Mammary Epithelial-Mesenchymal Interaction Regulates Fibronectin Alternative Splicing via Phosphatidylinositol 3-Kinase*

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The way alternative splicing is regulated within tissues is not understood. A relevant model of this process is provided by fibronectin, an important extracellular matrix protein that plays a key role in cell adhesion and migration and contains three alternatively spliced regions known as EDI, EDII, and IIICS. We used a cell culture system to simulate mammary epithelial-stromal communication, a process that is crucial for patterning and function of the mammary gland, and studied the effects of extracellular signals on the regulation of fibronectin pre-mRNA alternative splicing. We found that soluble factors from a mammary mesenchymal cell-conditioned medium, as well as the growth factors HGF/SF (hepatocyte growth factor/scatter factor), KGF (keratinocyte growth factor), and aFGF (acidic fibroblast growth factor), stimulate EDI and IIICS but not EDII inclusion into fibronectin mRNA in the mammary epithelial cell line SCg2, favoring fibronectin isoforms associated with proliferation, migration, and tissue remodeling. We explored the signaling pathways involved in this regulation and found that the mammary mesenchymal cell-conditioned medium and HGF/SF act through a phosphatidylinositol 3-kinase-dependent cascade to alter fibronectin alternative splicing. This splicing regulation is independent from promoter structure and de novo protein synthesis but does require two exonic elements within EDI. These results shed light on how extracellular stimuli are converted into changes in splicing patterns.

Pre-mRNA alternative splicing is a widespread process that regulates gene expression and is the most important source of protein diversity in vertebrates (1). Fibronectin (FN), the best characterized extracellular matrix (ECM) glycoprotein, plays a key role in cell adhesive and migratory behavior related to fundamental processes such as embryogenesis, wound healing, maintenance of tissue integrity, and malignancy. Different FN polypeptides arise through an intricate pattern of alternative splicing in three regions of the primary transcript, (from 5′ to 3′) extra domain II (EDI), extra domain I (EDI), and type III connecting segment (IIICS) (also called EDB or EIIIB, EDI, and V region, respectively), resulting in up to 12 variants in rodents. FN alternative splicing is modulated in a cell type-, development-, and age-specific manner and therefore constitutes a paradigm for studying the regulation of this complex process (2). 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 due to the presence of an internal 3′ splice site within this exon.

In vivo EDI+ FN is poorly represented in the ECM of adult normal tissues. However, this variant is over-expressed in developing embryos, wound healing, liver fibrosis, ovary granulosa cell proliferation, and some tumors (2, 3). It has been proposed that EDI inclusion may mediate a conformational change in the whole molecule that would result in an increased exposure of the RGD motif, therefore increasing cell-ECM interaction through the binding to α5β1 integrin. Furthermore EDI inclusion potentiates the ability of FN to promote cell cycle progression (4). However, a growing body of data suggests that EDI per se is active. It has been shown that this region is responsible for the conversion of lipocytes into myofibroblasts in fibrotic liver (5) as well as for the induction of several matrix metalloproteinases (MMP-1, -3, and -9) required for cell migration and tissue remodeling (6). Recently, Muro et al. (7) generated mice devoid of EDI exon-regulated splicing and demonstrated that EDI splice regulation is required for proper skin wound healing and normal life span.

The abbreviations used are: FN, fibronectin; Act D, actinomycin D; aFGF, acidic fibroblast growth factor; bFGF, basic fibroblast growth factor; CHX, cycloheximide; ECM, extracellular matrix; EDI, extra domain I; EDII, extra domain II; EGF, epidermal growth factor; ERK, extracellular signal-regulated kinase; ESE, exonic splicing enhancer; ESS, exonic splicing silencer; g6CM, conditioned medium from SCg6 cells; HGF/SF, hepatocyte growth factor/scatter factor; IIICS, type III repeat connecting segment; JNK, c-Jun N-terminal kinase; KGF, keratinocyte growth factor; LUC, luciferase; LY, LY294002; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; p2CM, conditioned medium from SCg2 cells; PD, PD98059; pAKT, phospho-AKT; pERK, phospho-ERK; PI, phosphatidylinositol; pJNK, phospho-JNK; TGFβ1, transforming growth factor β1; mFN, mutant FN; RT, reverse transcriptase; CMV, cytomegalovirus; DMEM, Dulbecco’s modified Eagle’s medium; SR, serine/arginine-rich.

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EDII inclusion correlates with that of EDI in several cases. EDII is excluded from FN in normal adult tissue and included during development and pathological conditions such as inflammation, major trauma, wound healing, and tumors (8, 9). Nevertheless, the biological function of EDII is unknown, and the mechanisms that regulate its alternative splicing differ from those for EDI (10–12).

