Transforming growth factor-β regulates the splicing pattern of fibronectin messenger RNA precursor

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Fibronectin (FN) polymorphism is caused by alternative splicing patterns in at least three regions (ED-A, ED-B and IIICS) of the primary transcript of a single gene. Using monoclonal antibodies, we previously demonstrated that transforming growth factor-β (TGF-β) preferentially increases the accumulation of the FN isoforms containing the ED-A sequence in cultured normal human fibroblasts [Balza et al., (1988) FEBS Lett. 228, 42–44]. To determine the basis of this effect, we have examined through S1 nuclease analysis, the levels of ED-A- and ED-B-containing mRNAs in cultured normal human skin fibroblasts before and after TGF-β treatment. These experiments have shown that TGF-β increases the relative amount of m-RNA for ED-A- and ED-B-containing FN isoforms. These data demonstrate that a growth factor may regulate the splicing pattern of a pre-mRNA.

Transforming growth factor-β; Fibronectin isoform; Pre-mRNA splicing regulation

1. INTRODUCTION

Fibronectins (FNs) are high-molecular-mass adhesive glycoproteins present in soluble form in plasma and other body fluids and in insoluble form in the extracellular matrices. FN molecules are involved in diverse biological phenomena, including the establishment and maintenance of normal cell morphology, cell migration, hemostatis and thrombosis, wound healing, and oncogenic transformation [1–4].

FN polymorphism is caused by alternative splicing patterns in three regions of the primary transcript of a single gene which may generate 20 different FN subunit isoforms (fig.1) [5–7]. The alternative splicing of FN pre-mRNA is regulated in a cell-, tissue- and developmentally specific manner [7–11]. Furthermore, it has recently been demonstrated that the splicing pattern of FN pre-mRNA is deregulated in transformed cells and in malignancies [7,12]. In fact, IIICS, ED-A and ED-B sequences are expressed in a greater degree in transformed human cells as well as in tumor tissues with respect to their normal counterparts [7,13–16].

It has been shown that transforming growth factor-β (TGF-β) increases FN levels in both media and extracellular matrix of a variety of cultured cells [17] and, using monoclonal antibodies, we have recently demonstrated that TGF-β preferentially increases the accumulation of the ED-A-containing FN isoform [18]. To investigate the molecular basis of this phenomenon,

we have studied through S1 nuclease protection analysis, the levels of the ED-A and ED-B-containing FN mRNAs in three different lines of cultured human skin fibroblasts treated with TGF-β and in untreated controls. The results demonstrate that the percentages of ED-A- and ED-B-containing FN mRNA increase from about 10 to 20 and from about 1 to 4.5, respectively, after TGF-β treatment.

2. MATERIALS AND METHODS

Cultured normal fibroblasts from fetal (GM-5386) and non-fetal (GM-5659, GM3440) human skin were purchased from NIGMS (New Jersey, USA), and grown as in [18]. Cells were grown to confluence, then were treated for three days with a low concentration (0.3%) of fetal calf serum (FCS) medium. Subsequently, cells were treated for 24 h with FCS-free medium containing 450 pM human platelet-derived TGF-β_1 (R & D Systems, Minneapolis, MN) while the set of control cells was treated only with FCS-free medium. Cells were then washed and total RNA was extracted as in [19].

