The Transforming Growth Factor-β-inducible Matrix Protein βig-h3 Interacts with Fibronectin*

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Proper growth and development require the orderly synthesis and deposition of individual components of the extracellular matrix (ECM) into well ordered networks. Once formed, the ECM maintains tissue structure and houses resident cells. One ECM component, βig-h3, is a highly conserved transforming growth factor-β-inducible protein that has been hypothesized to function as a bifunctional linker between individual matrix components and resident cells. To gain insights into its physiological function, full-length βig-h3 protein was produced using a baculovirus expression system and purified under native conditions. Human fibroblasts attached and spread on βig-h3-coated plates and developed actin stress fibers. Purified βig-h3 binds fibronectin (FN) and type I collagen (Col I) but does not bind gelatin. Using defined fragments of FN, we localized the βig-h3 recognition region to the gelatin/collagen binding domain present in the N-terminal region of the FN molecule. Our results identify FN and Col I as two ligands of βig-h3 in the ECM.

The ECM is composed of an extensive array of macromolecules, which specifically interact to form a highly organized structural network (reviewed in Ref. 1). This matrix is synthesized in a highly regulated fashion (2), and once assembled, it serves as a framework to maintain tissue architecture and house resident cells. Changes in ECM composition can alter matrix structure and consequently modify matrix-matrix and matrix-cell interactions, impairing organ function (3–5). To prevent as well as improve the treatment and management of fibrotic conditions, it is necessary to identify matrix components, establish their interactions with one another and resident cells, and ascertain changes in the ECM that occur as a consequence of specific pathological conditions.

Transforming growth factor-β (TGF-β)-inducible gene-h3 (βig-h3) is a matrix component originally obtained from fetal bovine nuchal ligament by reductive saline extraction (designated MP78) (6) and hypothesized to be a microfibrillar protein. More recently, βig-h3 was cloned from TGF-β-stimulated A549 cells (7, 8) and hence name (TGF-β-inducible gene-h3). The nascently transcribed protein contains a secretory signal sequence (residues 1–23), four homologous internal domains, and a cell attachment (RGD) site (7–10). The porcine homologue, which shares a 92% identity at the amino acid level with the human protein, has been purified from a collagen fiber-rich fraction of pig cartilage and designated RGD-CAP (9).

Several studies have revealed that βig-h3 is a widely expressed component of the ECM in a number of organ systems where it associates with other matrix macromolecules. In bovine tissues, βig-h3 was found with collagen fibers in developing nuchal ligament, aorta, lung, and mature cornea (11). It was also present in capsule and tubule basement membranes of developing kidney and reticular fibers in fetal spleen. We recently reported that βig-h3 is expressed by primary human lung and bladder smooth muscle cells and fibroblast cells in vitro where it is secreted into the culture medium and incorporated in the matrix (12, 13). In human tissue, the protein is deposited in the matrix surrounding individual smooth muscle cells in the bladder detrusor (13), in septal tips of alveolar ducts, and in the walls of alveoli and bronchioles in lung (12).

Alterations in βig-h3 have been directly linked to human disease. Mutations in the coding region of βig-h3 have been identified in patients with hereditary corneal dystrophies (14). These mutations map to positions 124 (Arg → Cys or His) and 555 (Arg → Gln or Trp) and are believed to cause the protein to denature, resulting in the formation of amyloidogenic intermediates that eventually precipitate (14, 15). At the present time, it is not known whether these mutations produce other clinical manifestations in addition to their effect on vision. Kim et al. (16) demonstrated that βig-h3 is down-regulated in melorheostosis, a rare disorder characterized by hyperostosis. Interestingly, the addition of exogenous βig-h3 to osteoblasts in culture inhibited bone nodule formation, suggesting that it plays a direct role in osteogenesis (16). Furthermore, periostin, which is closely related to βig-h3, is expressed by TGF-β-stimulated primary osteoblast cells and supports cell attachment and spreading in vitro (17). In tissue, periostin localizes to the periosteum and periodontal ligament and is speculated to play a role in the adherence of osteoblast precursor cells in the periosteum (17). Consequently, in addition to being a structural component of the ECM, βig-h3 and its relatives may regulate bone production by osteoblasts.