Splicing of the IIICS can generate three different variants in rodents, referred to as IIICS-0, IIICS-95, and IIICS-120 according to their lengths in the encoded amino acid residues. IIICS inclusion is higher in all fetal versus adult tissues, and this region is required for secretion of FN dimers during biosynthesis (13, 14).

Alternative splicing of several pre-mRNAs can be regulated by extracellular signals such as growth factors, cytokines, hormones, and stress stimuli (15). Accordingly, we have shown previously that a basement membrane-like ECM, as well as its two main protein components, modulates FN EDI alternative splicing in a hepatoma cell line (16). Nevertheless, only a few cases of the transducing components linking the cell surface with the nuclear splicing machinery have been investigated. For example, it has been demonstrated that the MAPK kinase 3/6-p38 pathway modifies the subcellular distribution of the heterogeneous nuclear ribonucleoprotein A1 and modulates the alternative splicing of transcripts derived from an adenovirus E1A reporter minigene. There is evidence suggesting a role for protein kinase C, Ras, and phosphatidylinositol 3-kinase (PI 3-kinase) in alternative splicing regulation (15). Recent reports linked the activation of the extracellular signal-regulated kinase (ERK) 1/2 to the regulation of CD44 alternative splicing triggered by T-cell activation. The STAR (signal transduction and activation of RNA) protein, SAM68, has been postulated as the final step of this cascade (17).

The mammary gland comprises stromal and epithelial cells that communicate with each other through the ECM. Disruption of this dynamic communication can both induce and promote breast cancer. Cross-talk between the mammary epithelium and stroma is also crucial for the proper patterning and function of the normal mammary gland. Unlike other organs, the mammary gland undergoes most of its growth and morphogenesis in the adult animal, and certain stages of the postnatal gland exhibit embryonic- and tumor-like features (18, 19). As EDI exon inclusion is linked to development, tissue remodeling, and tumorigenesis, the mammary gland represents a useful physiological context in which to study the regulation of FN alternative splicing by the cellular microenvironment, in particular by cell-cell interaction.

In this study we investigated the effects of different growth factors and cytokines on FN alternative splicing in a functionally normal mouse mammary epithelial cell line (SCp2) and show that hepatocyte growth factor/scatter factor (HGF/SF), keratinocyte growth factor (KGF), and acidic FGF (aFGF) stimulate EDI and IIICS inclusion but not EDII into mature FN mRNA. Furthermore, we demonstrate that soluble factors secreted by a mouse mammary mesenchymal cell line (SCg6) present in the conditioned medium from these cells (g6CM) also regulate FN EDI and IIICS alternative splicing in SCp2 cells, indicating that epithelial-mesenchymal interaction influences pre-mRNA alternative splicing. We explored the signaling pathways involved in this regulatory phenomenon, showing that g6CM as well as HGF/SF act through a PI 3-kinase-dependent cascade to alter FN splicing pattern in this cellular context.

This work defines different extracellular cues that regulate FN alternative splicing, increasing FN isoforms preferentially involved in cell adhesion, proliferation, migration, metastasis, and tissue remodeling. Furthermore, the data presented here provide new insights into how the cellular microenvironment can influence gene expression.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—SCp2 and SCg6 cells were grown in Dulbecco’s modified Eagle’s medium:F-12 (DMEM:F-12; Invitrogen) supplemented with 2% fetal bovine serum, insulin (5 μg/ml, Sigma) and gentamicin (50 μg/ml, Invitrogen).

Cell co-culture was performed using Falcon Tissue Culture Inserts bearing a 0.4-μm-pore size P.E.T membrane (Becton Dickinson Labware). Approximately, 1–2 × 10^5 cells were placed in DMEM:F-12 supplemented with 2% fetal bovine serum into 35-mm tissue culture wells. After 24 h, cell monolayers were rinsed, an insert was placed inside each well, and 1–2 × 10^5 cells were placed on top of the insert filter. Cells in the bottom well and in the insert were cultured together for 48 h.

**Cell Treatments**—Approximately 1–2 × 10^5 cells were placed in DMEM:F-12 supplemented with 2% fetal bovine serum into 35-mm tissue culture wells. After 24 h, the medium was replaced by serum-free DMEM:F-12, and cells were grown for another 24 h before treatment with growth factors (20 ng/ml), g6CM, SCp2 conditioned medium (p2CM) or serum-free DMEM:F-12 (control).

g6CM was obtained by placing 1 × 10^5 cells into 100-mm tissue culture dishes in 2% fetal serum medium and replacing it with serum-free DMEM:F-12 after 24 h. Cell supernatant was collected 24 h later and centrifuged to discard cell debris. Alternatively, g6CM was filtered through a 0.45-μm porous filter to obtain identical results. The same protocol was carried out with SCp2 cells to obtain p2CM.