The probe for the ED-A region was prepared as follows: a 752-base *Acc*I-*Eco*RV fragment of the pFH111 clone, kindly provided by Prof. F.E. Baralle (Istituto Sieroterapico Belfante, Milano, Italy) [8] was subcloned into *Acc*I-*Sma*I sites of pGEM3Z (Progema, Madison, WI); the 613-base probe containing a FN sequence of 481 bases, including the complete ED-A sequence, plus 132 bases from the vector, was obtained by *Ava*II-*Pvu*II digestion (figs.1 and 2A). The ED-B probe was prepared starting from the FN *Eco*RI insert of the clone λ F2 [15] which contains the complete ED-B sequence plus 294 bases upstream and 252 bases downstream. This fragment was subcloned into the M13mp19 vector. The 821-base double-stranded DNA probe, containing a FN sequence of 784 bases and 37 bases of the vector, was obtained by *Acc*I digestion (figs.1 and 3A). RNA S1 nuclease protection analysis was carried out as in [20] using the ED-A probe, labelled with ^{32}P at the 3′ end by end-filling as in [21] and ED-B probe 5′ end-labeled with ^{32}P using T4 polynucleatide kinase as in [22]. The nuclease-resistant fragments were analyzed on a 6% polyacrilamide gel containing 8 M urea, followed by autoradiography and double-
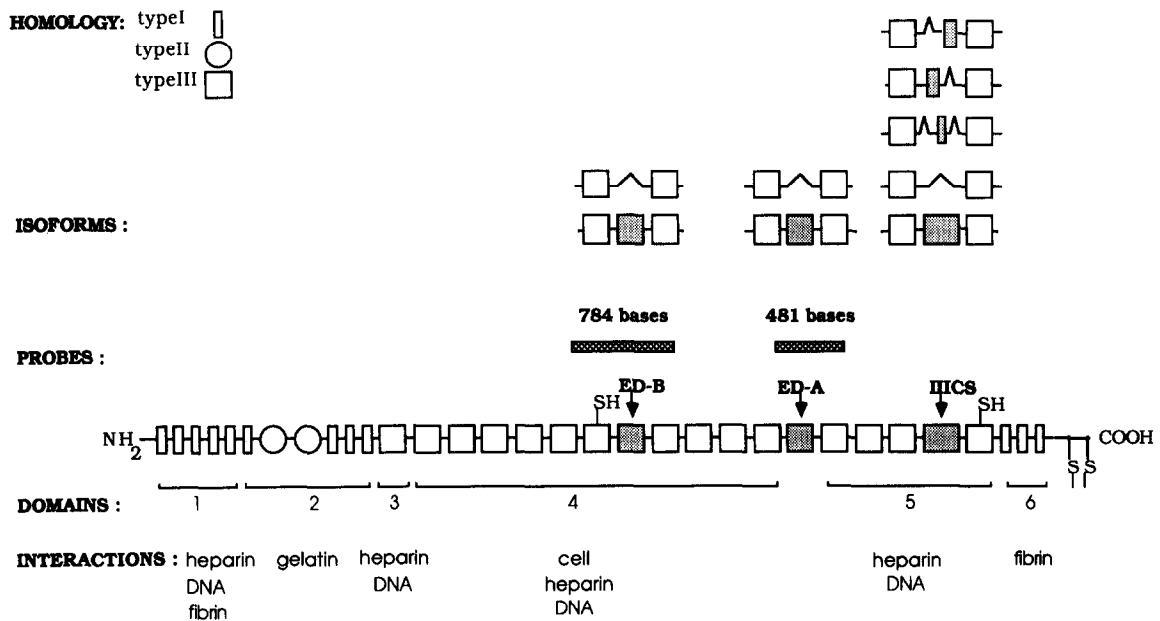
Fig.1. Model of the domain structure of a human FN subunit. Arrows indicate the regions of variability due to alternative splicing of the FN mRNA precursors. The figure also indicates the internal homologies, macromolecules interacting with the various FN domains, the possible isoforms generated by alternative splicing of the pre-mRNA and the FN sequences covered by the probe used to study, by S1 nuclease analysis, the splicing patterns of the ED-A and ED-B exons.

dimension analysis of the resulting film by an LKB Ultroscan XL laser densitometer.

3. RESULTS

Fig.1 shows a schematic representation of the domain structure of the human FN, the possible isoforms generated by alternative splicing of the primary transcript and the sequences covered by the two probes used to study, by S1 nuclease analysis, the splicing patterns of the ED-A and ED-B exons.

Fig.2A shows an autoradiogram of the S1 nuclease-resistant fragments of the cDNA probe, used to study the expression of the ED-A exon, protected by the hybridization with RNAs from a TGF-β-treated normal human skin fibroblast cell line (GM-5386) and from untreated control cells. The results of similar studies using three different normal human fibroblast cell lines, show (fig.2B) that there is nearly a 2.0–2.5-fold increase in the relative amount of the ED-A-containing FN mRNA molecules with respect to the controls, depending on the cell line.

