Cross-talk between Signaling Pathways Regulates Alternative Splicing

A NOVEL ROLE FOR JNK

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The regulation of alternative splicing by extracellular signals represents a key event in the control of gene expression. There is increasing evidence showing that many extracellular cues regulate alternative splicing. Nevertheless, the broad picture regarding the role of different signaling pathways and their interaction remains incomplete. Using the fibronectin gene as a model, we show that a laminin-rich basement membrane regulates the alternative splicing of two out of three regions of the transcript (extra domain I and type III connecting segment) in mammary epithelial cells, through a non-stress c-Jun N-terminal kinase (JNK) signaling pathway. We propose that dephosphorylation of the extracellular signal-regulated kinase is involved in this regulatory process. Furthermore, the laminin-rich basement membrane blocks the effect of a mammary mesenchymal cell-conditioned medium, which stimulates the inclusion of extra domain I and type III connecting segment through a phosphatidylinositol 3-kinase-dependent cascade, indicating that JNK signaling can inhibit the phosphatidylinositol 3-kinase-mediated splicing regulation. These results implicate JNK in the regulation of alternative splicing and provide new evidence on how extracellular stimuli are converted into changes in splicing patterns, strengthening the view that the control of alternative splicing is as complex and relevant as transcriptional control, together accounting for the spatiotemporal requirements of gene expression.

Alternative pre-mRNA splicing is the most important source of protein diversity (1, 2). Alternative splice site utilization is controlled in a developmental stage-, sex-, or tissue-specific manner and is influenced by the way the cells respond to their surrounding microenvironment. Accordingly, a growing body of evidence shows that signaling induced by growth factors, cytokines, hormones, and membrane depolarization can change the splicing pattern of several pre-mRNAs (3–5). The transducing components that link the cell surface with the nuclear splicing machinery are now being unraveled, implicating the mitogen-activated protein kinase/extracellular signal-regulated kinase (MEK)3/6-p38 pathway (6), protein kinase C (7), the extracellular signal-regulated kinase (ERK) cascade (8, 9), and calcium/calmodulin-dependent protein kinase IV (10) in signal-induced splicing regulation. Within this line of evidence, we have shown recently that a mammary mesenchymal cell-conditioned medium acts through a phosphatidylinositol (PI) 3-kinase-dependent cascade to regulate alternative splicing in a mammary epithelial cell line (11).

Fibronectin (FN) is the best characterized extracellular matrix (ECM) glycoprotein and plays a key role in cell adhesion and migratory behavior related to fundamental processes such as embryogenesis, wound healing, maintenance of tissue integrity, and malignancy. Alternative splicing in three different regions of the FN pre-mRNA referred to as extra domain II (EDII, also known as EDB or EIIB), extra domain I (EDI, also known as EDA or EIISA), and type III connecting segment (IIICS or V region) gives rise to 12 isoforms in rodents (12). FN alternative splicing is modulated in a cell type-, development-, and age-specific manner, and therefore it becomes paradigmatic to study the regulation of this complex process by extracellular cues. EDI and EDII are cassette exons, either excluded from or included into the mature FN mRNA. The third site of alternative splicing, IIICS, is subject to total inclusion, partial inclusion, or total exclusion because of the presence of three alternative 3′-splice sites, generating the variants referred to as IIICS-120, IIICS-95, and IIICS-0 according to their lengths (13, 14).

EDI has a role in cellular migration (13) and cell cycle progression (14). This exon was also shown to be responsible for the conversion of lipocytes into myofibroblasts in fibrotic liver as well as for the induction of several matrix metalloproteinases (MMP-1, MMP-3, MMP-9) required for cell migration and tissue remodeling (15, 16). Recently, Muro et al. (17) generated

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The abbreviations used are: MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase; ERK, extracellular signal regulated kinase; PI 3-kinase, phosphatidylinositol 3-kinase; FN, fibronectin; BM, basement membrane; Irb-BM, laminin-rich BM; ECM, extracellular matrix; ED, extra domain; IIICS, type III repeat connecting segment; JNK, c-Jun N-terminal kinase; MMP, matrix metalloproteinase; RT-PCR, reverse transcription-PCR; LUC, luciferase; SP, S6000125; SEK, stress-activated protein kinase/ERK kinase; PD, PD98059; siRNA, small interfering RNA; g6CM, conditioned medium from the mammary mesenchymal cell line SCG6.