**Reagents**—Growth factors and cytokines utilized were: epidermal growth factor (EGF), KGF, basic fibroblast growth factor (bFGF), aFGF, and HGF/SF from Sigma and transforming growth factor β1 (TGFβ1) and leukemia inhibitory factor (LIF) from R&D Systems, Inc. Kinase inhibitors were from Calbiochem. actinomycin D (Act D) was from Invitrogen and cycloheximide (CHX) from Sigma.

**Plasmids and Transfections**—EDI mini-ex plasmids were as follows: pSVEDA/Tot (α-globin promoter (20); pSVEDA/wtFN (wild type FN promoter (21)); pSVEDA/MnFN (mutant FN promoter (21)); pSVEDA/CMV (CMV promoter (21)); pSVEDA/Ant (α-globin promoter, mutated ESS (20)); pSVEDA/Ant/mutFN (mutant FN promoter, mutated ESS (11)), and pSVEDA/32e/mutFN (mutant FN promoter, mutated ESE (11)).

Plasmids used for luciferase assays were pGL5-Luc, pGal4-Elk1, and pGBBD/Xgal4-A52. Experiments with RasV12 included pCEV-RasV12 or empty vector (pBluescript, Stratagene).

Transfections were performed 24 h after plating using FuGENE 6 (Roche Applied Science). Approximately 2 × 10^5 cells were transfected with 3 μl of FuGENE 6 and 2 μg of total plasmid DNA in 35-mm tissue culture wells. Co-transfection of pCMVβgal allowed standardization by transfection efficiencies. Cells were stimulated 24 h after transfection. Luciferase Assays—Luciferase (LUC) activity in cell lysates was measured using the Luciferase Assay System (Promega). Cells were washed with phosphate-buffered saline before lysis with 100 μl of reporter lysis buffer (Promega). Cell extracts were centrifuged, and 50 μl of the supernatant was mixed with 100 μl of luciferase assay buffer II (Promega). LUC activity was tested with a junior luminometer (Berthold, Bad Wildbad, Germany).

**RNA Isolation and Radioactive RT-PCR Amplification**—Total RNA purification from cultured cells and RT-PCR analysis were carried out as described (11). The set of primers used for FN EDI (22), FN EDI minigene (20), and FN IIICS (23) were described previously. The set of primers used for EDII amplification was hEDB-dir (16) and mEDBrev (5’-CACT-GGACAGTGAGTGTG-3’).

Radioactive 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 according to the Cerenkov method (11).

**Western Blot Analysis**—After treatment, cells were lysed in boiling 2× sample buffer (4% SDS, 20% glycerol, 120 mM Tris, pH 6.8, 0.002% bromphenol blue, 200 mM β-mercaptoethanol) at the time points indicated. Proteins were separated (12%) on SDS-PAGE, transferred to nitrocellulose paper using a semidry blotter. Blots were blocked with 5% nonfat dry milk in TBS and 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 (pERK), anti-phospho-JNK (pJNK), anti ERK2, anti-JNK, and anti-AKT antibodies were from Santa Cruz Biotechnology. Anti-phospho-AKT (pAKT) was from Cell Signaling Technology.
of EDI alternative splicing, TGF (data not shown). Surprisingly, the widely reported regulator 1B). This effect was seen as early as 5 h and peaked at 24–48 h. Treatment with TGF (50 ng/ml; data not shown) as a positive control, did not up-regulate EDI isoform in these cells, even at the wide range of concentrations and HGF/SF increased the EDI/EDI ratio up to 4-fold (Fig. 1B). This effect was seen as early as 5 h and peaked at 24–48 h (data not shown). As a positive control, TGFβ1 stimulated EDI inclusion in Hep3B cells. Furthermore, leukemia inhibitory factor also failed to modify the EDI+/EDI− ratio (data not shown).

To explore whether the growth factors that regulate EDI inclusion also affect the splicing of the other two FN alternative regions, we performed specific RT-PCR for EDII and IIICS (Fig. 1A). Alternative splicing of IIICS was also modified upon treatment of SCp2 cells with different growth factors (Fig. 1C). Interestingly, aFGF, KGF, and HGF/SF up-regulated IIICS-120, the IIICS isoform containing the LDV motif. This motif has been shown to promote cell adhesion by interacting with integrins associated with cell adhesion, proliferation, migration, and matrix metalloproteinase induction (4, 6, 27–29). Conversely, the EDII FN isoform was not detected in RNA from untreated cells, and none of the described stimuli altered this splicing pattern (Fig. 1D).