Fig.3A shows an autoradiogram of the S1 nuclease-resistant fragments of the cDNA probe, used to study the expression of the ED-B exon, protected by the hybridization with RNAs from a TGF-β treated normal human skin fibroblast cell line (GM-5386) and from untreated control cells. The results, obtained with similar experiments on RNAs from three different normal human skin fibroblast cell lines are shown in Fig.3B. The amount of FN mRNA molecules containing the ED-B sequence in TGF-β-treated cells increases 4–5

times with respect to the controls, depending on the cell line.

4. DISCUSSION

Alternative RNA splicing is an important flexible mechanism able to generate diversity in a reversible fashion in response to developmental and environmental cues without requiring the expression of new genes [24–25]. Nothing is known, however, about the extracellular microenvironmental factors responsible for the modulation of splicing patterns of primary transcripts.

It has been demonstrated that TGF-β, released by platelet lysis, is sufficent to initiate the cascade of events resulting in the formation, of granulation tissue in wound healing and that the secretion of TGF-β by tumor cells may have a similar effect on stromal elements ([26] and references therein), thus, suggesting that TGF-β may be partially responsible for the higher expression of ED-A- and ED-B-containing FN isoforms observed in wound healing and tumor stroma compared to normal tissues [7,12–16,23]. In our previous studies, addressed to verify this hypothesis, we have demonstrated, using monoclonal antibodies, that in cultured human fibroblasts, the relative amount of ED-A-containing FN increases about 2–3 times after TGF-β treatment [18]. Here we have now demonstrated that TGF-β also increases, in cultured human fibroblasts, the levels of ED-A- and ED-B-containing FN mRNA. On the contrary, dexamethasone, which also increases the total level of FN [27], does not change either the
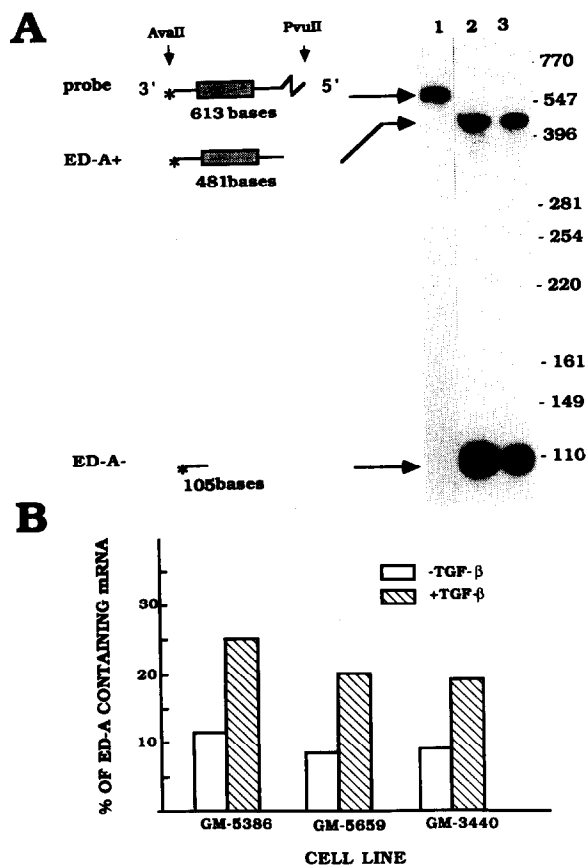
Fig.2. Effect of TGF-β on the expression of the ED-A exon in normal human skin fibroblasts. (A) On the left we have depicted a schematic representation of the probe (see section 2) used to study, by S1 nuclease analysis, the splicing patterns of the ED-A exon. The sequence deriving from the cloning vector at the 5' end is indicated by a wavy line. The asterisk marks the 3' ^{32}P-labelled end. The probe fragments protected by the FN mRNA species containing (ED-A+) or not containing (ED-A −) the ED-A sequences are also shown. The autoradiogram shows an S1 nuclease analysis of mRNA from TGF-β-treated (lane 3) and untreated (lane 2) GM-5386 normal human fibroblasts. Lane 1: undigested probe. The numbers on the right of the autoradiogram indicate the size (in nucleotides) of the molecular weight markers. (B) The relative abundance of ED-A-containing FN mRNA in three different cell lines of normal human skin fibroblasts treated (dashed areas) and untreated (white areas) with TGF-β. These results, obtained by laser densitometric scanning of autoradiograms deriving from experiments similar to those shown in A, represent the average of three independent experiments in which the values differed by no more than 10%.

