which recapitulates upregulation of exon v5 inclusion after phorbol-ester stimulation (König *et al.*, 1998). Similarly to primary T cells, phorbol-ester treatment of LB-17 cells led to phosphorylation of ERK, JNK and p38 (Figure 2). Thus,

Fig. 1. Induction of CD44 variant expression by anti-CD3e/anti-CD28 antibodies in murine primary T cells is repressed by inhibition of the ERK pathway. (A) Simplified scheme of the murine CD44 gene organization indicating the positions of PCR primers (arrows) used for exon-specific RT-PCR. Open boxes represent constant exons, filled boxes denote variant exons, thin lines represent intervening intron sequences. (B–F) Exon-specific RT-PCR analysis of CD44 variant expression. Murine primary T cells were either left untreated (B), induced with TPA (40 ng/ml) (C) or stimulated by anti-CD3e/anti-CD28 antibodies coated to plates for 6 h (D–F). In addition, cells were treated either with the solvent dimethylsulfoxide (DMSO) (D), the MEK inhibitor U0126 (E) or the p38 inhibitor SB203580 (F). For exon-specific RT-PCR 5' primers hybridizing to either the 5' constant region (C13, lane 1) or to the variant exons (lanes 2–11) were used together with a primer (C2A) recognizing the 3' constant region. Asterisks mark an unspecific PCR product (König *et al.*, 1996). Identity of bands in lanes 5, 6 and 11 was confirmed by sequencing. The lower band in (D) lane 11 represented a PCR artifact that was only seen in some experiments. (G) Activation of ERK and p38 MAP-kinase pathways: phosphorylation of ERK 1/2 (p42/p44) and p38 was examined by western blotting with phosphospecific antibodies after pre-treatment with U0126 or SB203580 for 15 min and subsequent induction with anti-CD3e/anti-CD28 antibodies for 10 min. (H) Activation of the JNK MAP-kinase pathway: phosphorylation of JNK (arrow) was examined by western blotting with an anti-phospho-JNK antibody 10 min, 1 h, 2 h and 6 h after induction with anti-CD3e/anti-CD28 or after treatment with TPA (40 ng/ml) for 10 min as a positive control.

LB-17 cells behave like primary T cells with respect to activation of MAP-kinase pathways.

In order to rapidly and quantitatively measure changes of alternative splicing, we developed a novel luciferase-based splice reporter system by modifying the CD44 v5 minigene pETv5 (Figure 3A). The principle of the luciferase reporter gene, pETCatEBLucv5, is illustrated in Figure 3A. Translation only gives rise to a luciferase fusion protein if CD44 exon v5 is included in the reporter gene mRNA. To show that the reporter gene translates exon v5 inclusion into luciferase activity, we transfected the construct into LB-17 cells and stimulated the cells with TPA. We then analyzed the splicing pattern of the minigene mRNAs and, in parallel, measured luciferase activity. RT-PCR analysis of RNA from cells transfected with the luciferase-containing minigene pETCatEBLucv5 (Figure 3B, lanes 1 and 2) gave very similar results when compared with the original pETv5 minigene (Figure 3B, lanes 3 and 4). Whereas the smaller PCR fragment, indicating exon skipping, predominated without induction, TPA treatment resulted in a shift to the larger fragment, indicating exon inclusion. This shift in exon inclusion was accompanied by a 2-fold increase of luciferase activity compared with non-treated cells (Figure 3C), suggesting that luciferase activity reliably reflects exon inclusion.