These results extend the number of growth factors that stimulate EDI inclusion, bringing the FGF family into the scene for the first time and suggesting that a number of mitogenic agents, but not every cytokine, are key mediators of extracellular up-regulation of EDI inclusion in mammary epithelial cells. Furthermore, a strong correlation between EDI and IIICS splicing regulation emerged from these experiments, whereas EDII splicing pattern remained unaffected by these treatments. This is consistent with the idea that alternative splicing of EDI and EDII exons is controlled by different molecular mechanisms (10–12).

Soluble Factors Secreted by Mammary Mesenchymal Cells Regulate FN Alternative Splicing in Mammary Epithelial Cells—The epithelial-mesenchymal interaction is mediated by growth factors secreted by both cell types. We have shown that many of these growth factors affect FN alternative splicing. However, these experiments performed with recombinant factors are far from representing the actual extracellular milieu. To simulate the epithelial-mesenchymal interaction and evaluate its influence on FN alternative splicing, we chose a cell culture model composed of two mouse mammary cell lines, the epithelial line SCp2 and the mesenchymal line SCg6. Both cell lines derive from the same parental cell strain, CID-9 (30). As shown in Fig. 2, SCg6 cells express higher levels of EDI+, EDII+, and IIICS-120 FN than SCp2 cells.

Co-culture experiments were performed with tissue culture inserts that allow the interchange of soluble factors without direct cell-cell contact. The presence of SCg6 cells stimulated
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A number of studies have associated extracellular stimuli with alternative splicing by extracellular stimuli. The endogenous FN alternative splicing pattern was analyzed by specific RT-PCR for all three alternative exons in two mouse mammary cell lines, one epithelial (SCp2 or p2) and one mesenchymal (SCg6 or g6), both derived from the heterogeneous cell strain CID-9. The splicing patterns of EDI (left), EDII (center), and IIICS (right) for these cell lines are shown.

EDI exon inclusion in SCp2 cells, increasing the EDI+/EDI− ratio by 3–4-fold compared with that of SCp2 cells cultured with SCp2Cells on the other side of the filter (Fig. 3A). In contrast, EDI splicing in SCg6 was not affected by the coculture with SCp2 cells (data not shown).

To assess whether a dynamic cell-cell communication was essential for this effect, we tested the influence of g6CM on EDI alternative splicing in SCp2 cells. Although treatment with conditioned medium from SCp2 (p2CM) did not change EDI inclusion, treatment with g6CM elicited an increase in the EDI+/EDI− ratio already observed after 5 h and more pronounced after 24 and 48 h (Fig. 3B). Furthermore, IIICS regulation was again correlated with that of EDI, showing a marked preference for the IIICS-120 isoform (Fig. 3B), whereas EDII alternative splicing was not affected by this treatment (data not shown). These results show a novel regulation of FN alternative splicing by cell-cell interaction and provide a physiological context for studying the regulation of alternative splicing by extracellular stimuli.

Signal Transduction Pathways Activated by g6CM and HGF/SF—A number of studies have associated extracellular stimuli and the regulation of FN alternative splicing. Nevertheless, the signaling cascades that lead to these effects remain unexplored (15).

The pathways that link extracellular stimuli and cellular effects are classically grouped into kinase cascades activated by either mitogenic (MAPK pathways) or stress (SAPK (stress-activated protein kinase) pathways) stimuli (31). It is well known that most growth factors activate the Ras-Raf-MAPK kinase (MEK)-ERK1/2 pathway (32). To confirm that this pathway was activated in response to g6CM and/or growth factors, we first performed a luciferase-based GAL-ELK1 reporter assay. SCp2 cells were transiently transfected with two plasmids. One plasmid carried the LUC reporter gene driven by a minimal promoter fused to binding sites for the yeast transcription factor GAL4, and the other coded for a fusion protein containing the GAL4 DNA binding domain plus the activation domain of ATF2 transcription factor. ATF2 is known to be phosphorylated and therefore activated by ERK1/2. Approximately a 10-fold activation of the reporter system was observed in cells treated with both g6CM and HGF/SF (Fig. 4A).

The stress kinases p38 and c-Jun N-terminal kinase (JNK), which are occasionally activated by growth factors (33), can also activate the transcription factor ELK1 in some cell lines. Therefore, we performed a LUC-based GAL-ATF2 reporter assay that only differs from the one described above in that the second plasmid codes for GAL4 binding domain fused to the activation domain of ATF2 transcription factor. ATF2 is known to be activated by JNK and p38 but not by ERK1/2. Upon treatment of SCp2 cells with g6CM or HGF/SF, a 1.5-fold activation of the reporter was observed, indicating that the JNK and/or p38 pathway are activated but to a lesser extent compared with the ERK1/2 pathway (Fig. 4B). To confirm and dissect these results, we performed Western blots for pERK and pJNK. We observed a robust increase in pERK and a clear augmentation in pJNK after 30 min of treatment (Fig. 4C). These activations were sustained in time, with high levels of pJNK and pERK still seen at 2 and 5 h, respectively (data not shown). Conversely, we detected little or no phosphorylation of p38 after 30 min of treatment with g6CM or HGF/SF, which contrasted with the strong activation induced by anisomycin, a known activator of p38 (data not shown).