1 The abbreviations used are: MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase; ERK, extracellular signal regulated kinase; PI 3-kinase, phosphatidylinositol 3-kinase; FN, fibronectin; BM, basement membrane; Irb-BM, laminin-rich BM; ECM, extracellular matrix; ED, extra domain; IIICS, type III repeat connecting segment; JNK, c-Jun N-terminal kinase; MMP, matrix metalloproteinase; RT-PCR, reverse transcription-PCR; LUC, luciferase; SP, S6000125; SEK, stress-activated protein kinase/ERK kinase; PD, PD98059; siRNA, small interfering RNA; g6CM, conditioned medium from the mammary mesenchymal cell line SCG6.

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mice devoid of EDI exon-regulated splicing and demonstrated that EDI splicing regulation is required for proper skin wound healing and a normal lifespan. On the other hand, IIICS inclusion is higher in all fetal versus adult tissues, and this region is required for the secretion of FN dimers during biosynthesis (18). The IIICS-120 isoform bears an LDL acid amino motif, which, like EDI, is a ligand for the α4β1 integrin (19, 20) supporting the idea that alternative splicing can regulate several cellular processes such as adhesion, migration, and invasion (20–22).

Integrins are a large family of cell surface receptors that mediate cell adhesion to ECM components, creating a link between the outside and the inside of the cell. The ECM not only provides a scaffold for the organization of cells in tissues, but it is also known to exert extraordinary control on cell behavior (23). This is particularly evident in the mammary gland, where stromal and epithelial cells communicate with each other through a basement membrane (BM)-like ECM. This BM influences mammary epithelial cell differentiation both in vivo and in culture, initiating a plethora of signaling processes including those leading to the expression of milk proteins (24–26). Upon binding to different BM components, integrins trigger a variety of signal transduction pathways (27). In this regard, integrin-mediated signaling can lead to the activation of the ERK or the c-Jun N-terminal kinase (JNK) cascades, and it has been shown that this integrin-initiated JNK activation does not occur through the classical stress pathway (23, 28, 29).

We have shown previously that a laminin-rich BM (lr-BM) is able to modulate FN EDI alternative splicing in the hematopoietic cell line Hep3B (30). Considering its physiology and the availability of a culture system that mimics the epithelial differentiation process observed in vivo, the mammary gland represents an interesting model to study the regulation of alternative splicing by extracellular signals (11). In this context, we now extend the effects observed in Hep3B cells upon treatment with a lr-BM to the functionally normal mouse mammary epithelial cell line EpH4. Furthermore, this treatment favors the use of more distal 3'-splice sites within IIICS, increasing the proportion of the LDV-lacking isoforms. As for the intracellular mechanism involved in this signal-dependent splicing regulation, we show that the lr-BM acts through a JNK cascade to change FN EDI and IIICS splicing patterns. We also present evidence supporting a role for ERK dephosphorylation in this process, suggesting that different signaling pathways regulating alternative splicing might interact with each other. Indeed, the presence of the lr-BM completely inhibits the ability of a mammary mesenchymal cell-conditioned medium to stimulate EDI and IIICS inclusion, consistent with the negative cross-talk reported between the PI 3-kinase-Akt and the JNK signaling pathways (31, 32).

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Treatments—**EpH4 cells were grown in Dulbecco’s modified Eagle’s medium:F-12 (Invitrogen) supplemented with 2% fetal bovine serum, insulin (5 μg/ml Sigma), and gentamicin (50 μg/ml Invitrogen). Hep3B cells were maintained and treated as described previously (30).

