Polarized Regulation of Fibronectin Secretion and Alternative Splicing by Transforming Growth Factor β*

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Fibronectin is a multifunctional protein that is synthesized in several different forms that result from alternative splicing of mRNA. Although expression of splicing variants appears to be both developmentally regulated and tissue-specific, the functional significance of these isoforms is largely unknown. We found that cultured airway epithelial cells vectorially secrete two distinct species of fibronectin, one of which contains the alternatively spliced EIIIA region (EIIIA+) and one in which the EIIIA segment is spliced out (EIIIA−). Fibronectin containing the EIIIA region is preferentially secreted apically. Although both apical and basal stimulation with transforming growth factor β1 increased fibronectin synthesis, the secretory response differed depending on which surface was being stimulated. Apical secretion of fibronectin and expression of EIIIA+ fibronectin mRNA increased only after apical stimulation. These data demonstrate a novel mechanism for the polarized regulation of targeted secretion and alternative splicing of fibronectin and suggest that the EIIIA segment may act as a targeting signal for the vectorial secretion of fibronectin.

Epithelial cells are characterized, in part, by their ability to sort and vectorially secrete proteins (1). Several mechanisms for polarized transport and targeting of transmembrane proteins have been identified (2). In many cases, the signal responsible for preferentially targeting a given protein to a given cell surface appears to be encoded by the protein’s amino acid sequence. Many proteins can be secreted out both surfaces of polarized epithelial cells, but little is known about the mechanisms of differential targeting of secretion (3). If the same protein performs different functions at the apical or basolateral surfaces, differential regulation of secretion could be biologically important. In human airways, fibronectin is present not only within the connective tissue of the airway wall but also within the airway lumen (4). Although the precise role(s) for luminal fibronectin have not been defined, they are presumed to differ from those associated with its presence in the extracellular matrix. Luminal fibronectin binds avidly to a number of microorganisms found in the airways and may be important in the clearance of these organisms and therefore the prevention of respiratory infections (5–7).

Transforming growth factor β (TGF-β1) is a family of regulatory proteins important in embryogenesis and wound healing that stimulates fibronectin synthesis and secretion in a wide variety of cells (8, 9). TGF-β1 is synthesized and secreted by cells present within the airway lumen (e.g. macrophages) and within the airway wall (e.g. monocytes) (10–12). In vitro, we found that when cultured guinea pig tracheal epithelial cells are grown to confluence on microporous membranes, they secrete fibronectin from both apical and basal surfaces. We hypothesized that apical and basal secretion of fibronectin might be differentially regulated in airway epithelial cells and examined the effects of TGF-β1 on the regulation of polarized fibronectin secretion.

MATERIALS AND METHODS

Cell Culture—Guinea pig tracheal epithelial cells were harvested from male Hartley outbred guinea pigs obtained from a Ceasarern-originated, barrier-sustained colony (Charles River Breeding Laboratory, Stoneridge, NY). Tracheal epithelial cells were harvested as previously described and cultured according to a modification of Adler’s protocol (13, 14). Cells cultured in this manner possessed intercellular tight junctions by electron microscopy. Immunofluorescence staining demonstrated the presence of keratin, consistent with a population of differentiated epithelial cells. Confluent monolayers were studied between days 14 and 16 of growth.

Metabolic Labeling and TGF-β Stimulation—Confluent monolayers were washed with unsupplemented Ham’s F-12 medium. Apical and basal media were replaced with serum-free growth media. In addition, certain monolayers were stimulated from either the apical or basal surface with 250 pM TGF-β1 (R & D Systems Inc., Minneapolis, MN). Following 18 h of stimulation, cells were washed above and below with methionine-free Ham’s F-12 and metabolically labeled with [35S]methionine (Expre35S) Protein Labeling Mix, Du Pont) at 250 μCi/ml for 3 h. The TGF-β concentrations were maintained as described. After 3 h, labeled media were collected from apical and basal compartments. Cells and debris were removed by centrifugation and samples placed immediately on ice. Monolayers were lysed in Laemmli sample buffer. Polyacrylamide Gel Electrophoresis and Densitometry—Conditioned culture media from each compartment were adjusted to Laemmli sample buffer conditions and boiled for 2 min. All samples were then analyzed via one-dimensional 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with a 5% stacking gel under reducing conditions (15). Gels were fluorographed (16) and autoradiographed (X-Omat, Eastman Kodak Co., Rochester, NY). Densitometry was performed using a Technology Resources, Inc. densitometer. A total of 11 dishes from four separate experiments were analyzed and a percent increase over control calculated (mean ± S.E.). Results were analyzed by one-way analysis of variance and the Newman-Keuls test.