Because PI 3-kinase-AKT, analogous to Ras-Raf-MEK-ERK1/2, is a typically HGF/SF-activated pathway (34), we measured the levels of pAKT in SCp2 cells. Both HGF/SF and g6CM activated this cascade, as reflected by the increased levels of pAKT (Fig. 4C). In conclusion, we found that g6CM and HGF/SF activate the classical pathways that lead to phosphorylation of ERK and AKT concomitantly with the activation of the stress kinase JNK.

Up-regulation of EDI+ and IIICS-120 FN Occurs Mainly through the PI 3-Kinase Pathway—To determine whether there was one particular pathway that could account for the effects exerted by g6CM or HGF/SF on FN alternative splicing, we first studied the influence of different pharmacological kinase inhibitors on the regulation of EDI alternative splicing.

Blocking MEK activity with PD98059 (PD) caused a 25% decrease in EDI+/EDI− ratio in untreated cells (Fig. 5A). However, HGF/SF still increased this ratio in similar proportions to those in cells with a functional MEK. Similar results were seen when the stimulation was performed with g6CM (Fig. 5B) and U0126, another MEK inhibitor (data not shown). Because PD inhibited the g6CM-induced activation of the GAL-ELK1 reporter (Fig. 5C), we concluded that MEK activity is not necessary, in this cellular context, for the described effects of HGF/SF and g6CM on EDI splicing regulation. On the other hand, blocking PI 3-kinase activity with the inhibitor LY294002 (LY) totally abrogated HGF/SF-induced effects on EDI alternative splicing (Fig. 5A). Similarly, inhibiting PI 3-kinase activity drastically inhibited g6CM-mediated increase of EDI+/EDI− ratio; this inhibition was potentiated by PD (Fig. 5B), suggesting a minor but significant role for MEK only in a PI 3-kinase activity-deficient context. As expected, LY blocked g6CM-induced AKT phosphorylation (Fig. 5D) whereas GAL-ELK1 reporter activation remained unaffected (Fig. 5C). Blocking p38 or JNK activity with the inhibitors SB202190 or SP600125, respectively, did not inhibit the studied EDI splicing regulation (data not shown).

In agreement with the proposed correlation between FN IIICS and EDI splicing regulation, we found that LY inhibited up to 50% of the IIICS splicing regulation exerted by HGF/SF, whereas PD and SP600125 did not block either this effect or that mediated by g6CM (Fig. 5E and data not shown). As already mentioned for EDI, PD modified both the basal and HGF/SF-stimulated LDV+/LDV− ratios to the same extent, leading to a similar HGF/SF-mediated -fold increase compared with that observed in the absence of PD. These results strongly support a dominant contribution of the PI 3-kinase survival pathway in converting extracellular stimuli into changes in FN splicing patterns.

Extracellular Regulation of FN EDI Alternative Splicing Is Also Observed in Transfected Minigenes—The SR family of proteins comprises RNA-binding proteins that are essential for both constitutive and alternative splicing. Regulation of alternative splicing can be achieved by changes in relative abundance, phosphorylation, and subcellular localization of these splicing factors (35). Changes in promoter structure and occupation as well as chromatin structure remodeling have been shown also to modify splicing patterns, indicating a coupling
between transcription and alternative splicing (11, 12, 21). To study the involvement of cis and trans acting factors and the influence of promoter structure and/or chromatin organization on the described splicing regulation, we used a reporter system for EDI alternative splicing (11, 12, 21).

We first analyzed whether the splicing regulation exerted by g6CM was also seen over the transcripts generated from the transfected minigene bearing only the −220/+44 region of the FN promoter upstream of EDI and its flanking introns and exons. SCp2 cells were transfected with this construct and treated with g6CM or left untreated. In such a context, g6CM stimulated EDI relative inclusion by 2-fold (Fig. 6A). Moreover, this stimulation was observed when transcription was carried out by a FN promoter that harbors point mutations in the transcription factor binding sites CRE and CCAAT (mFN promoter) or by completely different promoters (α-globin or CMV) (Fig. 6B). Hence, EDI splicing regulation can still be seen in a different chromatin structure scenario, as it is a transiently transfected plasmid and cannot be prevented either by alteration of transcription factor binding sequences or by promoter swapping. Therefore, the described regulation does not seem to be the consequence of a specific chromatin structure remodeling phenomenon or a process mediated by a specific transcription factor.