To perform the corresponding treatments, ~1 × 10⁵ EpH4 cells were plated in Dulbecco’s modified Eagle’s medium:F-12 supplemented with 2% fetal bovine serum into 35-mm tissue culture wells. After 24 h, cells were treated with 1.5% (v/v) reconstituted basement membrane (Matrigel, Collaborative Biomedical Products) in Dulbecco’s modified Eagle’s medium:F-12 without serum. Alternatively, 35-mm tissue culture wells were precoated on ice with 0.2 ml of cold Matrigel, which was allowed to gel in a humidified incubator for 15 min at 37°C. Cells were immediately plated on top of it.

Mammary mesenchymal cell-conditioned medium (g6CM) was obtained by plating 1 × 10⁵ SCgé cells into 100-mm tissue culture dishes in 2% fetal bovine serum medium and replacing it by serum-free Dulbecco’s modified Eagle’s medium:F-12 after 24 h. The cell supernantant was collected 24 h later and centrifuged to discard cell debris. For the experiments with kinase inhibitors, 24 h after plating, the cells were preincubated with the corresponding inhibitor or an equal volume of Me₂SO (vehicle) for 2–4 h and then treated with Matrigel or left untreated in the presence of the inhibitor for 24 h. Kinase inhibitors used were from Calbiochem.

**Transfections and Plasmids—**Transfection of EpH4 and Hep3B cells were performed 24 h after plating using FuGENE 6 (Roche Applied Science) or Lipofectamine (Invitrogen). Approximately 1 × 10⁵ EpH4 or 2 × 10⁵ Hep3B cells were transfected with 3 μg of FuGENE 6 or 6 μg of Lipofectamine and 2 μg of total plasmid DNA in 35-mm tissue culture wells. Cells were stimulated 24 h after transfection with the corresponding treatment.

The g6bovine/FN EDI minigene is described elsewhere (31). The following expression vectors, pCEFI.HA-p38, pCEFI.HA-ERK2, pCEFI.HA-MKK6α(EE), pCEFI.HA-MEKK1(EE), pCEFY.MEEKK, pCDNA3.0cDNA3 Rac1Q7, pEGB SEK, pEGB SEK-KR, and empty vectors were obtained from Dr. Omar Coso (IFIBYNE-CONICET), pCDNA3 Flag-JNK1, pCDNA3 Flag-JNK2, pCDNA3 Flag-JNK1α(af), and pCDNA3 Flag-JNK2α(af) were provided by Dr. Roger Davis (Howard Hughes Medical Institute, University of Massachusetts Medical School). Overexpression experiments were performed in Hep3B cells.

**RNA Isolation and Radioactive RT-PCR Amplification—**Total RNA purification from cultured cells, RT-PCR analysis, and sets of primers used were described previously (11). RT-PCR products were electrophoresed in 6% (w/v) polyacrylamide native gels and detected by autoradiography. Radioactivity in the bands was measured in a scintillation counter by the Cerenkov method.

**Western Blot Analysis—**After treatment, cells were lysed in boiling 2× sample buffer (4% SDS, 20% glycerol, 120 μM Tris, pH 6.8, 0.002% bromphenol blue, 200 μM β-mercaptoethanol) at the time points indicated. Proteins were electrophoresed (12% acrylamide SDS-PAGE), blotted, probed with specific antibodies, and visualized by enhanced chemiluminescence detection using horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology) and Luminol (Sigma). Anti-phospho-ERK, anti-phospho-JNK, anti-phospho-p38α, anti-ERK, anti-JNK, anti-p38, and anti-Akt antibodies were from Santa Cruz Biotechnology. Anti-phospho-Akt was from Cell Signaling Technology. Luciferase Assays—Luciferase (LUC) activity in cell lysates was measured using the luciferase assay system (Promega). 24 h posttransfection the cells were treated with Matrigel for 8 h and then washed with phosphate-buffered saline before lysis with 100 μl of reporter lysis buffer (Promega). Cell extracts were centrifuged, and 30 μl of the supernatant were mixed with 100 μl of luciferase assay buffer II (Promega). LUC activity was tested with a Junior luminometer (Berthold, Bad Wildbad, Germany).