To exclude the possibility that increased transcription from the splice reporter plasmid induced by TPA might

account for the increase in luciferase activity, we cloned the luciferase coding sequence immediately downstream of the Rous sarcoma virus (RSV) promoter within the vector backbone of the splice reporter (pETLuc, Figure 3A). In LB-17 cells transfected with this construct, luciferase activity after stimulation with TPA was only marginally higher than in unstimulated cells (Figure 3C). To rule out the possibility that varying amounts of primary transcripts expressed from the transfected plasmid changed the ratio of exon skipping and exon inclusion, we transfected increasing amounts of pETCatEBLucv5 into the LB-17 cells. Although increasing amounts of transfected plasmid gave rise to increasing transcript levels, no shift from exon skipping to exon inclusion was observed in the RT-PCR assay (data not shown). Thus, the luciferase reporter construct reliably monitors changes in exon v5 inclusion and recapitulates regulation of alternative splicing of the endogenous CD44 exon v5.

Regulation of CD44 exon v5 inclusion by the Raf-MEK-ERK signaling cascade

In primary T cells inclusion of exon v5 sequence in mature mRNA required the activation of MAP-kinase pathways. To confirm this result for LB-17 cells and to individually test the participation of signaling components of these pathways we co-transfected the luciferase reporter gene together with dominant-active versions of signaling

Fig. 2. Phosphorylation of ERK, JNK and p38 after phorbol-ester treatment of LB-17 mouse T-lymphoma cells. LB-17 cells (5×10^6 per well) were induced with TPA (40 ng/ml) for 10 min or treated with an equivalent volume of DMSO (solvent) as control, harvested and subjected to SDS-PAGE followed by western blotting with phospho-specific and pan-antibodies against ERK (A), JNK (B) and p38 (C).

components into LB-17 cells and determined their effect on splicing. Activation of the ERK and the JNK pathways by these constructs were monitored in parallel by co-transfections with luciferase-based Gal-Elk and Gal-Jun reporter systems (Hibi *et al.*, 1993; Kortjenann *et al.*, 1994). Co-transfection of an expression construct encoding a constitutively active form of Ras, HaRas L61 (Medema *et al.*, 1991), induced CD44 exon v5 usage similarly to TPA stimulation (Figure 4A and B; see also König *et al.*, 1998). HaRas L61 activated the ERK pathway (Gal4-luciferase was activated by co-transfected Gal-Elk; Figure 4C) and to a minor extent the JNK pathway (Gal4-luciferase was activated by co-transfected Gal-Jun; Figure 4D) and also led to phosphorylation of p38 (not shown). Co-transfection of constructs expressing dominant-active mutants of Raf (Raf-BxB) (Bruder *et al.*, 1992), MEK1 (MEK1-DD) (Mansour *et al.*, 1994) and a MEK-ERK fusion construct (MEK1-ERK2-LA) generating a constitutively active ERK kinase (Robinson *et al.*, 1998) had only a marginal effect on JNK but activated the Gal-Elk reporter 10- to 50-fold (Figure 4C) and induced exon inclusion of CD44 exon v5 as demonstrated by upregulation of luciferase activity (Figure 4A) and by a shift from the small (-v5) to the large RT-PCR fragment (+v5) in the RT-PCR assay (Figure 4B).

HaRas L61 and to some extent the MEK-ERK fusion and Raf-BxB activated, in addition to the ERK pathway, the JNK pathway as indicated by Gal-Jun activation (Figure 4D). To dissect the relative contributions of MAP-kinase pathway activation, we measured the effect of activated Ras and activated Raf on v5 exon inclusion in the absence or presence of the MEK inhibitor U0126. U0126 inhibited induction of v5 exon inclusion upon expression of activated Ras or activated Raf by 55–70% (see