Although the precise physiological function of βig-h3 is not known, it has been suggested that it interconnects different matrix components and resident cells (11–13) and consequently serves as a bifunctional linker protein. In this report, we demonstrate that βig-h3 supports cell attachment and spreading,
induces actin stress fiber formation, and binds fibronectin, an abundant glycoprotein of the extracellular matrix.

**MATERIALS AND METHODS**

**Chemicals and Reagents**—Affi-Gel-10 affinity chromatography support was purchased from Bio-Rad. Renaissance chemiluminescence reagents were obtained from PerkinElmer Life Sciences. Goat anti-rabbit IgG horseradish peroxidase conjugate, purified human fibronectin (FN), FN proteolytic fragments, and toluidine blue were purchased from Sigma. Novex SDS-polyacrylamide minigels, oligonucleotide primers, and SF-900 serum-free insect cell culture medium were supplied by Invitrogen. Talon cobalt affinity chromatography resin was obtained from CLONTECH. BA-85 nitrocellulose membranes were supplied by Schleicher & Schuell. SP-Sepharose cation exchange resin was obtained from Amersham Biosciences.

**Antibody Production and Purification**—Anti-βig-h3 antibodies 1073 and 1077 were developed in rabbits against synthetic peptides present in the N- and C-terminal regions of human βig-h3 and affinity-purified as described previously (12). For immobilization, anti-βig-h3 antibody 1077 and preimmune rabbit IgG were covalently attached to Affi-Gel-10 support resin following the protocols recommended by the manufacturer.

**Western Blot Analysis**—Proteins were resolved on Novex 8–16% polyacrylamide gradient gels under reducing conditions and transferred to nitrocellulose membranes (12, 13). After transfer, the membranes were blocked in PBST and 5% nonfat dry milk and subsequently incubated with anti-βig-h3 antibodies diluted 1:2000 in PBST, 0.3% bovine serum albumin. Bound antibody was detected with goat anti-rabbit horseradish peroxidase conjugate and developed with chemiluminescence or chloronaphthol substrate.

**Production of βig-h3 Protein**—Recombinant βig-h3 was expressed using the BV system employing established protocols (for review see Ref. 18). The cDNA containing the entire open reading frame for βig-h3 (comprising amino acid residues 24–683, the sequence of the full-length secreted protein) was amplified by PCR. The forward and reverse primers were (for 5′/rev 683/3′) 5′-GGCATGA TCAT GGT CCC GCC AAA AGC CAC CCA ACG GGC and 5′-GGCATGA TCAT GGT CCC GCC AAA AGC CAC CCA ACG GGC, respectively. The PCR product targets the recombinant open reading frame to the polyhedron locus of the BV genome. To facilitate purification, His6 residues were placed on the C-terminus of the protein. The construct was sequenced to verify its accuracy and subsequently transfected along with BV DNA (BaculoGold, BD Pharmingen) into insect cells. Recombinant virus was plaque puriﬁed, and used for protein expression. Using this system, the expressed protein is secreted into the culture medium. For the production of recombinant βig-h3, Spodoptera frugiperda 9 (SF-9) insect cells were infected with virus (4 plaque-forming units/cell) and grown in suspension culture in Sf-900 medium for 3 days (~70% survival). The cells were pelleted by centrifugation, and βig-h3 was purified from the medium using affinity chromatography.

**Cell Culture and Fractionation of Conditions**—Primary human bladder fibroblast cells were isolated as described previously (19) and grown in Dulbecco’s modified Eagle’s medium/Ham’s F-12 medium (1:1) containing 10% fetal bovine serum and penicillin/streptomycin at 37 °C in an atmosphere of 95% air, 5% CO2. Conditioned medium was cleared by centrifugation at 10,000 × g for 30 min at 4 °C and loaded onto a Hitrap Mono S (strong cation exchange) column (Amersham Biosciences), the column was washed with 10 ml of 50 mM phosphate (pH 6.5), 50 mM NaCl, and bound proteins were eluted with a linear NaCl salt gradient (0.05–1 M) in the same buffer. Aliquots from each fraction were analyzed on Western blots.

**Solid Phase Binding Assays**—Microtiter plates were coated with purified matrix component, or other proteins (50 μg/ml) diluted in PBS overnight at 4 °C. The plates were washed three times with PBST and blocked with PBST containing 1% BSA for 1 h at room temperature.