**Small Interfering RNA (siRNA)—**Duplexed RNA oligonucleotides (Stealth RNAi) were synthesized by Invitrogen. Hep3B cells were transfected with plasmid DNA in combination with 40 pmol of different siRNAs using Lipofectamine or Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocols, and they were treated 5 h posttransfection. The sequences of the siRNAs are (sense strands, 5′–3′) JNK1, GGGCCUCAGGAGGACUGCUAUU and JNK2, GAUGUC-UACUAUGCACAGCUAUU. The siRNA targeting the LUC gene used as a control has been described (33).

**RESULTS**

A Laminin-rich Basement Membrane Regulates FN EDI and IIICS Splicing in Mammary Epithelial Cells—We have shown previously that a lr-BM inhibited FN EDI inclusion but did not regulate FN EDII exon splicing in Hep3B cells (30). These results led us to study ECM-dependent regulation of FN alternative splicing in the functionally normal mouse mammary epithelial cell line EpH4. These cells undergo morphological and functional differentiation when cultured on a reconstituted lr-BM, a process characterized by the formation of three-dimensional structures that resemble mammary alveoli and the production of milk proteins in the presence of lactogenic hormones (24).

EpH4 cells were cultured with or without an overlay of a lr-BM. Alternatively, the lr-BM was used as a substratum, and the cells were plated on top of a thick BM gel. In both cases, cells were harvested after different time points, RNA was pu-
rified and subjected to RT-PCR with different primer pairs designed to compare the relative proportion of the different FN mRNA isoforms, EDI/H11001 versus EDI/H11002, EDII/H11001 versus EDII/H11002, or IIICS-120 versus IIICS-95/H11001 IIICS-0 (Fig. 1A).

Similar to what we observed in Hep3B cells, the addition of a lr-BM inhibited the inclusion of the EDI exon by 2-fold (Fig. 1B) but had no effect on EDII splicing in EpH4 cells (Fig. 1C). Down-regulation of EDI inclusion was clearly observed after 24 h of treatment and persisted for at least 3 days (data not shown). Interestingly, IIICS alternative splicing was also altered by the presence of the lr-BM; the ratio between the LDV-containing isoform (IIICS-120) versus the LDV-lacking isoforms (IIICS-0 and IIICS-95) decreased by 3-fold (Fig. 1D).

**Signal Transduction Pathways Activated by a lr-BM**—The effects of the ECM are basically mediated by integrins, a large family of cell surface receptors that anchor cells to different ECM proteins and transduce mechanical and biochemical signals through the cell membrane (23). Integrins activate various protein kinases including focal adhesion kinase (24, 34), Src-family kinases (35), Abl (36), and integrin-linked kinase (37). These kinases can then in turn phosphorylate a variety of downstream targets, activating different signaling cascades such as JNK, ERK, or PI 3-kinase (35, 38–41).

To uncover the signaling pathways involved in transducing the information from the lr-BM to the splicing machinery, we first analyzed whether different known protein kinases were activated or inhibited by the treatment with a lr-BM. We performed Western blot analysis with antibodies against the phosphorylated isoforms of ERK, JNK, p38, and Akt, a classical PI 3-kinase target. We observed a robust activation of JNK (Fig. 2A) and ERK (Fig. 2B) after a 5-min treatment with lr-BM, whereas the phosphorylation levels of p38 and Akt remained unchanged (Fig. 2, C and D).

These results were confirmed by a LUC-based reporter assay. EpH4 cells were transiently transfected with a combination of two plasmids. One plasmid carrying the LUC reporter gene driven by a minimal promoter fused to GAL4 binding sites (GAL4-LUC) and the other plasmid coding for a fusion protein containing the GAL4 DNA binding domain plus the transactivation domain of c-Jun, a transcription factor that is phosphorylated and therefore activated by JNK (GAL4-c-JunTAD). Treatment with a lr-BM induced a 3-fold increase in LUC activity, confirming the activation of the JNK pathway under these culture conditions (Fig. 3C).