Fig. 3. A luciferase-based splice reporter assay for exon inclusion of CD44 v5. (A) Schematic representations of the original pETv5 splice reporter construct (König *et al.*, 1998), the luciferase splice reporter construct (pETCatEBLucv5) and the construct used as a control for transcriptional activation (pETLuc). The scheme for pETCatEBLucv5 illustrates the principle of the assay. Skipping of the v5 exon sequence leads to a reading frame that ends before the luciferase coding sequence (STOP). Inclusion of the v5 exon generates a reading frame for a luciferase fusion protein, ending after the luciferase coding sequence (Stop). Arrow, RSV LTR promoter; ATG, start codon for the fusion protein; gray boxes, insulin exon 2 and 3 sequences; open boxes, CD44 exon v5, and luciferase sequence, respectively; black boxes, SV40 poly(A) signal. Thin black lines, intron sequences; gray lines indicate splice pattern. (B) RT-PCR analysis of LB-17 cells transiently transfected with splice reporter constructs. Cells were harvested and assayed 24 h after transfection with either 2 μ g of the splice reporters pETCatEBLucv5 (lanes 1 and 2) or pETv5 (König *et al.*, 1998) (lanes 3 and 4) and treatment with DMSO or TPA (40 ng/ml) for 6 h as indicated. RT-PCR analysis of minigene transcripts resulted in a short fragment (pETv5: 244bp; pETCatEBLucv5: 253 bp) when CD44 exon v5 was skipped, and in a large fragment (pETv5: 354 bp; pETCatEBLucv5: 370 bp) when CD44 exon v5 was included. Identity of PCR fragments was confirmed by sequencing. Schematic drawings of the splice reporter constructs indicating the positions of the primers (arrows) used for PCR are given below the corresponding lanes (see legend to A). M, DNA molecular size marker (100 bp ladder; Gibco-BRL). (C) Detection of luciferase activity. LB-17 cells were transiently transfected with 2 μ g of the luciferase splice reporter construct pETCatEBLucv5 or the control reporter for promoter activation, pETLuc. Eighteen hours after transfection, the cells were treated with TPA (40 ng/ml) or DMSO for 6 h before they were harvested and subjected to luciferase assays. Luciferase light unit counts of stimulated cells were divided by counts measured in unstimulated cells to obtain values of fold induction. Each bar represents data of three independent experiments and standard deviations are indicated on top of the bars.

Figure 5A; RT-PCR not shown). The U0126 inhibitor effectively blocked Gal-Elk (Figure 5B) but not Gal-Jun activation (Figure 5C). We interpret this result to indicate a major contribution of MEK-ERK signaling and a minor contribution of most likely JNK or p38 to the regulation of alternative splicing of CD44 v5 by Ras signaling.

A dominant-active form of MAP kinase kinase 6 (MKK6), MKK6-E, described to exclusively activate the p38 MAP kinase pathway (Raignaud *et al.*, 1996), activated only marginally the Gal-Jun and Gal-Elk reporters (Figure 6A and B) but efficiently phosphorylated a co-transfected Flag-tagged p38 protein (Figure 6C). MKK6-E, however, had only marginal effects on CD44 v5 exon inclusion (Figure 6D; RT-PCR not shown). Thus, p38 activation does not suffice for upregulation of alternative splicing of CD44 v5 in LB-17 cells.

The small GTPase Rac has been described to activate the JNK and the p38 pathways (Lamarche *et al.*, 1996; Genot *et al.*, 1998). It is therefore possible that Ras acts through Rac onto JNK to regulate alternative splicing. In a co-transfection experiment with a dominant-active form of Rac (Rac L61), the JNK reporter indeed responded (Figure 6A), while the ERK pathway was not activated (Figure 6B). When co-transfected with the luciferase splice reporter pETCatEBLucv5, constitutively active Rac upregulated luciferase activity similarly to activated Ras (Figure 6D) and induced a shift from the small (-v5) to the large RT-PCR fragment (+v5) (data not shown). This result is consistent with the possibility that activation of the JNK pathway can suffice to upregulate CD44 exon inclusion in LB-17 cells, and that it may contribute to the induction of CD44 v5 exon usage by Ras.

Taken together, we have shown that in both primary T cells and in the LB-17 T-lymphoma cell line induction of CD44 v5 exon inclusion is regulated by the ERK MAP-kinase pathway. Although activation of the JNK pathway may contribute to the regulation of CD44 alternative splicing in the LB-17 cell line, co-stimulation of primary T cells does not lead to JNK pathway activation. Thus, in activated T cells the ERK MAP-kinase pathway is the key pathway for regulation of alternative splicing of CD44, which is in agreement with experiments using pathway-

specific inhibitors. Components of the ERK pathway actively contributing to exon inclusion are Ras, Raf, MEK and ERK.