**RESULTS**

Production and Characterization of Recombinant βig-h3—To obtain sufficient quantities of protein for biochemical studies, full-length βig-h3 cDNA was cloned into BV and expressed in...
Sf-9 cells. The BV-expressed protein is secreted into the culture medium and isolated by a combination of cation exchange and metal chelate affinity chromatography (18). The purified protein has a mass of \(70\) kDa on SDS-polyacrylamide gels, close to the theoretical value derived from its amino acid sequence, and reacted well with our anti-\(\beta\)ig-h3 antibody (Fig. 1). To verify that the recombinant protein is biologically active, we assessed its ability to serve as a support for cell attachment. Human fibroblasts were seeded into microtiter plates coated with purified matrix protein. After \(1\) h, the cells attached and began spreading on wells coated with \(\beta\)ig-h3, Col I, and FN (Fig. 2A). In contrast, the cells seeded in BSA-coated wells were poorly attached and readily washed from the wells (Fig. 2A). Cell binding was also examined using a quantitative binding assay. The cells were seeded into microtiter plates coated with purified matrix components. After a \(1\)-h incubation, the plates were washed, and attached cells were stained with toluidine blue. The cells exhibited comparable levels of attachment to \(\beta\)ig-h3, Col I, and FN but did not adhere to BSA-coated wells (Fig. 2B).

One response of cells following adhesion to the ECM is actin polymerization, which results in stress fiber formation (22–24). To determine the effect of \(\beta\)ig-h3 on intracellular actin, the cells were seeded into coated wells in serum-free medium, allowed to attach, and stained with phalloidin. Prominent actin stress fibers were present in cells seeded onto \(\beta\)ig-h3 and Col I and FN (Fig. 3). Therefore, recombinant \(\beta\)ig-h3 supports cell attachment, spreading, and actin stress fiber formation.

Interaction of \(\beta\)ig-h3 with Collagen and Fibronectin—Several studies have reported that \(\beta\)ig-h3 is a component of the ECM, but its ability to interact with specific matrix components has not been examined in a detailed manner. The binding studies were performed by first coating microtiter plates with BSA, gelatin, Col I, or FN. The plates were washed, blocked, and subsequently incubated with \(\beta\)ig-h3. No binding was detected to wells coated with gelatin (Fig. 4) or BSA (data not shown). In contrast, we observed the saturable binding of \(\beta\)ig-h3 to Col I and FN (Fig. 4). The calculated \(K_d\) for \(\beta\)ig-h3-FN interaction was \(72\) nM. These results verify that \(\beta\)ig-h3 is capable of interacting with matrix proteins and exhibits relatively high affinity binding to FN. Additional experiments were undertaken to examine these interactions in greater detail. To establish specificity, competitive binding assays were performed by assessing \(\beta\)ig-h3 binding to immobilized FN in the presence of increasing amounts of soluble FN. \(\beta\)ig-h3 binding was inhibited with soluble competitor FN (Fig. 5). To assess the stability of this interaction, plastic beads were coated with FN.
and subsequently incubated with βig-h3. The beads were washed next with buffer containing increasing concentrations of NaCl, and bound protein remaining on the beads was eluted and analyzed on Western blots. This analysis revealed that the FN-βig-h3 complex was stable at ≥250 mM NaCl (Fig. 6).

FN contains several well characterized domains, which interact with specific matrix components (25, 26). The heparin binding fragment is present as a 30-kDa fragment on the N-terminal region of the FN molecule, whereas the collagen/gelatin binding fragment is contained within a 40-kDa fragment adjacent to the heparin binding domain. These fragments are generated by mild proteolytic digestion of human fibronectin and purified by ion-exchange chromatography (27). Both fragments were obtained from Sigma and are >95% pure based on SDS-polyacrylamide gel electrophoresis (Fig. 7). For binding studies, each peptide was immobilized on microtiter plates for use in solid phase binding assays. We found high affinity binding of βig-h3 to the collagen/gelatin domain (Fig. 8). In contrast, inconsistent binding to the heparin recognition site was observed (data not shown).