Immunoprecipitation and Immunoblotting—Immunoprecipitation buffer (RIPA+) contained 0.1% SDS (Bio-Rad), 1% Triton (Bio-Rad), and 1% deoxycholic acid (Sigma) in phosphate-buffered saline. Denaturing immunoprecipitations were performed on conditioned media or cell lysates boiled in Laemmli sample buffer and subsequently adjusted to RIPA+ conditions. All samples were preabsorbed

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1 The abbreviations used are: TGF, transforming growth factor; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction.
with protein A-Sepharose beads (Pharmacia LKB Biotechnology Inc.) and then incubated with a rabbit anti-rat fibronectin polyclonal antibody (Calbiochem, 1:1000 dilution). Samples were remixed with protein A-Sepharose beads. After washing with RIPA+, the final sample was eluted by boiling the beads in Laemmli sample buffer. Rabbit anti-mouse IgG (Nordic) was used as a negative control. Immunoblotting was performed by transferring the proteins onto nitrocellulose (Schleicher & Schuell) using a Hoefer transfer apparatus, followed by blotting with the rabbit anti-rat fibronectin polyclonal antibody (Calbiochem, 1:1000 dilution) or a mouse anti-human fibronectin monoclonal antibody (IST-9) directed against the EIIIA domain (17, 18) (gift of Dr. L. Zardi, 1:200 dilution).

Polymerase Chain Reaction—cDNA was prepared as follows. 10 μg of total RNA and 100 pmol of random hexadeoxynucleotide were added to 20 μl of PCR buffer (1 mM each of dATP, dCTP, dGTP, and dTTP, 50 mM KCl, 20 mM Tris, pH 8.4, 1.5 mM MgCl2, and 10 mg/ml bovine serum albumin) and heated to 95 °C for 2 min. After cooling, 200 units of Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories) was added. The mixture was then incubated at 42 °C for 30–60 min followed by heating to 95 °C for 10 min. One μl of the reverse transcription reaction was used in each PCR. PCR buffer contained approximately 1 mM each of the primer mixtures and 25 units/ml Taq DNA polymerase. Amplification was performed in a thermal cycler (Ericomp, San Diego, CA) as follows: initial denaturation at 95 °C for 4 min, 30 cycles of amplification (95 °C for 45 s, 45 °C for 45 s, 72 °C for 60 s), and final extension at 72 °C for 10 min. DNA fragments were purified after separation in low melting temperature agarose, cloned into pBluescript, and sequenced from both strands.

RESULTS AND DISCUSSION

Confluent monolayers of guinea pig tracheal epithelial cells were stimulated via the apical or basal surface with TGF-β, and conditioned media were analyzed as described (19). 32P-Labeled probes were constructed using the random primer method (20). Hybridizations were performed in 5 × SSC, 40% formamide, 5 × Denhardt’s solution, 20 mM Tris, pH 7.4, 10% dextran sulfate, and 100 μg/ml salmon sperm DNA overnight at 50 °C. Filters were washed in 2 × SSC and 0.1% SDS at 50 °C, and then exposed to film for 16 h at −70 °C with an intensifying screen.

Northern Analysis—Northern analysis was performed as previously described (19). 32P-Labeled probes were constructed using the random primer method (20). Hybridizations were performed in 5 × SSC, 40% formamide, 5 × Denhardt’s solution, 20 mM Tris, pH 7.4, 10% dextran sulfate, and 100 μg/ml salmon sperm DNA overnight at 50 °C. Filters were washed in 2 × SSC and 0.1% SDS at 50 °C, and then exposed to film for 16 h at −70 °C with an intensifying screen.