Inclusion of EDI is stimulated by the binding of different SR proteins to the exonic splicing enhancer (ESE) present in EDI. This exon also carries an exonic splicing silencer (ESS) downstream of the ESE (20). To evaluate whether the g6CM-mediated increase of EDI+/EDI− ratio relies on the integrity of the ESE and the ESS, we transfected SCp2 cells with minigenes carrying mutations in the ESE (ΔESE) or the ESS (ΔESS) downstream of the mFN promoter. Disruption of the ESE diminished EDI+/EDI− ratio by more than 100-fold (Fig. 6C). Under these conditions, g6CM stimulation of EDI+/EDI− ratio was totally inhibited. Similarly, mutation of the ESS also abrogated the effects of g6CM on EDI splicing at the same time that it increased basal levels of EDI+/EDI− ratio. Because the EDI− isoform was barely detectable, we performed the same...
experiment but with the minigene driven by the α-globin promoter, which generates transcripts with lower EDI/EDI ratio (Fig. 6B; Ref. 21). With this construct, mutation of the ESS also inhibited the g6CM-triggered stimulation of EDI inclusion (Fig. 6C). These results show that the extracellular regulation of FN EDI splicing strictly depends on the integrity of EDI splicing enhancer and silencer.

A Constitutively Active Form of Ras Stimulates EDI Relative Inclusion—We demonstrated that g6CM increased the EDI+/EDI− ratio of mRNAs transcribed from a FN-EDI minigene. Transcripts from this minigene were also responsive to HGF/SF effects on EDI alternative splicing (data not shown).

Ras is a small GTPase protein activated by growth factors and constitutively activated under certain pathological conditions such as in human tumors (36). When activated, Ras in turn stimulates the Raf-MEK-ERK and PI 3-kinase pathways (34). Considering that high levels of EDI inclusion are also associated with proliferation and pathological conditions, to-
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Regression of Fibronectin Alternative Splicing by g6CM Is Not Mediated by a Change in mRNA Stability nor Does It Require de Novo Protein Synthesis—We then asked whether the signaling pathways activated by g6CM were acting by favoring the production of mature FN EDI+ mRNA over FN EDI− mRNA or, alternatively, by altering the relative stability of these two isoforms. Therefore, we evaluated the effect of g6CM on FN alternative splicing in cells pretreated with the transcription inhibitor actinomycin D, a situation in which the only means of altering the isoform pattern is by changing mRNA stability. The stimulation period selected was long enough to observe a robust regulation of FN EDI alternative splicing by g6CM without affecting cell survival. After a 16-h treatment with g6CM, the endogenous EDI+/EDI− ratio increased more than 60%, an effect that was totally abrogated by Act D (Fig. 8). Although this result argues against a change in mRNA stability to account for the observed regulation, we could not rule out the possibility that Act D was inhibiting the synthesis of a protein necessary for changing the relative stability of both isoforms. However, we observed a 4.5-fold increase in the EDI+/EDI− ratio when the treatment with g6CM was performed in the presence of the translation inhibitor cycloheximide. This result not only invalidated the previous hypothesis but also demonstrated that this regulation of FN EDI alternative splicing is independent on de novo protein synthesis and is potentiated by CHX. Furthermore, Act D totally blocked the synergistic effect caused by the addition of g6CM plus CHX (Fig. 8).

Interestingly, similar results were obtained for LDV+/LDV− ratios when analyzing IIICS splicing under these conditions, expanding the correlation between regulation of EDI and IIICS alternative splicing even further (data not shown).

These results show that the regulation of FN alternative splicing by g6CM does not require de novo protein synthesis nor does it involve a change in relative mRNA stability. Therefore, this regulation should rely on a change in the activity of the preexisting splicing machinery and/or in the relative efficiency of mRNA export from the nucleus to the cytoplasm.

**DISCUSSION**

We show here that different growth factors such as HGF/SF, KGF, and aFGF, as well as the conditioned medium from the mouse mammary mesenchymal cells SCg6, up-regulate the inclusion of EDI and IIICS, two of three alternative regions of the FN primary transcript, in the mouse mammary epithelial cell line SCp2. All of the growth factors and cytokines tested in the present work are involved in mammary gland morphogenesis (37). Although g6CM may contain several of these factors, only KGF and HGF/SF are known to be secreted exclusively by mammary stroma and to bind their receptors in epithelial cells (38). Furthermore, HGF/SF is the only one that induced changes in FN alternative splicing as well as cell scattering similar to g6CM in SCp2 cells (data not shown). When g6CM was passed through a 50,000 molecular weight cut-off ultrafiltration device, the activity, evaluated as the ability to increase FN EDI relative inclusion, remained in the retentate. This indicates that the responsible factor(s) should be larger than 50

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**Fig. 6.** g6CM-mediated FN EDI inclusion does not depend on promoter structure and requires intact exonic elements. A, FN EDI alternative splicing regulation by g6CM on transcripts generated from a transfected minigene (EDI exon with its flanking introns and exons) driven by a fragment of the human FN promoter (~230/+44). B, effect of g6CM on FN EDI alternative splicing of transcripts generated from the minigene under different promoters. C, effect of g6CM on alternative splicing of wild-type (wt), ESE-disrupted (ΔESE), and ESS-disrupted (ΔESS) EDI exon. Cells were treated with g6CM for 48 h.