Alternatively, cells were co-transfected with the GAL4-LUC construct together with a plasmid that codes for the GAL4 DNA binding domain fused to the transactivation domain of ATF-2, a transcription factor that could be activated either by JNK or p38 depending on the cellular context. Treatment of co-transfected EpH4 cells with a lr-BM did not induce LUC activity (data not shown), indicating that ATF-2 is not a downstream target of JNK in this context and, furthermore, confirming that p38 is not activated by this treatment as already observed by Western blot analysis. Altogether, these results indicate that a
lr-BM induces the activation of JNK and ERK, two classical signal transduction kinases already reported to be activated in response to integrin signaling (23, 34, 38).

JNK Is Required for the lr-BM-dependent Regulation of FN Alternative Splicing—Signal transduction pathways are known to alter splice site selection (5). Accordingly, we have shown recently that a mammary mesenchymal cell-conditioned medium stimulates EDI and IIICS-120 inclusion into mature FN mRNA via a PI 3-kinase-dependent pathway (11). Other extracellular stimuli have been shown to regulate alternative splicing through protein kinase C/Ras (7), Ras-Raf-MEK-ERK (8, 9), Src (42), calcium/calmodulin-dependent protein kinase IV (10), and p38 (6).

To determine whether any of the kinases that were phosphorylated in response to the lr-BM could account for the signal-dependent changes in FN alternative splicing, we studied the influence of different pharmacological kinase inhibitors on the lr-BM regulation of EDI and IIICS alternative splicing. Blocking JNK activity with SP600125 (SP) inhibited up to 75% of the lr-BM-regulated EDI exon inclusion (Fig. 3A) and caused up to 100% inhibition of the lr-BM effect on IIICS inclusion (Fig. 3B). The observed effect of SP was dose-dependent, already seen at a concentration of 10 μM and reaching the maximum inhibition at 50 μM (data not shown). As expected, SP totally inhibited the lr-BM-mediated JNK activation (Fig. 3C), although it neither inhibited lr-BM-induced ERK phosphorylation nor MLK3-induced p38 activation (data not shown), demonstrating its functionality as well as its specificity in this context. These results demonstrate that JNK activation is necessary for the signal-dependent splicing regulation exerted by the lr-BM, implicating this pathway in the regulation of alternative splicing.

JNK Regulates Alternative Splicing in Transient Transfection Assays—The results shown in Fig. 3 revealed a role for a JNK-dependent cascade in the lr-BM-mediated splicing regulation. To further confirm those results, we made use of a previously reported system consisting in the transfection of an h-globin/FN EDI minigene (30). A basic scheme of this minigene is depicted in Fig. 4A. The regulation of FN alternative splicing by the lr-BM is also observed in transcripts derived from this minigene (30).

To confirm the requirement of JNK signaling for the lr-BM-induced splicing regulation, we designed siRNAs targeting JNK. Transfection of the JNK1 siRNA completely knocked down the expression of JNK1 and did not affect the levels of JNK2 (Fig. 4B). Conversely, JNK2 siRNA effectively knocked down JNK2 without affecting the levels of JNK1 (Fig. 4B). We then tested the lr-BM effect in the presence of these siRNAs and, in agreement with the results obtained using the JNK...
PI 3-Kinase and p38 Are Not Involved in the lr-BM-induced Splicing Regulation—Earlier reports indicate that PI 3-kinase connects Ras to Rac, leading to the activation of JNK (43–45). Therefore, it would be conceivable that the activation of JNK produced by the lr-BM could involve PI 3-kinase in an Akt-independent manner, as Akt is not phosphorylated in response to the lr-BM treatment (Fig. 2D). To determine whether PI 3-kinase has a role in the lr-BM-mediated splicing regulation, we made use of a specific PI 3-kinase inhibitor, LY294002. Blocking PI 3-kinase activity drastically decreased EDI (Fig. 4A) and LDV+/LDV− (Fig. 4B) ratios in untreated cells, consistent with our previous observations (11). In this context, the lr-BM was still able to activate JNK (data not shown) and to inhibit exon inclusion to the same extent as in the absence of the inhibitor (Fig. 5, A and B).