Fibronectin Alternative Splicing

The effect of TGF-β on protein secretion varied depending on the protein being examined. Secretion of a 97-kDa protein from both apical and basal surfaces was decreased after stimulation by TGF-β, whereas secretion of several other proteins was increased. A few secreted proteins were unaffected by TGF-β. One of the most prominent secretory products of both apical and basal surfaces was an approximately 220-kDa protein identified by immunoprecipitation as fibronectin (Figs. 1, B and C). Fibronectin synthesis increased after apical or basal stimulation by TGF-β (Fig. 1B), but the secretory response to TGF-β stimulation differed depending on which surface was being stimulated (Fig. 1D). Basal secretion of fibronectin increased following both apical and basal stimulation by TGF-β. In contrast, apical secretion of fibronectin increased significantly after apical but not basal stimulation. Since N-glycosylation has been implicated in the apical targeting of certain proteins (21) and also accounts for some of the heterogeneity observed in fibronectin (22), we examined whether targeting of fibronectin was due to this particular form of post-translational modification. Inhibition of N-glycosylation with tunicamycin decreased fibronectin secretion from both apical and basal surfaces, but the polarized response to TGF-β was preserved (data not shown).

Alternative splicing occurs at three regions in the fibronectin gene, and several alternatively spliced isoforms of fibronectin have previously been described (23–27). Two segments (EIIIA and EIIIB) are either spliced in or out. A third segment (IIICS) is spliced in varying lengths depending on the cell type and species being studied. One explanation for our results was that the primary sequence of apical fibronectin differed from that of basal fibronectin and that the directional response to TGF-β stimulation might be mediated via differential alternative splicing.

To test this hypothesis, we designed degenerate mixtures of oligonucleotides based on published DNA sequences for human, chicken, and rat fibronectins (23–27). The primers were designed to anneal with nucleotide sequences flanking the EIIIA and EIIIB domains in order to detect all possible splicing variations in these regions. The dashed ovals in Fig. 2B denote the sequences used to construct primers for the EIIIA region. The product of the PCR reaction performed using the primers flanking the EIIIB region was a single band of approximately 160 nucleotides in length, the size predicted for fibronectin cDNA in which the EIIIB region was absent (EIIIB'). The product of the PCR reaction performed using

FIG. 1. Polarized secretion of fibronectin by guinea pig tracheal epithelial cells. Confluent monolayers of guinea pig tracheal epithelial cells were incubated with or without TGF-β. Conditioned media were harvested from apical and basal compartments and subjected to 10% SDS-PAGE and fluorography. A, media from the apical (lanes 1–8) and basal (lanes 9–16) chambers of eight separate dishes were harvested and analyzed individually following no stimulation (lanes 1, 2, 9, 10), apical stimulation (lanes 3–5, 11–13), and basal stimulation (lanes 6–8, 14–16). The large arrow denotes the 220-kDa protein whose secretion was decreased by TGF-β, and a 38-kDa protein whose secretion was unaffected. Equal volume percentages of apical and basal compartments were analyzed. B, cell lysates following no stimulation (lane 1), apical stimulation (lane 2), or basal stimulation (lane 3) were harvested and subjected to immunoprecipitation using a polyclonal anti-rat fibronectin antibody. C, immunoprecipitates from apical conditioned media (labeled “apical”) and basal conditioned media (labeled “basal”) following no stimulation (lanes 4, 5), apical stimulation (lanes 2, 5), or basal stimulation (lanes 3, 6). D, fibronectin secretion from each surface was quantified via densitometry of the 220-kDa fibronectin band from autoradiograms of conditioned media. *, the effect of basal stimulation on apical secretion of fibronectin differed significantly from the effects of both basal stimulation on basal secretion and apical stimulation on apical secretion (p < 0.001).
Fibronectin Alternative Splicing

A.

\[
\text{NH}_{2}-\text{GAL} \rightarrow \text{VSWAQNPSGESQPLVQTAVT}-\text{COOH}
\]

B. BEFORE EIIIA-A REGION

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<td>VSVIAGHSSEQPLVTQNT</td>
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C. T R G

\[ \text{I} \quad \text{II} \quad \text{III} \quad \text{IV} \quad \text{V} \quad \text{VI} \quad \text{VII} \quad \text{VIII} \quad \text{IX} \]