Analysis of Conditioned Medium—We have previously reported that human lung and bladder cells secrete βig-h3 into culture medium (12, 13). An analysis of conditioned medium from these cells by Bio Gel P200 gel filtration chromatography (exclusion limit ≥200 kDa) revealed that the bulk of βig-h3 elutes with the void volume and an apparent Mr of 200 (data not shown). This was unexpected given that the protein has mass of 70 kDa (see Fig. 1) and suggests that βig-h3 is present as a complex with itself or other proteins. To clarify this observation, the conditioned medium was fractionated by ion-exchange chromatography, and each fraction was analyzed on SDS-polyacrylamide gels and Western blots. βig-h3 was found to elute with 0.5 M NaCl (Fig. 9A). Subsequent incubation of the blots with an antibody to FN revealed that it eluted with the same ionic strength as βig-h3, suggesting that these proteins associate with each other in conditioned medium.
were washed with 10 mM phosphate (pH 7.4) containing 0.1% Tween 20 bated with gelatin- (lane 2 extracted and analyzed on Western blots.

As expected, IgG. Hence, FN immunoprecipitates with resin. No binding of either protein was observed to preimmune components (1 lane 2). To confirm that these proteins interact, additional experiments were undertaken. Anti-βig-h3 antibody 1077 was covalently attached to Affi-Gel-10. The conditioned medium was incubated with the immobilized antibody resin, the resin was washed extensively with PBS, and bound proteins were eluted and examined on Western blots probed with anti-βig-h3 and anti-FN antibodies. As expected, βig-h3 binds to the immobilized βig-h3 antibody (Fig. 9B). In addition, FN also binds this resin. No binding of either protein was observed to preimmune IgG. Hence, FN immunoprecipitates with βig-h3.

**DISCUSSION**

The structural integrity of the ECM is necessary for proper organ development and function. Matrix structure is in turn determined by precise interactions among individual matrix components (1–5). βig-h3 is a recently described matrix protein present in the ECM of bladder, lung, and other organ systems

![Fig. 5. Competitive binding assay. Microtiter plates were coated with FN and incubated with βig-h3 in the presence of increasing amounts of soluble (competitor) FN. Note reduced βig-h3 binding in the presence of competitor.](image)

![Fig. 6. Stability of βig-h3-FN complex. βig-h3 (500 ng) was incubated with gelatin- (lane 2) or FN-coated beads (lanes 3–7). The beads were washed with 10 mM phosphate (pH 7.4) containing 0.1% Tween 20 and 0.15–1 M NaCl. Bound protein, which remained on the beads was extracted and analyzed on Western blots. Lane 1, input βig-h3 (25 ng).](image)

To confirm that these proteins interact, additional experiments were undertaken. Anti-βig-h3 antibody 1077 was covalently attached to Affi-Gel-10. The conditioned medium was incubated with the immobilized antibody resin, the resin was washed extensively with PBS, and bound proteins were eluted and examined on Western blots probed with anti-βig-h3 and anti-FN antibodies. As expected, βig-h3 binds to the immobilized βig-h3 antibody (Fig. 9B). In addition, FN also binds this resin. No binding of either protein was observed to preimmune IgG. Hence, FN immunoprecipitates with βig-h3.

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The structural integrity of the ECM is necessary for proper organ development and function. Matrix structure is in turn determined by precise interactions among individual matrix components (1–5). βig-h3 is a recently described matrix protein present in the ECM of bladder, lung, and other organ systems

![Fig. 7. Analysis of FN domains. Specific domains of FN were obtained from Sigma and analyzed on SDS-polyacrylamide gels for purity. Lane 1, Heparin binding domain; lane 2, collagen/gelatin binding domain. 1 μg of each fragment was run in each lane. Numbers on the left show molecular masses in kDa.](image)

(7, 12, 13). A primary goal of this study was to investigate the interaction of purified βig-h3 with specific components of the ECM.

Full-length recombinant human βig-h3 was produced under native conditions in the baculovirus expression system. The purified protein supported the attachment and spreading of human fibroblasts. Similar results have been reported with dermal fibroblasts (28) and corneal epithelial cells (29), suggesting that βig-h3 serves as an attachment factor for these cells as well. We also observed the formation of actin stress fibers in cells seeded on βig-h3-coated wells. The appearance of these fibers was similar to those observed in cells plated on Col I. The binding of cell surface integrins to specific ECM components activates intracellular GTPases and downstream effectors, resulting in actin polymerization and stress fiber formation (22–24). βig-h3 contains an RGD motif and four internal repeated domains. Ohno et al. (30) demonstrated that antibodies to α5β1 integrin block the attachment of chick cells to bacterially expressed βig-h3. Surprisingly, a recent report identified the pentapeptides NKDIL and EPDIM present in the second and fourth domains of βig-h3 as mediating corneal epithelial cell attachment to βig-h3 via α5β1 integrin (29). Taken together, these results suggest that cell attachment, spreading, and actin stress fiber formation are probably a consequence of βig-h3 engagement of specific cell surface integrins.