On the other hand, blocking p38 activity with SB202190 also inhibited both EDI and IIICS exon inclusion in untreated cells but did not affect the signal-induced splicing regulation seen upon treatment with a lr-BM (Fig. 5, C and D). In addition, overexpression of p38, together with its kinase MKK6, stimulated EDI exon inclusion by 2.6-fold (Fig. 5E). These results, together with our previous work, demonstrate that whereas inhibition of the PI 3-kinase and p38 pathways inhibits exon inclusion, activation of these pathways stimulates exon inclusion, further confirming that the lr-BM-mediated splicing regulation does not involve PI 3-kinase or p38.

A Role for the ERK Pathway in the lr-BM Signal-mediated Splicing Regulation—The results shown in Fig. 2B demonstrate that ERK is phosphorylated upon treatment with a lr-BM for 5 min. To study a possible contribution of the ERK pathway in the lr-BM-mediated splicing regulation, similar experiments to the ones shown in Figs. 3 and 5 were performed using the MEK inhibitor, PD98059 (PD).

Consistent with our previous results (11), treatment with PD drastically inhibits EDI and IIICS inclusion in untreated cells, indicating that ERK, like PI 3-kinase and p38, actively contributes to maintaining basal inclusion levels of EDI and IIICS. In this context, the lr-BM effect was inhibited by ~50% (Fig. 6, A and B). As shown in Fig. 6C, PD inhibited the lr-BM-stimulated phosphorylation. It is worth noting that the decrease in the extracellular signal-induced splicing regulation could not be due to the mere inhibition of EDI and IIICS inclusion levels observed upon treatment with PD, as LY294002 and SB202190 also inhibit both EDI and IIICS inclusion but do not inhibit the splicing regulation exerted by the lr-BM.

Interestingly, Paumelle et al. (46) have reported that treatment of Madin-Darby canine kidney cells with hepatocyte growth factor/scatter factor activates both ERK and JNK pathways and sustained activation of the former results in the inactivation of the latter. In addition, earlier reports show that JNK induces the expression of mitogen-activated protein ki-
nase phosphatase 1 causing dephosphorylation of ERK (47), and on the other hand, a scaffold protein in the JNK signaling pathway suppresses the ERK pathway (48). Taking into account that integrin signaling is known to produce a protracted activation of JNK (34), it would be possible to speculate that the decrease in EDI and IIICS inclusion levels triggered by the lr-BM could be the result of a sustained activation of JNK that would shut off the ERK cascade. Following this line, treatment with PD would cause the same effect as treatment with the lr-BM, explaining the lack of effect of the latter in a dephosphorylated-ERK context. To test this hypothesis, we monitored the levels of ERK and JNK phosphorylation over a lr-BM time-course treatment. Fig. 6 shows that JNK is activated at 5 min upon treatment, and this activation augments at 15 min lasting for at least 3 h. In the case of ERK, it becomes activated after a 5-min treatment and clearly decreases even below basal levels as JNK phosphorylation increases. This dephosphorylation of ERK lasts for at least 3 h (Fig. 6, D and E). This is consistent with the fact that overexpression of ERK, together with its kinase MEK, stimulated exon inclusion by 3.5-fold (Fig. 6F).

These results support the notion that the lr-BM might exert part of its effect by down-regulating the ERK pathway, which has a positive role in EDI and IIICS exon inclusion. This effect might be JNK-dependent or, alternatively, the two pathways could have independent roles in this signal-dependent splicing regulation, both being necessary for the final outcome, the down-regulation of EDI and IIICS inclusion levels. Moreover, these results could explain the lack of sufficiency of the JNK signaling pathway to mimic the splicing regulation exerted by the lr-BM.