ERK-induced CD44 v5 exon inclusion depends on exonic silencer sequences

To test whether sequences of CD44 v5 that we had previously identified to be necessary for phorbol-ester-activated exon inclusion (König *et al.*, 1998) are also required for activation of v5 usage by the ERK pathway, we made use of v5 exon mutants. In these mutants exonic silencer sequences in the left part (L) or the middle part (M) of the exon were replaced by a heterologous sequence that confers splice silencing comparable to the wild-type sequence but impairs phorbol-ester-induced exon

Fig. 4. Inclusion of CD44 exon v5 is induced by activation of the ERK MAP-kinase pathway. (A) Detection of CD44 v5 exon inclusion by luciferase activity. LB-17 cells were co-transfected with 2 μ g of the pETCatEBLucv5 splice reporter construct and 2 μ g of an expression vector for constitutively active HaRas L61, Raf-BxB, MEK1-DD or MEK1-ERK2-LA (filled bars) or the corresponding empty vectors (open bars). Each bar represents values of three independent experiments. Standard deviations are indicated on top of the bars. (B) Detection of CD44 v5 exon inclusion by RT-PCR analysis. LB-17 were co-transfected with 2 μ g of pETv5 and 2 μ g of constitutively active signaling mutants or vector controls as indicated (PRC-CMV, pKRSPA, pcDNA3, pCMV5). RT-PCR bands were quantified densitometrically after scanning using the Fuji Aida programme. For calculating percentage relative exon inclusion the value of % exon inclusion of control vectors was set to 100%. (C and D) To test for activation of the ERK and JNK pathways, LB-17 cells were co-transfected with 0.2 μ g of a construct expressing the Gal-Elk fusion protein or 0.8 μ g of a Gal-Jun expression vector and 0.8 μ g of the pG5.E4A38lux3 luciferase reporter plus 2 μ g of control vectors (left bars, open) or expression constructs for the signaling mutants indicated (right bars, filled). Luciferase assays were performed 24 h after transfection.

Fig. 5. Ras- and Raf-induced alternative splicing depends on MEK activity. (A) LB-17 cells were co-transfected with 2 μ g of pETCatEBLucv5 and 2 μ g of expression vectors for constitutively active HaRas L61 and Raf-BxB, respectively, or corresponding empty vectors (pRC-CMV or pkRSPA). U0126 (10 μ M) was added 3 h after transfection. Cells were harvested 24 h later and luciferase assays were performed. (B and C) ERK and JNK pathway activation was assayed after co-transfection of the Gal-Elk or Gal-Jun luciferase reporter system with 2 μ g of expression plasmids for constitutively active HaRas L61, Raf-BxB or a control vector. Inhibitor treatment was performed as described in (A).

inclusion (König *et al.*, 1998). After co-transfection of luciferase-linked versions of these mutants (Figure 7A) and an expression construct for constitutively active MEK (MEK1-DD) into LB-17 cells we found that the increase in luciferase activity induced by ERK activation was significantly reduced in the L and M mutant as compared with wild-type v5 (Figure 7B). As expected, no increase in exon inclusion as measured by luciferase activity was detected in cells transfected with a mutant in which the right part of the exon (R) was mutated, which comprises a splice enhancer necessary for exon recognition (König *et al.*, 1998). Consistent with these results, the L and M mutants also showed a lower level of induced exon inclusion in the RT-PCR assay as compared with the wild-type exon (Figure 7C, compare lanes 5–7). The R mutant did not show any v5 exon inclusion either in the non-induced or in the induced LB-17 cells (Figure 7C, lanes 4 and 8). Thus, the findings suggest that inclusion of the v5 exon in response to activation of the ERK pathway