Fig.3. Effect of TGF-β on the expression of the ED-B exon in normal human skin fibroblasts. (A) On the left there is a schematic representation of the probe (see section 2) used to study, by S1 nuclease analysis, the splicing pattern of the ED-B exon. The sequence derived from the cloning vector, at the 3' end, is indicated by a wavy line. The asterisk marks the 5'-labelled end. The probe fragments protected by FN mRNA species containing (ED-B+), and not containing (ED-B −) the ED-B sequence are also shown. The autoradiogram shows an S1 nuclease analysis of RNA from TGF-β-treated (lane 3) and untreated (lane 2) GM-5386 normal human fibroblasts. Lane 1: undigested probe. The numbers on the right of the autoradiogram indicate the size (in nucleotides) of the molecular weight markers. (B) The relative abundance of ED-B-containing FN mRNA in three different cell lines of normal human skin fibroblasts, treated (dashed areas) and untreated (white areas) with TGF-β. These results, obtained by laser densitometric scanning of autoradiograms deriving from experiments similar to those shown in A, represent the average of three independent experiments in which the values differed by no more than 20%.

relative amount of ED-A-containing FN [18] or the relative amount of ED-A-containing FN mRNA (data not shown).

The data reported here suggest that TGF-β may control the formation of stroma in granulation and tumor tissues, not only by increasing the accumulation of FN and other extracellular matrix proteins [26], but also by modifying the relative amount of the different FN isoforms. This is achieved through the modulation of the splicing of FN pre-mRNA, and possibly pre-mRNA of other extracellular matrix proteins as well, with a
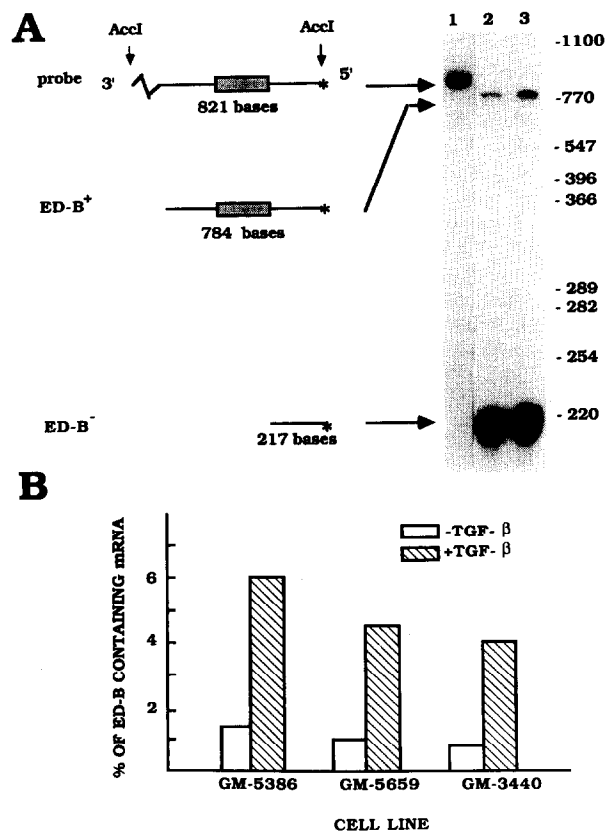
consequent heightened expression of isoforms containing extra sequences which may have specific biological functions.

Furthermore, these data demonstrate for the first time, to our knowledge, that a growth factor may modulate the splicing pattern of a primary transcript.

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