The fractionation of conditioned medium by ion-exchange chromatography revealed that FN has a similar elution profile to βig-h3 (Fig. 9A). One interpretation of this finding is that these proteins associate with each other. Indeed, the results from this study support this hypothesis. FN was immunoprecipitated with βig-h3 from this same medium. Furthermore, recombinant βig-h3 exhibited high affinity binding to FN with a Kd ~70 nM (Fig. 4). Once formed, the βig-h3-FN complex was stable in >250 mM salt. By using peptides encompassing defined regions of FN in solid phase binding assays, we mapped one binding site for βig-h3 to the collagen/gelatin domain in the N-terminal region of FN (Fig. 8). Hence, these studies provide strong evidence that βig-h3 directly interacts with FN.

A surprising result obtained in the course of our studies is that we were unable to completely block βig-h3 binding to immobilized FN with soluble competitor. This may be a reflection of differences in the interaction between βig-h3 and immo-
Fig. 8. βig-h3 binding to FN domain. Microtiter plates were coated with the collagen/gelatin binding fragment of FN, washed, and incubated with βig-h3 (15 h at 4°C). The plates were washed, and bound βig-h3 was detected with Ab1077. Inset, double reciprocal plot of binding data. βig-h3 binds with an apparent $K_d = 50$ nM.

FN is a dimeric glycoprotein ($M_r = 440$) and an abundant...

FIG. 9. A, analysis of conditioned medium. Conditioned medium was applied to a HiTrap SP cation exchange column in 25 mM phosphate (pH 6.5), 0.05 M NaCl and eluted with a linear salt gradient (0.05–1 M NaCl) in the same buffer. Aliquots from each fraction were analyzed on Western blots probed with antibodies to βig-h3 and FN. Numbers on left show molecular masses in kDa. Note that βig-h3 and FN have a similar elution profile from the column (fractions 8–15). Input, conditioned medium; numbers 1–20, individual fractions from SP column. The position of βig-h3 and FN are indicated on the right. B, immunoprecipitation with anti-Big-h3 antibody. Conditioned medium was incubated with immobilized anti-βig-h3 Ab1077 or preimmune IgG, the resin was washed, and bound proteins were eluted and examined on Western blots probed with anti-FN or anti-βig-h3 antibodies. Note that FN and βig-h3 bind immobilized Ab1077.

bilized and soluble FN. The immobilization of FN may reveal βig-h3 binding sites that are not well exposed in soluble FN. Alternatively, there may be a nonspecific binding component in bound FN that cannot be totally eliminated with soluble competitor FN.

FN is a dimeric glycoprotein ($M_r = 440$) and an abundant...
ECM component. It is involved in a wide range of physiological functions including cell attachment and spreading, wound healing, and vascularization. FN contains several well defined structural domains that interact with a number of macromolecules including collagen, fibrin, heparin, laminin, and cell surface integrins (for review see Ref. 25). Several isoforms of FN arise via alternative splicing. These isoforms are expressed in a tissue-specific manner (25, 26) and differentially expressed during embryonic development and in certain pathological conditions (31, 32). The ability of FN to interact with a wide range of cell types and matrix proteins enables it to perform a wide range of functions.

The results presented in this report strongly suggest that FN is a candidate ligand for βig-h3 in the ECM, allowing βig-h3 to serve a linker function. In this capacity, βig-h3 could directly interact with cells via α,βi integrins and other matrix components via FN. Alterations in the primary sequence of βig-h3 such as those observed in individuals with hereditary corneal dystrophies (14) could potentially affect its interactions with FN or other ECM components, altering the corneal matrix structure. In addition, βig-h3 has a theoretical isoelectric point of ~7.3, and alterations in its primary structure may make the protein more prone to precipitating out of solution. This would be expected to have extreme effects on βig-h3-cell and βig-h3-matrix interactions. The results presented in the current report should provide a framework to address these issues in future studies.

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