**FIG. 5.** The lr-BM-mediated splicing regulation does not involve PI 3-kinase or p38 pathways. The lr-BM effect on FN EDI (A and C) and IIICS (B and D) alternative splicing is not affected by treatment with the PI 3-kinase inhibitor LY294002 (LY) 25 μM (A and B) nor by the p38 inhibitor SB202190 (SB) 20 μM (C and D). EpH4 cells were pretreated with the corresponding inhibitor or an equal volume of vehicle (DMSO) for 2–4 h and then left untreated (−) or stimulated with Matrigel 1.5% v/v (lr-BM), in the presence of the inhibitors. After 24 h, RNA was extracted and subjected to RT-PCR. Values represent the mean ± S.E. E, the α-globin/FN EDI minigene was co-transfected with empty vector (empty) or a combination of MKK6(EE) and p38 expression vectors (1 μg each, MKK6-p38).
The lr-BM Inhibits the Effect of Mesenchymal Growth Factors on FN Alternative Splicing—A growing body of data shows that the JNK signaling pathway negatively cross-talks with the PI 3-kinase-Akt and the ERK signaling cascades (31, 32, 49–51). We have shown recently that soluble factors present in the conditioned medium of the mammary mesenchymal cell line SCg6 (g6CM), as well as the hepatocyte growth factor/scatter factor stimulate the inclusion of EDI and IIICS exon in mammary epithelial cells via a PI 3-kinase-dependent pathway, with a minor but significant contribution of the ERK pathway (11). These data, together with our current results showing that JNK activation along with ERK dephosphorylation are involved in the lr-BM-mediated down-regulation of EDI and IIICS inclusion, suggest that there could be a negative interaction between the lr-BM-mediated and the g6CM-mediated splicing regulation.

To address this issue, EpH4 cells were plated either on tissue culture plastic or on top of the lr-BM and then treated with g6CM or growth factors or left untreated. The presence of a lr-BM abrogated the ability of g6CM (Fig. 7) or growth factors (data not shown) to increase EDI and IIICS inclusion levels, showing a preponderance of the JNK-mediated splicing regulation in this context. Altogether, our results show that JNK and PI 3-kinase have opposite roles in the regulation of FN alternative splicing extending the known antagonism between these two signaling pathways to the field of splicing regulation.

**DISCUSSION**

We showed here that a laminin-rich basement membrane inhibits the inclusion of EDI and IIICS into mature FN mRNA in mammary epithelial cells, whereas it has no effect on the third alternative region, EDII. The present work extends our previous studies about the influence of the ECM on alternative splicing (30) to a different cellular and physiological context, the mammary epithelial differentiation, which is known to be extremely dependent on signals emanated from the basement membrane (25, 26, 52). Furthermore, we showed that the IIICS...
Values represent the mean ± S.E.

Fig. 7. The stimulating effect of mesenchymal growth factors on EDI and IIICS splicing is inhibited by the lr-BM. EpH4 cells were plated either on tissue culture plastic (-) or on top of Matrigel-coated wells (lr-BM). After 24 h in serum-free medium, cells were either left untreated (-) or treated with a mammary mesenchymal cell-conditioned medium (g6CM) for another 24 h before RNA extraction and RT-PCR. Values represent the mean ± S.E.
the epithelial cells would become insensitive to them, turning into a situation in which signals from the BM would become predominant. BM signaling would now provoke down-regulation of EDI and IICS inclusion with a concomitant decrease in MMP expression and cell proliferation and provide a set of Fn molecules more characteristic of the stationary phenotype required during lactation.

In summary, this work provides new insights into how extracellular signals can alter splice site selection, bringing JNK to the scene. Furthermore, it defines how different signaling cascades that regulate alternative splicing may influence each other, highlighting the fact that the splicing pattern of a single transcript is the read out of an intricate network of different signaling pathways.

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