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from the minigene. RNA was extracted 48 h post-transfection. Bars as a dose-dependent manner (0, 0.5, and 1 µg) of the constitutively active form of Ras (RasV12) stimulates EDI inclusion in inclusion. A

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vector), cells were treated with PD (50 µM), LY (25 µM), or a combination of both. After 24 h, RNA was extracted and subjected to RT-PCR. kDa. When either recombinant HGF/SF or recombinant KGF was subjected to the same procedure, the activity remained in the retentate for the former but was recovered in the filtrate fraction for the latter (data not shown). It has been reported previously that HGF/SF not only induces scattering of various epithelial cells in culture but also increases the expression of the EDI FN isoform in Madin-Darby canine kidney cells (24) and in human colorectal carcinoma cells (25). These reports and our findings lead us to speculate that HGF/SF could be one of the factors present in g6CM and involved in the observed regulation of FN alternative splicing in mammary epithelial cells. However, cell scattering is not a prerequisite for a change in FN splicing, as other factors such as bFGF, aFGF, KGF, and EGF induce the latter without affecting cell morphology. On the other hand, TGFβ1, proposed as one of the main regulators of EDI splicing, must be excluded as the factor responsible for the conditioned medium effect because EDI splicing was unresponsive to TGFβ1 in Scp2 cells. The unexpected lack of effect of TGFβ1 is consistent with the variety of effects (e.g. proliferative and antiproliferative) exerted by this factor under different conditions (39) and emphasizes the importance of tissue specificity and context in signal transduction pathways (40).

HGF/SF acts through the receptor tyrosine kinase Met. It is known that PI 3-kinase is activated downstream of Met and is required for HGF-induced tubulogenesis and cellular motility. However, branching morphogenesis induced by a constitutively active PI 3-kinase is still dependent on the ERK pathway. Furthermore, activation of Ras-ERK downstream of Met is required for HGF-induced adherents junction disassembly (41). Certainly, the orchestration of complex cellular responses requires the activation of different signaling cascades and extensive cross-talk between them may occur, making the dissection of each individual pathway involved a difficult task. Our results reveal the involvement of PI 3-kinase signaling as the key pathway connecting HGF/SF with the nuclear splicing machinery. We show a drastic abrogation of the effects elicited by g6CM or HGF/SF on FN EDI and IIICS alternative splicing by a PI 3-kinase inhibitor, demonstrating the prevalence of a PI 3-kinase-mediated pathway and unraveling part of the hitherto unknown signaling cascade mediating FN alternative splicing regulation. The fact that the g6CM effect is not totally inhibited by LY in contrast with the HGF/SF-mediated effect, together with the observation that blocking the MEK/ERK pathway exerted a cooperative inhibitory effect only in the former case, suggests that other soluble factors present in g6CM apart from an HGF-like activity must also be participating in the regulation of FN alternative splicing in this cellular context.

Insulin is known to activate PI 3-kinase (42). Accordingly, we found that insulin depletion decreases the EDI+/EDI− ratio as well as the LDV+/LDV− ratio for the IIICS region in Scp2 cells in a dose-dependent manner (data not shown). It has also been shown that insulin down-regulates the JNK pathway through PI 3-kinase-AKT cascade, indicating a negative cross-talk between these two pathways (43). In agreement with this proposed cross-talk, we found that inhibition of the JNK pathway with SP600125 potentiates the stimulatory effect of HGF/SF and g6CM on FN EDI and IIICS-120 inclusion (unpublished data).

Conceivably, many of these extracellular cues may act through different signaling transduction cascades, leading to changes in relative abundance, subcellular localization, or the activation state of splicing factors such as SR proteins. In this context, it is possible to speculate that the extracellular stimuli studied in the present work would modulate the phosphorylation of SR proteins. By using an EDI exon reporter system and promoter swapping, we show that the splicing regulation of FN EDI exon by g6CM does not depend on promoter architecture; but it does require the integrity of two exonic elements, the splicing enhancer and splicing silencer, defined within EDI,

Fig. 7. A constitutively active form of Ras induces EDI exon inclusion. A, co-transfection of an expression construct coding for a constitutively active form of Ras (RasV12) stimulates EDI inclusion in a dose-dependent manner (0, 0.5, and 1 µg) in transcripts generated from the minigene. RNA was extracted 48 h post-transfection. Bars in the histogram represent the EDI+/EDI− ratio, and the line represents the LUC activity of the GAL-ELK1 reporter. B, inhibition of RasV12 effect on EDI splicing, 24 h post-transfection (1 µg of RasV12 or empty vector), cells were treated with PD (50 µM), LY (25 µM), or a combination of both. After 24 h, RNA was extracted and subjected to RT-PCR.