Fig. 6. Inclusion of CD44 exon v5 can be induced by constitutively active Rac. (A and B) JNK kinase pathway (A) and ERK pathway (B) activity was determined by co-transfection of LB-17 cells with the Gal-Jun or Gal-Elk luciferase reporter system and 2 μ g of plasmids expressing constitutively active Ras (HaRas L61), Rac (Rac L61) or MKK6 (MKK6-E) (right bars, filled), or with a corresponding empty vector (left bars, open). (C) Activation of p38 MAP kinase after co-transfection of MKK6-E. LB-17 cells were co-transfected with 20 μ g of a Flag-p38 expression construct (lanes 2 and 3) and 20 μ g of a plasmid expressing constitutively active MKK6-E or pcDNA3 as control. Flag-tagged p38 (arrow) has a lower electrophoretic mobility compared with endogenous p38 (compare with sample where a control vector has been transfected instead of the Flag-p38 construct, lane 1). (D) Activation of CD44 v5 exon inclusion upon expression of constitutively activated Rac. Two micrograms of the luciferase splice reporter pETCatEBLucv5 were co-transfected with 2 μ g of plasmids expressing HaRas L61, Rac L61 or MKK6-E (filled bars), or with an empty expression plasmid (open bars). Cells were harvested 24 h after transfection and luciferase activity was determined.

depends on signal-responsive elements in the left and middle part of the exon and that silencing factors are inactivated.

Discussion

To establish stage- or tissue-specific splice patterns, cells require information from extracellular cues that must be transmitted to the splicing machinery in the nucleus. Several lines of evidence indicate involvement of PKC and Ras signaling in the regulation of alternative splicing of CD44 and CD45 pre-mRNAs after TCR stimulation (König *et al.*, 1998; Lynch and Weiss, 2000). Using a novel luciferase-based splice reporter system we have identified here signaling components by which Ras relays signals to the regulatory splice components. In the inducible T-cell line LB-17 phorbol-ester, constitutively active mutants of Ras, Rac, Raf, MEK and ERK induce the inclusion of CD44 v5 sequences into mature mRNA depending on signal-responsive exonic silencer sequences. Furthermore, we show that activation of the MEK-ERK pathway or of the JNK pathway suffices to inactivate splice silencing. The p38 pathway is not linked to CD44 splicing.

In primary T cells activated by TCR stimulation, only the signal transduction through ERK appears to be relevant since inhibition of MEK, but not of p38, reduced v5 inclusion significantly, and since the JNK pathway is not activated within the time window (Weiss *et al.*, 2000; see Figure 1H).

TCR-induced exon v5 inclusion in primary T cells (this study) and TPA-induced exon v5 inclusion in LB-17 cells (König *et al.*, 1998) do not depend on protein synthesis. These data propose post-translational modification such as signal-induced phosphorylation of regulatory splice factors as the regulatory principle.

U0126 inhibits the inclusion of several variant exon sequences. It is therefore possible that the same signaling pathway addresses the regulatory components of multiple exons in T cells. There is, however, specificity in splice regulation, e.g. T cells synthesize predominantly single-variant-exon CD44 proteins. Epithelial cells of the skin produce a single CD44 protein carrying all variant exon sequences, CD44 v1-v10, while colorectal epithelial cells produce CD44 v8-v10 unless in a stage of active proliferation when CD44 v3-v10 appears on the cell surface (Heider *et al.*, 1993; Naor *et al.*, 1997). Specificity in variant exon selection could be caused by differences in the equipment of cell types with certain RNA-binding splice regulatory proteins and/or by the activation of distinct signaling pathways, which might address exons differentially through their individual regulatory sequences. We have recently shown that skipping of certain exons, namely v5 and v6, can be induced by over-expression of hnRNP A1 in NIH 3T3, whereas inclusion of exon v4 and v7 is not affected (Matter *et al.*, 2000). A conclusive solution to the problem of specificity must await complete identification of the exon-specific regulatory mechanisms.