Primer Digestion of the Fragment with the Restriction Endonuclease

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<td>CTCTCGAG</td>
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<tr>
<td>EcoRI</td>
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**Fig. 2. Analysis of fibronectin splicing variants in guinea pig tracheal epithelial cells.** A, Schematic diagram of the fibronectin molecule showing the locations of the alternatively spliced EIIIA, EIIIB, and IICS domains. B, The deduced amino acid sequence of the EIIIA region of guinea pig fibronectin (G) is compared with the corresponding sequence in human (H), chicken (C), and rat (R). Only those amino acids that differ from the human sequence are listed. The single dashed overlines denote the nucleotide sequences used to construct primers for the EIIIA region. The double dashed underlines correspond to the 181-nucleotide fragment used as a template to generate the \(^{32}P\)-labeled cDNA probe used for Northern hybridization. C, Northern blot using the EIIIA\(^{+}\) probe on RNA from cells that were not stimulated (lanes 1–3) or apically (lanes 4–6) or basally (lanes 7–9) stimulated with 250 pm TGF-β. A band of approximately 7.5 kilobases was obtained that corresponded to the predicted size for fibronectin RNA containing the EIIIA region (arrow). Equal amounts of total RNA (20 μg) were loaded per lane; the intensity of the ribosomal bands after ethidium bromide staining was equal in all lanes. **Fig. 3. Detection of EIIIA\(^{+}\) fibronectin.** Cells were stimulated with 0 or 250 pm TGF-β as described. Conditioned media from apical (labeled “apical”) and basal (labeled “basal”) compartments were subjected to 10% SDS-PAGE followed by immunoblotting using either a polyclonal rabbit anti-rat fibronectin antibody (A) or monoclonal mouse anti-human fibronectin antibody directed against the EIIIA region (B). Cells were either not stimulated (lanes 1, 4) or stimulated apically (lanes 2, 5) or basally (lanes 3, 6) with TGF-β. and that fibronectin containing the EIIIA domain is preferentially secreted from the apical surface. The functions of the EIIIA domain are unknown; however, its expression appears to be both developmentally regulated and cell-specific. The principal form of fibronectin in plasma is EIIIA\(^{+}/\)EIIIB\(^{+}\). Both EIIIA\(^{+}\) and EIIIA\(^{-}\) forms have been found in the extracellular matrix of normal adult tissues, but the EIIIA\(^{+}\) form may be preferentially present in injured or fetal tissues. EIIIA\(^{+}/\)EIIIB\(^{+}\) forms of fibronectin are usually restricted to the developing embryo but are also re-expressed in healing wounds (28, 29). Previous studies involving assays for cellular adhesion, spreading, cytoskeletal organization, and migration have shown little or no differences between fibronectin isoforms containing or lacking the EIIIA domain (30, 31). Our findings suggest that alternative splicing of the EIIIA segment of fibronectin may be involved in the apical targeting of fibronectin secretion from polarized cells. One possible explanation is that the primary sequence of the EIIIA domain itself contains targeting information. However, the data presented here do not exclude other differences between apically and basally secreted fibronectin.
basally secreted fibronectin that could be responsible for targeting.

TGF-β has been previously shown to preferentially increase secretion and mRNA levels of EIIIA\(^+\) fibronectin from nonpolarized airway epithelial cells, such as fibroblasts (32, 33). We show that in polarized airway epithelial cells, this is a surface-specific effect; only apical stimulation by TGF-β\(_1\) increased levels of EIIIA\(^+\) fibronectin mRNA and secretion of the EIIIA\(^+\) isoform of fibronectin. The differential effect of apical versus basal stimulation on apical secretion of fibronectin suggests that TGF-β\(_1\) can differentially regulate both alternative splicing and secretion of fibronectin depending on which surface it is stimulating. The differential response of the apical and basal surfaces to TGF-β\(_1\) could be explained by the presence of different TGF-β\(_1\) receptors on each surface. Three cell surface TGF-β\(_1\)-binding proteins have been identified via cross-linking studies (34–37), and examination of TGF-β\(_1\)-resistant cell mutants suggests that at least two of these, types I and II, are functionally active receptors (38). While these receptors appear to mediate a multitude of cellular responses including those associated with cell adhesion, the nature of the signaling pathway(s) involved is unknown and may differ according to cell type and response. Different signal transduction or activation mechanisms may exist for apical versus basal TGF-β\(_1\) receptors. Release and activation of TGF-β\(_1\) may occur at either epithelial surface in association with airway injury or inflammation. The ability of epithelial cells to respond differently to injury occurring at either the apical (lumenal) or the basal (erosional) surface may be integral to the airway epithelium’s function as an interface between the underlying mesenchyme and the external environment.

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REFERENCES