Fig. 8. Regulation of FN alternative splicing by g6CM does not depend on mRNA stability or de novo protein synthesis. After a 2-h pretreatment with Act D (5 µg/ml) or vehicle (ethanol) plus CHX (2.5 µg/ml) or vehicle (MeSO), Scp2 cells were either left untreated (light gray bars) or treated with g6CM (dark gray bars). Cells were harvested 16 h after treatment with g6CM. RNA was extracted and subjected to RT-PCR to evaluate endogenous FN EDI+ and EDI− isoforms as described above.
suggesting the involvement of SR proteins in the depicted regulation. It has been proposed recently that the splicing silencer could help to ensure an RNA secondary structure associated with the display of the enhancer (44). Based on this model, it is possible to speculate that not only the splicing enhancer but also the silencer participate in SR-dependent processes. The results from the experiment with Act D allow us to rule out the involvement of differential mRNA stability in the g6CM-mediated effect on FN splicing. Furthermore, the kinetics of the change in the pattern of EDI alternative splicing in response to g6CM or HGF/SF is consistent with a rapid modification in SR protein activity. Accordingly, we found that the g6CM effect does not depend on de novo protein synthesis, as it is not suppressed by CHX. The fact that CHX potentiates this effect suggests that it could be activating a synergistic signaling pathway or, alternatively, blocking the synthesis of an inhibitory protein. Further experiments would be required to confirm whether modulation of SR protein activity is a key step in the studied regulatory phenomenon.

ECM modulates tissue homeostasis of the mammary gland and growth, differentiation, and apoptosis of mammary cells in culture (40, 45). Regulation of these processes begins at the cell surface, where different ECM components interact with their receptors, the integrins (46). FN is the ligand for at least a dozen members of the integrin receptor family. EDI itself binds to αβ1 and αβ2 (29), and IICS contains binding sites for αβ1 and αβ2 (47). Recently, a novel glycosaminoglycan-binding site has been identified within the FN IICS, showing that cooperation between cell surface proteoglycans and integrins is important for mediating the adhesion of cells to FN (48). Cooperative signaling by αβ1 and αβ2 integrins has been proposed to modulate the expression of matrix metalloproteinases such as collagenase, 92-kDa gelatinase, and stromelysin-1 in response to FN (27). Therefore, the inclusion or exclusion of EDI and IICS into mature FN mRNA by alternative splicing can add or remove integrin recognition sites from the FN molecule, altering cell behavior.

FN is known to be synthesized in large amounts mainly by mesenchymal cells in vivo. However, Sakai et al. (49) found high concentrations of FN mRNA restricted to salivary epithelial cells in eel regions and demonstrated the involvement of these FN molecules in regulating salivary gland branching morphogenesis. Concomitantly, a decrease in both mRNA and protein levels of E-cadherin was discovered, postulating a cooperative regulation of FN mRNA levels of E-cadherin was discovered, postulating a con- lial cells in cleft regions and demonstrated the involvement of high concentrations of FN mRNA restricted to salivary epithelial cells in vivo mesenchymal cells modulate the expression of matrix metalloproteinases such us modification in SR protein activity. Accordingly, we found that in response to g6CM or HGF/SF is consistent with a rapid kinetics of the change in the pattern of EDI alternative splicing the g6CM-mediated effect on FN splicing. Furthermore, the enhancer but also the silencer participate in SR-dependent regulation. It has been proposed recently that the splicing silencer could help to ensure an RNA secondary structure associated with the display of the enhancer (44). Based on this model, it is possible to speculate that not only the splicing enhancer but also the silencer participate in SR-dependent processes. The results from the experiment with Act D allow us to rule out the involvement of differential mRNA stability in the g6CM-mediated effect on FN splicing. Furthermore, the kinetics of the change in the pattern of EDI alternative splicing in response to g6CM or HGF/SF is consistent with a rapid modification in SR protein activity. Accordingly, we found that the g6CM effect does not depend on de novo protein synthesis, as it is not suppressed by CHX. The fact that CHX potentiates this effect suggests that it could be activating a synergistic signaling pathway or, alternatively, blocking the synthesis of an inhibitory protein. Further experiments would be required to confirm whether modulation of SR protein activity is a key step in the studied regulatory phenomenon.

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Mammary Epithelial-Mesenchymal Interaction Regulates Fibronectin Alternative Splicing via Phosphatidylinositol 3-Kinase
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