Specificity in exon usage also exists in response to one and the same extracellular signal. For instance, TCR stimulation results in changes of alternative splicing of both CD44 and CD45 pre-mRNAs. Whereas TCR stimulation leads to inclusion of exons in CD44 mRNA, in the case of CD45 mRNA exons are skipped (Screaton *et al.*, 1995; Lynch and Weiss, 2000). Both splicing modes involve PKC and Ras, suggesting that signal transduction

Fig. 7. Silencer sequences of CD44 v5 respond to activation of the ERK pathway. (A) Scheme of the wild-type CD44 v5 luciferase splice reporter and the mutants used in (B) and (C). Arrow, RSV LTR promoter; ATG, start codon for the fusion protein; gray boxes, insulin exon 2 and 3 sequences; open boxes, CD44 exon v5, and luciferase sequence, respectively; black boxes, SV40 poly(A) signal. Thin black lines, intron sequences; gray lines indicate splice pattern. Black boxes indicate the part (left, L; middle, M; or right, R) of the CD44 exon v5 that was replaced by a heterologous sequence (König *et al.*, 1998). (B) MEK-induced usage of wild-type and mutant CD44 exon sequences. Two micrograms of the wild-type or the indicated mutant splice reporter were co-transfected with 2 μg of a plasmid expressing MEK1-DD (filled bars), or with the empty expression plasmid pcDNA3 (open bars) into LB-17 cells. Cells were harvested and assayed for luciferase activity 24 h after transfection. (C) RT-PCR analysis of CD44 v5 exon inclusion. LB-17 cells were co-transfected with 2 μg of wild-type and mutant splice reporters and 2 μg of constitutively active MEK or vector control (pcDNA3). Twenty-four hours after transfection cells were harvested and RT-PCR analysis was performed. Quantification of RT-PCR bands was performed as described in the legend to Figure 4B.

in T cells leads to changes in splice factor activity specific for the usage of certain exons rather than to unspecific changes in the overall activity of the splicing machinery.

Constitutive Ras activation with subsequent activation of the downstream oncogenic signaling pathways is also a common feature of epithelial and hematopoietic malignancies, including malignant lymphomas (Weijzen *et al.*, 1999). Upregulation of CD44 variants has been reported in a large number of epithelial and hematopoietic tumors including high grade malignant lymphomas and has been shown to be associated with poor prognosis (Stauder *et al.*, 1995). We propose that enhanced activity of oncogenic Ras addressing MAP-kinase pathways is a mechanism by which lymphoma cells and possibly also other tumor cells upregulate CD44 variant expression and thereby increase their metastatic potential. The linkage to alternative pre-mRNA splicing of a pathway conserved in all eukaryotes that regulates normal development as well as pathological processes such as oncogenesis, thus provides a way to convert extracellular stimuli into changes in splice patterns resulting in physiological responses or disease.

Materials and methods

Cell culture and transfections

Primary T cells were isolated from spleens of 8- to 16-week-old Balb/c mice (bred and maintained in our animal facility) using a commercially available kit for negative selection of T cells (mouse T-cell enrichment columns; R&D systems). Purity of T-cell preparation was >80% for all experiments as determined by fluorescence-activated cell sorting (FACS) after staining with phycoerythrin-labeled anti-CD3 ϵ (clone 145-2C11; PharminGen).

T cells were cultured in six-well plates (4×10^6 per well) in RPMI, supplemented with heat-inactivated fetal calf serum (10%), glutamine (2 mM), penicillin/streptomycin (100 U/ml), sodium pyruvate (1 mM), HEPES pH 7.4 (10 mM) and β -mercaptoethanol (50 μ M) at 37°C and 6% CO₂ for 6 h. Then the T cells were treated with TPA (Sigma; 40 ng/ml), or induced on six-well plates coated with anti-CD3 ϵ (10 μ g/ml) and anti-CD28 (10 μ g/ml, clone 37.51; PharminGen) for various times. MAP-kinase pathway inhibitors were used at a final concentration of 10 μ M for U0126 (Promega) or 25 μ M for SB 203580 (Calbiochem). Culture conditions for LB-17 2.3 cells, a subclone of the murine LB-17 T-lymphoma cell line, have been described elsewhere (Zahalka *et al.*, 1995).

5×10^6 LB-17 2.3 cells were transfected with 1–2 μ g of DNA of different reporter constructs, alone or together with 2 μ g of oncogenic signaling mutants or control vectors, respectively, using a polycationic transfection reagent (Superfect, Qiagen) according to the manufacturer's instructions.

For co-transfection experiments with constitutively active MKK6-E or pcDNA3 and an expression plasmid for Flag-tagged p38, 20 μ g of each construct were transfected into 1.5×10^7 LB-17 cells by electroporation (Gene Pulser, Bio-Rad; 220 V, 960 μ F, 0.2 cm gap cuvettes).

RT-PCR analysis

cDNA of transfected cells was prepared by standard procedures using DNase I-digested cytoplasmic RNA. Exon-specific RT-PCR of endogenous CD44 transcripts and of transfected minigene transcripts was essentially performed as described (König *et al.*, 1998). RT-PCR assays of transcripts from the luciferase splice reporter were carried out using the 5' primer N5Ins (König *et al.*, 1998) and the 3' primer Luc3' (5'-CCAGCGGATAGAATGGCGCCG-3') in a PCR with 38 cycles (60 s 95°C, 60 s 59°C, 90 s 72°C). PCRs were in the linear phase (not in the plateau phase) under these conditions, as confirmed by using different amounts of cDNA.

Luciferase-based splice reporter assay

A novel luciferase-based splice reporter involving the plasmid pETCatEBLucv5 was established, which allowed fast quantitative measurement of CD44 v5 exon inclusion. pETCatEBLucv5 (for scheme see Figure 3A) was generated using the following cloning strategy: (i) insertion of an oligonucleotide sequence containing a start codon and the first two codons of the bacterial chloramphenicol transferase (CAT) gene (upper strand: 5'-agcttctgctaaaatggagaaaaaacag-3'; lower strand: 5'-agacgattttacctctttttttgtc-3') into *HindIII*–*PvuII* of p53In (Mobictec);

(ii) insertion of an *EcoRI*-8mer linker sequence (5'-GGAATTC-3') into the *PvuII* site of the modified p53In; (iii) insertion of a *BglII* site following nucleotide 29 of the insulin exon 3 of p53In by PCR. This *BglII* site was used to introduce the luciferase coding sequence amplified by Pwo polymerase (Boehringer) from the plasmid pGL3 Basic (Promega) with the sense primer 5'-CCGAGATCTACGCCAAAAACATAAA-3' and the antisense primer 5'-TAAAGATCTTACACGGCGATCTTTTCCGC-3'; (iv) insertion of a single nucleotide (T) between nucleotides 103 and 104 of the CD44 exon v5 sequence of the original minigene construct pETv5 (König *et al.*, 1998) by site-directed mutagenesis (Quik-Change-Mutagenesis Kit, Stratagene) using the sense primer 5'-GAGGAGACCCACATTGCTACAAGCACAAG-3' and the antisense primer 5'-CTTGTGCTGTAGCAATGTGGGGTCTCTC-3'. The modified CD44 v5 sequence with adjacent introns from pETv5 was then inserted in the *BamHI* site of the luciferase-containing construct. Luciferase-based splice reporter mutants of the left (Δ Luc), the middle (Δ M Luc) and the right part of exon v5 (Δ R Luc, for a scheme see Figure 7A) were generated accordingly using the mutants pETv5 Δ La30 (Δ L), pETv5 Δ Ma30 (Δ M) and pETv5 Δ Ra30 (Δ R) (König *et al.*, 1998). To reconstitute the reading frame in the Δ R mutant, an additional nucleotide (T) was introduced in the *MluI* linker of the construct.

The control construct pETLuc was generated by inserting the luciferase coding sequence obtained from pGL3 basic after PCR amplification with Pwo polymerase (sense primer 5'-CTAAGTAAGCTTGGCATTCCG-TACTG-3', antisense primer 5'-TAAAGATCTTACACGGCGATCTTTCCGC-3') into *HindIII*–*PvuII*, downstream of the RSV promoter of p53In (for scheme see Figure 3A).

Cells were transfected with 2 μ g of pETCatEBLucv5 or pETLuc and treated with TPA or were co-transfected with pETCatEBLucv5 together with 2 μ g of signaling mutant constructs. They were harvested at 24 h after transfection, washed twice in ice-cold phosphate-buffered saline (without calcium and magnesium) and lysed on ice in 300 μ l of 1% Triton X-100, 0.1 M Tris acetate pH 7.5, 2 mM EDTA. Following clearance of the lysate by a centrifugation step (5 min, 12 000 g), luciferase assays were performed using 200 μ l of the cleared lysate in a Berthold luminometer (Lumat 9501) with an automatic injection device for 350 μ l of 25 mM glycylglycine, 15 mM MgSO₄, 4 mM EGTA, 1 mM dithiothreitol (DTT), 2 mM ATP and 100 μ l of 0.25 mM luciferin, 25 mM glycylglycine, 15 mM MgSO₄. Values were normalized for protein concentration in the lysates as measured by Bradford assay.

Luciferase assays to test for ERK and JNK pathway activation

Luciferase assays were performed to check for activation of ERK and JNK pathways in co-transfection experiments when endogenous ERK or JNK phosphorylation could not be assayed due to low transfection efficiencies. A construct (0.2 μ g) expressing a chimeric protein in which the transactivation domain of the transcription factor Elk-1 was fused to the DNA binding domain of the yeast transcription factor GAL4 (pSG-Gal4-Elk1), or 0.8 μ g of a similar construct containing the transactivation domain of c-Jun (Hibi *et al.*, 1993; Kortenjann *et al.*, 1994), was co-transfected with 0.8 μ g of the plasmid pG5.Ef Δ lux3 and 2 μ g of signaling mutant constructs. The reporter plasmid pG5.Ef Δ lux3 contains a pentamer of the GAL4 binding site fused to positions +1 to +38 of the adenovirus E4 promoter and a luciferase gene. Luciferase activity was determined 24 h after transfection as described above.

Western blot analysis

After induction, cells were lysed in 4% SDS, 20% glycerol, 120 mM Tris pH 6.8, 0.002% bromophenolblue, 200 mM DTT at the timepoints indicated. For transient transfections, cells were harvested 24 h after transfection. Genomic DNA was sheared by aspiration through a 26 G needle and lysates were cleared by centrifugation (5 min, 12 000 g). Proteins were resolved by 10% SDS-PAGE and blotted on Immobilon-P transfer membranes (Millipore) using a semidry blotting device. Membranes were blocked in 5% bovine serum albumin, 0.1% Tris-buffered saline, Tween-20 at room temperature for 1 h and then probed with rabbit anti-phospho-ERK, anti-phospho-JNK or anti-phospho-p38 antibodies (New England Biolabs) according to the instructions of the manufacturer. Bands on blots were visualized by enhanced chemiluminescence (Amersham) after incubation with a horseradish-peroxidase-coupled secondary anti-rabbit antibody (Sigma). Loading controls were conducted accordingly using rabbit anti-ERK (Clone K-23; Santa Cruz), rabbit anti-JNK (Clone C-17; Santa Cruz) and goat anti-p38 (Clone C-20; Santa Cruz) antibodies and the appropriate horseradish-peroxidase-coupled secondary antibodies (Sigma, DAKO).

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